EFFECTS OF ALCOHOL AND CAFFEINE ON RAT EMBRYOGENESIS

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Dedicated with love and respect

to my parents

LYMAN and ESTHER ROSS

who encouraged me, and never lost

their patience in my pursuit of knowledge.

I will praise Thee: for I am fearfully and wonderfully made.

Psalm CXXXIX 14

In the closest union there is some separate existence of component parts; in the most complete separation there is still a reminiscence of union.

The Notebooks of Samuel Butler

The grand aim of all science is to cover the greatest number of empirical facts by logical deduction from the smallest possible number of hypotheses or axioms.

Einstein

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LIST OF ABBREVIATIONS

AC	:	atrium commune
B1	:	first branchial bar
B2	:	second branchial bar
B3	:	third branchial bar
CP	:	cardiac primordia
CPN	:	closed posterior neuropore
DR	:	dorsal recess
DVS	:	developing ventricular system
E	:	embryo
F	:	forelimb bud
H	:	heart
IS	:	implantation site
MD	:	maternal decidua
ME	:	mesencephalon
O	:	otocyst
OL	:	olfactory plate
OP	:	optic primordium
PYS	:	parietal yolk sac
R	:	rhombencephalon
S	:	somite
TE	:	telencephalic evaginations
VA	:	vitelline artery
VV	:	vitelline vein
VYS	:	visceral yolk sac

ABSTRACT

The teratogenicity of alcohol and caffeine was investigated in Sprague-Dawley rats. All embryos were explanted on gestational Day 12 and twenty-one developmental endpoints (embryonic growth, central nervous system, cardiovascular system, musculoskeletal system and craniofacial region) were evaluated.

The first group of animals was treated with an aqueous solution of alcohol (12.5%^{v}/v), administered intraperitoneally on gestational Days 6 through 12. A significant increase in the incidence of resorptions and abnormal embryos (peculiar head shape, open neuropores, delay in development of the cardiac primordia, forelimb and branchial bars) was induced by alcohol treatment, compared to the controls. Embryonic growth was also affected.

Caffeine (25 mg/kg) was administered via the tail vein on gestational Day 10 to a second group of pregnant animals. The incidence of resorptions and abnormal embryos differed significantly from the controls. Overall embryonic growth was reduced. Developmental defects included unfused neural folds, open neuropores, distorted head shape, failure of the forelimb bud to develop and a reduction in somites.

The third group of pregnant rats received both alcohol and caffeine. Embryonic growth was severely affected. Malformed embryos displayed disturbances in flexion and rotation, as well as

in the development of the forebrain, midbrain, hindbrain regions and in the caudal neural tube. Several embryos had an under-developed yolk sac circulatory system; others possessed a primitive beating "s"-shaped cardiac tube instead of a differentiated heart. The development of the forelimb bud and branchial bars was also impaired.

Caffeine was found to be a coteratogen in the presence of alcohol because the incidence of resorptions, malformed embryos and the severity of the defects were increased when caffeine was administered together with alcohol compared to treatment with each substance individually.

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1. REVIEW OF RELATED LITERATURE

1. REVIEW OF RELATED LITERATURE

1.1 ALCOHOL

1.1.1 Physical Properties

Alcohol is one of a series of organic chemical compounds. In alcohols, the hydrogen of the hydrocarbon is substituted for by an hydroxyl (OH) group. This hydroxide of the hydrocarbon radical reacts with acids to form esters. Likewise the metallic hydroxide of the hydrocarbon reacts and forms salts. Pure ethyl alcohol or ethanol (CH_3CH_2OH) possesses a molecular weight of 46.07, with a melting point of -114.6^{o}C and a boiling point of 78.4^{o}C (Hodgman, 1959). Physically, ethanol has been described as possessing a weak odour and a strong burning taste.

1.1.2 Absorption and Distribution

The disposition of ethanol in the body in regards to its absorption and distribution was well characterized many years ago. The experiments of Mellanby (1919), Carpenter (1929) and Widmark (1932) were the pioneering investigations describing the absorption and distribution of alcohol. Berggren and Goldberg (1940) found that not only did the absorption of alcohol from the gastrointestinal tract follow the laws of simple diffusion, but because of the small size and relatively weak charge of the molecule, ethanol diffused quite easily through the cell membrane. Further insight into this interesting mode of distribution was the fact that although ethanol was able to easily penetrate the cell membrane by diffusion, it occurred quite slowly. This led researchers to inquire further into the manner in which alcohol distributes itself in the body. This

ultimately led to the interesting observation that the distribution of alcohol was accellerated by the degree of vascularization and blood flow. It was found that organs with a dense vascularization and a constant rich blood supply, such as the brain, lungs, liver, kidneys and placenta, equilibrated quickly with the circulating blood. Evidence for this rapid equilibration effect was realized by Crone (1965), when it was found that during a single passage through the brain, the extraction ratio of ethanol was of the magnitude of 93%.

Ethanol is absorbed from both the stomach and the small intestine. The greater amount of absorption of alcohol is from the small intestine. The rate of absorption is variable, depending on several factors. The two key factors affecting the rate at which the alcohol will be absorbed are: 1.) the condition of the gastrointestinal system and 2.) the concentration of alcohol ingested.

Alcohol tends to distribute itself in the body's fluids. This ultimately translates to the fact that most tissues are subjected to the same concentrations of alcohol which is present in the circulating blood. In the liver the concentrations tend to become higher. This is due to the fact that this organ receives a vast amount of blood via the portal vein. So that blood from both the stomach and the small intestine reach the sinusoids of the liver.

Small quantities of alcohol enter fatty tissue because of its poor blood supply. This aspect of alcohol distribution becomes increasingly important in the pregnant woman, because alcohol tends not to accumulate in the body fat, but rather it will be absorbed

and carried along the maternal vascular conduits. One major vascular area is the placenta and its associated vessels leading to the fetus. Because of the placental circulation and ethanol's inherent physical properties (such as small molecular weight, water lipid solubility and ionic nature), ethanol crosses quite freely the placental membrane and rapidly accumulates in the fetus. (Brien et al., 1983; Kaufman and Woollam, 1981).

1.1.3 Alcohol and Reproduction

The ingestion of alcohol by pregnant females has been known for many years to bring about detrimental effects in the offspring. Research has been carried out in different species of experimental animals, but has produced similar results. Alcohol adversely affects the developing conceptus.

Sandor and Elias (1968) found that ethanol induced in chick embryos a variety of malformations. In the pregnant rats ethanol caused fetal growth retardation following maternal treatment with ethanol (Sandor and Amels, 1971). Chernoff (1977) in a series of experiments reported that female mice exposed to a liquid diet prior to and throughout gestation produced offspring that would die prenately or displayed anomalous development.

Sandor (1968) showed that exposure of the chick embryo to ethanol resulted in cell necrosis of the neural tube and haphazard neuroblast formation in the primitive spinal cord. Sandor and Amels (1971) treated pregnant rats on Day 6, 7 and 8 of gestation and examined the embryos microscopically at Day 9.5 of gestation. Their results showed that a retardation had taken place in the development

of the somites, notocord and headfold. Brown et al. (1979) cultured Day 9.5 rat embryos in a serum medium containing ethanol and found that after 48 hours, the embryos displayed developmental delay and growth retardation.

Nakatsuji (1983) looked at the teratogenic effects of ethanol on Xenopus laevis embryos. These tadpole embryos were grown in a media that contained ethanol. Some of the embryos revealed facial malformations and a reduction of brain and body size. The cause of these malformations was explained in terms of the inhibition of cell migration during gastrulation which led ultimately to the formation of a smaller neural plate.

Studies performed on laboratory rodents assessing the effect of alcohol ingestion during pregnancy upon neonatal viability and development (Tze and Lee, 1975; Kronick, 1976) have given varying results. It was found that in general, the offspring of alcohol consuming mothers weighed less. Pilstrøm and Kiessling (1967) and Kronick (1976) found an increased mortality in litters of alcoholic treated rats and mice. Ethanol consumption by pregnant rodents was also found to cause intrauterine growth retardation (Øisund et al., 1978; Abel et al., 1981). Persaud (1983) showed that moderate consumption of ethanol during early embryonic development is not teratogenic in the rat, but increasing the dose of ethanol at a specific time period especially from gestational day 1 through 12 resulted in a significant increase of embryonic death and resorption in addition to intrauterine growth retardation.

In terms of developmental anomalies, ethanol when

administered at relatively high doses to pregnant mice was found to be teratogenic. (Sulik et al., 1981; Kronick, 1976; Chernoff, 1977; Randall et al., 1977; Boggan and Randall, 1979; Rasmussen and Christensen, 1980). Treatment of pregnant mammals with alcohol resulted in fetal or embryo death, fetal growth retardation and open neural tube defects. (Kronick, 1976; Randall and Taylor, 1979; Chernoff, 1977, 1980).

Brown et al. (1979) cultured rodent embryos in vitro and found that the development of untreated embryos was indistinguishable from growth in utero. They noticed that when embryos were cultured in the presence of ethanol at the critical period of organogenesis that both differentiation and growth were retarded, but no gross alterations in morphogenesis were induced (Brown et al., 1979). These results led them to believe that hypoplastic features displayed by infants exposed to alcohol in utero was caused by reduced embryonic cellular proliferation early in gestation.

The manner in which ethanol freely crosses the placenta and accumulates in fetal tissues and in amniotic fluid is well documented (Brien et al., 1983; Kaufman and Woollam, 1981). Jones et al. (1981) reported that placental blood flow is decreased as a result of pregnant female rats consuming alcohol in their drinking water. This decrease in placental blood flow will undoubtedly lead to a decrease in the transfer of nutrients to the fetus which might account for the intrauterine growth retardation.

Malformations are also present in human infants exposed to ethanol in utero. Jones et al. (1973) investigated offspring born

to alcoholic mothers. Detected in the offspring were smaller birth weight, microcephaly, reduction in width of the palpebral fissures and maxillary hypoplasia. Of the eight children examined, they found five with cardiac defects. Jones and Smith (1973) have estimated that approximately one-third of children born to chronic alcoholic mothers possess Fetal Alcohol Syndrome characteristics. In a subsequent study, Jones et al. (1974) found that in all the offspring born to alcoholic mothers, some type or form of developmental delay was apparent. Lemoine et al. (1968) found a high occurrence of cleft palates and cardiac anomalies amongst infants exposed to alcohol in utero.

1.1.4 Fetal Alcohol Effects

1.1.4.1 Historical Aspects of Fetal Alcohol Effects

Throughout history staunch statements towards the issue of maternal drinking during pregnancy have been made. Even in biblical times the detrimental effects of alcohol ingestion upon pregnancy was suspected. The quotation from Judges 13:7 which states, "Behold, thou shalt conceive and bear a son, and now drink no wine or strong drink." exemplifies the early condemnation of the consumption of alcohol during pregnancy.

Later on in time, during the Greco-Roman era, alcohol was thought to be a teratogenic agent. Vulcan, the god of fire and a blacksmith to the gods, was believed to be physically deformed as a result of his parents being intoxicated at the time of conception. Aristotle noted that "Foolish and drunken and harebrained women most often bring forth children like unto themselves, morose and languid." (Aristotle, cited in Burton, 1621).

Also, Carthaginian law apparently denied the newlyweds the drink of wine on their wedding night so as to prevent the conception of a developmentally disturbed child, (Haggard and Jellineck, 1942).

At the turn of the 18th century, the harmful effects of maternal drinking during pregnancy were recognized in the United Kingdom. It was during the gin epidemic that people began associating alcohol ingestion with maldevelopment of the fetus and child. In the 19th century the Alcohol Licensure Act (1834) was proclaimed, in which the government described the children born to alcoholic mothers as possessing a "starved, shrivelled and imperfect look", (Jones et al., 1973).

In an engraving done by William Hogarth in 1750, the detrimental effects of the ingestion of alcohol during pregnancy were characterized. His artistic representation of the slum at St. Giles Parish, Westminister titled "Gin Lane" depicted the detrimental effects of maternal alcohol consumption. The "Gin Lane"engraving is unique in the sense that the predominating two figures of the engraving are a mother and a falling child. Authorities working in the field of fetal alcohol effects have focused their attention toward the facial attributes of this falling child. The infant, as depicted by Hogarth, tends to possess eyes that are somewhat different. The eyes have been characterized as possessing shorter than normal palpebral fissures. These characteristic engraved eye features are not reproduced in other Hogarth engravings, thus it has been postulated that the artist was probably aware of the disharmonious relationship between maternal

alcohol ingestion and facial dysmorphology.

It was not until 1968 when Lemoine at al. examined 127 infants born to 69 mothers who drank alcohol excessively, showing a characteristic pattern of congenital abnormalities. Fetal Alcohol Syndrome is presently the term used to describe the children exposed to alcohol in utero. This nomenclature was proposed by Jones et al. (1973).

1.1.4.2 Fetal Alcohol Syndrome

Cases describing children suffering from Fetal Alcohol Syndrome are increasing in the medical literature. The prevalence of this syndrome varies from 1/1000 (Dehaene et al., 1977) to 1/600 (Olegard et al., 1975) live births worldwide. Considerable variability is apparent in the prevalence of this syndrome in different parts of the same country, due to the varying enthusiasm and knowledge of clinicians to diagnose this syndrome. It is estimated that Fetal Alcohol Syndrome contributes to the third leading cause of mental retardation in children (Wright et al., 1983).

In the appreciation of the effects of any teratogen an important principle is the variability of severity. Amongst medical agencies and centers worldwide, where large numbers of children affected by ethanol have been studied, a wide spectrum of the effects of alcohol on the fetus has been noted. At the most severe end of the spectrum are patients that possess the unique constellation of anomalies termed "Fetal Alcohol Syndrome". One must also note that there does exist individuals who fall along the rest of the continuum and hence possess every possible subcombination of the Fetal Alcohol

Syndrome anomalies.

The abnormalities most typically associated with alcohol teratogenicity can be grouped into 3 categories:

1.) Central Nervous System Dysfunction

2.) Growth Deficiencies

3.) Characteristic Pattern of Facial Abnormalities.

The variability of phenotype usually results from variable dose exposure at variable gestational timings. Also included in the variability of severity is the inherent genetic predisposition of the individual fetus.

Recent evidence suggests that chronic consumption of 89 ml of absolute alcohol per day, which translates to six hard drinks, is a major risk to the developing fetus. Lower levels of alcohol or less frequent use of alcohol possesses an unknown risk to the fetus. No absolute safe level of ethanol consumption has yet been established. According to the United States Surgeon General (Maugh, 1981), each patient should be told about the risk of alcohol consumption during pregnancy. The patient should be advised by her doctor to refrain from all alcoholic beverages and to be aware of the alcoholic content contained in certain foods and drugs.

1.1.4.3 Alcohol-induced Congenital Anomalies

Mental retardation has been one of the most common and serious problems of ethanol teratogenicity. Of 126 patients tested on a standardized test of performance, 107 (or 85%) scored more than two standard deviations below the mean (Hanson et al., 1976).

Shurygin (1974) in the Soviet Union compared twenty-three

children born to women who had been alcoholic during gestation to nineteen children born to women who became alcoholic after pregnancy. The children exposed to alcohol in utero displayed neonatal neurological impairments. Fourteen of the twenty-three were eventually diagnosed as being mentally retarded.

Impressive evidence for the prenatal effect of ethanol on the central nervous system has come from neuropathological investigations. Recently structural alterations have been demonstrated in the brains of infants exposed to alcohol in utero (Majewski and Bierich, 1978). In one series four brains showed similar malformations in that they each showed a failure or interruption in the migration of neuronal and glial cells. Consistently present were cerebellar dysplasia and heterotopic cell clusters. Malformations of the brain were readily seen since the brain surface had been affected. Ethanol was shown to interfere with brain organization and hence it was considered as an etiologic agent in the production of neural tube defects.

Chernoff (1977) as well as Randall and Taylor (1979) noted the condition of exencephaly in mice after in utero ethanol exposure. Clarren and Smith (1978) observed an alcoholic mother who gave birth to an anencephalic child. They also described other neural tube defects in children, examples of which were meningomyeloceles and lumbosacral lipomas.

Neurological abnormalities are frequently seen at birth in babies suffering from Fetal Alcohol Syndrome. Irritability and tremors characterize the babies. A poor suck reflex and auditory

disturbances have also been noted. These abnormalities may last for several weeks or months, depending upon the severity of expression of the syndrome. Mild dysfunction in cerebellar function (Hanson et al., 1976) and hypotonicity (Reinhold, 1975) have also been characterized.

The majority of infants with Fetal Alcohol Syndrome suffer from growth deficiency. Children with Fetal Alcohol Syndrome remain greater than two standard deviations below the mean for both height and weight. Of the decrease in height and weight, the weight is more severely affected. Also a constant feature noted in these cases was an overall decreased amount of adipose tissue.

The children affected by Fetal Alcohol Syndrome do not grow at a normal rate. This retardation of growth prompted many endocrinological studies. Root and Rieter (1975) found that infants suffering from Fetal Alcohol Syndrome displayed normal levels of growth hormone, cortisol and gonadotropins. Clarren and Smith (1978) believe that the deficient growth of this syndrome illustrates the prenatal damage to cell proliferation, hence leading to smaller fetal cell numbers and ultimately to reduced size.

Clarren and Smith (1978) described the principal facial characteristics of the Fetal Alcohol Syndrome from their study of two hundred and forty-five infants. They found the eyes to possess short palpebral fissures (>80% of patients). The nose was characterized as being short and upturned (>50% of patients) and also they possessed a hypoplastic philtrum (>80% of patients). The maxilla was found to be hypoplastic (>50% of patients). The upper lip was described as possessing a thinned upper vermillion border. In infancy retrogna-

thia was seen in more than 80% of patients, while in adolescence, micrognathia or relative prognathia occurred (>50% of patients). It is these characteristic facial features that unite these infants into a discernible clinical entity.

1.2 CAFFEINE

1.2.1 Introduction

Caffeine, 1,3,7 trimethylxanthine, is most frequently thought to be contained solely in coffee, but it is also contained in a number of common food products. It has been described as a neurally active substance present in most beverages such as tea, coffee, soft drinks as well as being present in food preservatives, chocolate and in some pharmaceutical formulations.

Concern recently has been directed toward this xanthine-derived substance in regards to its teratogenic effects in animals. In 1980, the Food and Drug Administration in the United States issued a number of notices to pregnant women, advising them to refrain from consuming caffeine-containing beverages during pregnancy.

1.2.2 Placental Transfer

The ability of caffeine to cross the placenta and enter fetal tissues has been demonstrated in experimental animals (Axelrod and Reichenthal, 1953; Sieber and Fabro, 1970) and in humans (Goldstein and Warren, 1962; Horning et al., 1973). It has been reported that caffeine, a xanthine alkaloid, distributes freely in the intracellular water of all tissues and equilibrates rapidly between maternal plasma and fetus (Goldstein and Warren, 1962; Horning et al., 1973). Since

this substance has been shown to cross the placental membrane quite easily, there is increasing concern about the potential teratogenic effects of caffeine.

1.2.3 Embryopathic Effects

Caffeine, dissolved in sterile water, was injected into the air sacs of fertile White Leghorn chick eggs at doses 100, 200, 300, 400, 500, 700, 900 and 1,100 µg per egg (Gilani et al., 1982). The embryos were treated at either 48, 72 or 96 hours incubation and evaluated on Day 9 of development. Major findings of this experiment were that treatment with 400 and 1,100 µg caffeine at 48 hours resulted in a significantly high incidence of abnormal embryos, compared to the controls. The most commonly seen malformations included reduced body size, micropthalmia, exencephaly, everted viscera and a short neck.

The toxic and teratologic effects of caffeine on chick embryos explanted at stages 4 - 7 and cultured for 19 - 22 hours were investigated (Lee et al., 1982). It was found that caffeine at dose levels of 200 - 300 µg/ml. significantly increased the incidence of neural tube defects. Concentrations of caffeine at levels of 500 µg/ml. or higher inhibited development or the morphogenesis of nearly all the organ primordia. The tissue most sensitive to the caffeine insult was the developing neuroepithelium. Concentrations of caffeine at 400 µg/ml. selectively inhibited the formation of the neural folds. Inhibition or inability of the neural folds to rise eventually led to a neural tube defect. These observations suggested that caffeine may cause neural tube defects if the embryo is exposed to it in suitable quantity and during a specific developmental period. Caffeine disrupts

the apical microfilament bundles in the developing neuro epithelial cells, thus inhibiting the contractile ability of these cells. It was suggested that inability of the cells to contract could cause the neural tube to remain open.

Nishimura and Nakai (1960) subjected 100 SMA strain mice to a single intraperitoneal injection of 1% caffeine once during the seventh to fifteenth days of pregnancy. The dosage level was 0.25 mg/g body weight. The mice were sacrificed near term or in mid pregnancy and evaluated in terms of their development. The treated animals were compared to a control group which consisted of fetuses from twenty-five normal pregnancies. An important observation made from this experiment was the fact that caffeine administration may result in embryonic death or in malformation of the fetuses. The incidence of embryonic death was highest when the injection was given between Day 7 and Day 12 of pregnancy. Malformations were observed in 18 to 43% of fetuses when the injection of caffeine was given between Day 10 and Day 14 of pregnancy.

In the study of Nishimura and Nakai (1960), the malformations that predominated were related to the skeletal system. These malformations normally manifested themselves as digital defects and cleft palate. The digital defects consisted of angulation, brachydactylism, syndactylism, adactylism and polydactylism. Clubfoot and joint malformations were also anomalies found. Subcutaneous hematomas were also observed in the general vicinity of the fetus' body where the anomaly appeared.

Thayer and Kensler (1973) administered caffeine to

CD/1 mice at doses up to 39 mg/kg body weight per day throughout four generations and upon recovery of the offspring found that many were underweight. Palm et al. (1978) administered to Sprague-Dawley rats 30 mg caffeine/kg/day throughout gestation and found a number of offspring with developmental anomalies. Malformations included kidney pelvis underdevelopment, decreased humerus density and significant reductions in organ to body weight ratios (in particular the brain, lungs and liver). No teratogenic effects were found in Sprague-Dawley rats while foetotoxicity appeared only as delayed ossification of sternebrae when Nolen (1981) administered a dose of up to 80 mg caffeine/kg/day. Kimmel et al. (1982) delivered a single intravenous dose of caffeine at a dose level of 37.5 mg/kg on Day 11 of gestation and found abnormal cartilage development as well as delayed ossification patterns. Female pregnant rodents during gestation given coffee to drink produced offspring with reduced body, liver and brain weights (Groisser et al., 1982).

1.2.4 Teratological Effects

Various routes of administration of caffeine have been studied. It has been shown that large doses of caffeine of the order of 100 mg/kg and more are teratogenic in mice if given parentally (Nishimura and Nakai, 1960; Fujii et al., 1969; Terada and Nishimura, 1975). Knoche and Konig (1964) and Groupe d'etude (1969) discovered that oral administration of caffeine at doses of 50 or 75 mg/kg produced only mild increases in malformations. Those malformations that were most commonly observed were those that involved the skeletal system. Defects occurred in the various limbs, digits and palate.

Small petechiae or hemorrhages were also noticed to be in close proximity to the area of the defect.

Teratological studies in rats with caffeine caused derangement of normal development. Rats that are given an oral dose of 75 mg/kg or more (Bertrand et al., 1965; Leuschner and Schwerdtfeger, 1968; Collins et al., 1980) commonly revealed a defect involving the skeletal system called ectrodactyly.

In other studies performed on Charles River CD-1 mice, a single dose of 100 mg/kg caffeine injected intraperitoneally on Day 14 of pregnancy caused a low incidence of cleft palate in the fetuses (Elmazar et al., 1981). Single oral doses of caffeine of 200 and 300 mg/kg on pregnancy Day 14 were also found to cause cleft palate in some of the fetuses. These doses were found to be toxic to the dams (Elmazar et al., 1981). Scott (1983) administered caffeine intraperitoneally to CD-1 mice on Days 11 and 12 of pregnancy at doses of 80 - 250 mg/kg. A dose related response pattern was evident from his results. Malformations most commonly observed included cleft palate, limb malformations and hematomas which occurred in the regions of the malformations. An unusual observation involving the limbs was made with regards to the teratogenic effect of caffeine. An asymmetric response was detected in that the left limbs were affected more often than the right (Scott, 1983). This teratogenic effect of caffeine on limb development has been reported by other investigators as well (Collins et al., 1981; Fujii and Nishimura, 1969; Bertrand et al., 1965).

1.3 COMBINED EFFECTS OF ALCOHOL AND CAFFEINE

Relatively few studies have been directed towards the combined effects of alcohol and caffeine on the developing embryo. Henderson and Schenker (1984) investigated the effects of ethanol and/or caffeine on fetal development. They found that ethanol (6%^{v}/v) incorporated in the diet and chronically administered to the maternal rat caused a 24% reduction in fetal survival. They also found that the mean weight of the offspring was also reduced 17%. The most interesting observation was that if caffeine was administered (concentration of which yielded 16 mg/kg/24 hours) together with ethanol that the reduction in fetal survival, fetal body size and fetal weight was decreased further.

Henderson and Schenker's (1984) study was appropriately designed, for it is known that in our present society many pregnant females are consuming a variety of drugs together with ethanol during pregnancy. It has been reported that high levels of caffeine ingestion may accompany ethanol ingestion (Streissguth et al., 1980). Caffeine and alcohol have been described as embryopathic (Oullette et al., 1977; Patwardhan et al., 1981) and for this reason, combinations of both agents represent a real threat to the developing embryo.

Ethanol was found to be teratogenic in laboratory animals (Henderson et al., 1979; Abel et al., 1981; Randall and Taylor, 1979). Henderson and Schenker (1984) have shown that either acute or chronic ethanol treatment of the pregnant rodent caused a reduction in both the viability and growth of the fetus. Explanations for these changes have included alterations in fetal protein synthesis and/or reductions

in placental amino acid transport (Henderson et al., 1979, 1980, 1981; Patwardhan, 1981).

Caffeine at a dose between 50 to 300 mg/kg has been shown to induce fetotoxic effects in animals (Martin, 1982; Palm et al., 1978). Caffeine has been shown to disrupt the fetoplacental unit in the sense that the placental weight in rats was decreased (Gilbert and Pistey, 1973).

Ethanol readily crosses the placenta (Kaufman and Woollam, 1981) and could directly affect the developing embryo. Likewise, caffeine and its metabolic byproducts are transmitted to the fetus. One difference between the two substances lies in their relative metabolism during pregnancy. It was noted that caffeine is poorly metabolized during pregnancy (Neims et al., 1979; Kling and Christensen, 1979). Accumulation of caffeine in the circulatory system certainly has focused researchers' attention towards caffeine's teratogenic potential (Martin, 1982).

Henderson and Schenker (1984) showed in the rat that ingestion of caffeine and alcohol at the same time potentiated the fetal effects of the ethanol itself. Several theories that attempt to explain the increase in severity of fetotoxicity have been advanced. Mitchell et al. (1983) have proposed that the presence of ethanol may impair the metabolism of caffeine, so that the concentration of caffeine increases and accumulates to toxic levels. Other explanations have lead to the suggestion that ethanol and caffeine once together may act in a synergistic way.

1.4 CHEMICAL INTERACTIONS

1.4.1 General Remarks

In humans, the ability for a teratogenic agent to act upon the developing embryo is seldom an isolated event, but rather it is influenced by a number of interactions between various chemical agents. Humans are exposed every day to chemical compounds that are detrimental to the developing embryo. These agents range from common over-the-counter pharmaceutical formulations to pharmacologically active agents, examples of which are alcohol, nicotine and caffeine. Skalko and Kwasigroch (1983) noticed that these various combinations of potentially embryotoxic agents are commonly ingested by pregnant women, and that some of these combinations of teratogenic agents have a detrimental effect on the development of the conceptus.

The influence of interactions of known teratogenic agents with other chemical agents has not been extensively studied. According to Skalko (1985), when one speaks of chemical interactions, one refers to the manner in which a drug or chemical may be altered by either being present prior to the administration of some other agent or even upon simultaneous administration. Brockman (1974), Warren and Bender (1977) and Valeriote (1979) found that in the case of multiple drug therapy in combating cancer, the interactions between drugs and chemical agents were beneficial. In other cases, Stolman (1967) discovered the lethal interaction between ethanol and barbiturates.

1.4.2 Site of Interaction

The chemical interactions between drugs may occur at different sites in the body. The interactions may occur in the gut and result in either a decrease or increase in the presence of the various agents. Interactions may occur along plasma protein binding sites, or in an organ such as the liver. Various effective receptor sites in which chemical interactions between agents would occur have been described (Hartshorn, 1976; Warren and Bender, 1977; Cadwallader, 1983).

1.4.3 Theoretical Basis

Wilson (1964) first described the phenomenon of chemical teratogenic interactions. Pregnant rodents, once subjected to chemical teratogenic agents at low and sometimes subthreshold dosages produced malformed offspring at a rate above which was expected if the one agent was administered alone.

Based on research done by Hartshorn (1976) and Cadwallader (1983), the following summary was devised in order to illustrate the various types of interactions between chemical compounds (Skalko, 1985).

Types of Interactions Between Chemicals

1. Homergic

   Two chemicals produce the same overt effect

   (a) Summation

   (b) Additive

2. Heterergic

Only one pair of chemicals produce an effect

(a) Synergism

(b) Antagonism

1.4.4 Nomenclature of Teratogens

Skalko (1985) has described the embryotoxic effects as being altered or modified by concurrent administration of other chemical agents, normal metabolites and altered physiological states. Concentrations of agents, dosages, time period of administration and length of time between treatment influences the ability of one agent to modify the response of another.

Skalko and Kwasigroch (1983) suggested that one of the agents used was to be designated as the "primary" teratogen. This "primary" teratogen was to be administered at embryotoxic doses. In comparison, Skalko and Kwasigroch (1983) believed that the "secondary" teratogen should be used at a relatively low dosage (not embryotoxic). By using these specific dosage restrictions, the researchers could gauge the ability of how the "secondary" teratogen could amplify the effects of the "primary" teratogen.

1.4.5 Antiteratogens

An antiteratogen is described as a chemical agent which, when it is found in association with a proven teratogen, does not allow the known teratogen to exert its deleterious effects on the developing embryo. Hence, an antiteratogen operates antagonistically to the known teratogen, and thus prevents the embryo from being malformed (Skalko, 1985). Many antiteratogens have been isolated and described in the

scientific literature. Beaudoin (1976) and Landauer (1979) described some normal metabolites as being antiteratogenic. β-adrenergic blocker propanolol was found by Fujii and Nishimura (1974) to belong to the antiteratogenic family of compounds. Nomura et al. (1983) found caffeine and retinoic acid, which alone are well known teratogens, to possess antiteratogenic properties.

1.4.6 Coteratogen

While caffeine was found by Nomura et al. (1983) to be an antiteratogen, it is also a well known teratogen. Caffeine, depending upon the different chemical environment that it may be found, will display what is called coteratogenic abilities. Current evidence has suggested that caffeine potentiates the deleterious embryopathic effects of other "primary" teratogens. Caffeine has been shown in animal experiments to potentiate the embryotoxic effects of hydroxyurea and acetaxolamide (Ritter et al., 1982), chlorambucil (Fujii and Nakatsuka, 1983), mitomycin C (Nakatsuka et al., 1983), and phenytoin (Skalko et al., 1984). According to Skalko (1985), caffeine displays both heterergic effects: synergism and antagonism. Caffeine may be designated a coteratogen because of its ability to potentiate the embryotoxicity of a known "primary" teratogen and hence this synergistic effect is called a positive coteratogen. However, in other circumstances caffeine produces an antagonistic (antiteratogenic) effect, which then would classify it as a negative coteratogen. Currently caffeine has been shown to work in both a positive and negative coteratogenic manner, depending upon the chemical environment in which it is found. Substances already found in the chemical environment

will interact with caffeine. Ultimately, the nature of the primary teratogen determines the type of coteratogenic effect (postitive or negative) that caffeine will display.

1.5 TERATOLOGICAL EVALUATION

Currently researchers gauge toxicity testing of drugs during pregnancy according to teratologic effects. Embryos treated with a specific agent are compared to the well documented pattern of normally developed embryos. Certain agents may disrupt the development of an embryo and hence, in order to observe and evaluate these discrepancies of growth and development, the normal developmental charts are of importance.

In order for the normal developmental charts to be useful in teratological studies, the different stages must be clearly characterized. In some cases only certain stages of development are described (Van Beneden and Julin, 1884; Waterman, 1943), while in others, the method of classifying development into stages is unsuitable (Minot, 1905; Huber, 1915; Butcher, 1929; Henneberg, 1937; Burlingame and Lone, 1939; Brock and Kreybig, 1964)(Edwards, 1968). Some of these listed were unsuitable in the sense that the various stages were not characterized in sufficient detail.

In past years developmental studies have been performed on rabbits, rats and mice. The developmental parameters used were usually gestational age and somite number, but as time progressed and further research had been done, it was found that these criteria were inadequate. Gestational age was not a valid criterion because there is considerable variation in the stage of development reached, both

between litter mates and amongst litters of the same gestational age. With regards to rats, this point becomes amplified in that the actual mating of the rat is normally unobserved and consequently an exact time of mating is unknown. The use of somite number to classify embryos and group them into specific developmental stages is unsatisfactory for a number of reasons. Burlingham and Long (1939) discovered that the number of somites could vary up to six points at any particular stage of development. They also reported that somite number was not always readily visible and that the total number were not always seen throughout development because they gradually become obliterated.

Taking into consideration these problems, a method designating the various defined stages of the embryo according to external features was needed. Classifications that rely on external features without using chronological age or somite number have been described for the chick (Hamburger and Hamilton, 1951), the mouse (Gruneberg, 1943) and the rat (Christie, 1964). The systems of classification of development used by these researchers depend on the appearance of readily visible features that ultimately depict development into specific defined stages.

Brown and Fabro (1981) developed a morphological scoring system for the evaluation of the embryos. These investigators have designed a morphological scoring sheet in which 17 parameters are outlined and categorically separated. Each parameter consists of different levels of development, and consequently a different score is assigned to each level. The increase in score reflects a higher level or degree of development achieved by the embryo. Some parameters

possess the gradation of scoring number with a range of 0 to 5; others are between 0 to 4; 0 to 3; 0 to 2 and even 1 parameter possesses the range of 0 to 1. One must keep in mind that in this scoring system as one increases the value from one number to a corresponding higher number, this reflects a change in development. One must also note that with these values, a one-half value is allowed to be given if the embryo is found to be developmentally in between stages.

On these morphological scoring sheets certain parameters are listed on the side. These parameters consist of categories that depend upon a linear measurement for their determination. These categories are Crown-Rump Length, Head Length and Yolk Sac Diameter. These measurements are obtained by use of a micrometer located in the eyepiece of the dissecting microscope. Based upon the magnification used to observe the embryo, the measurement may be adjusted in order to properly measure the embryo.

Somite number is another parameter that is collected and recorded. Brown and Fabro (1981) have developed a relatively simple rule to use once the problem of indistinct somites become apparent. They stated that one may use the somite adjacent to the mid-point of the forelimb bud as number 11 when cephalic somites become indistinct. Hence, by using this rule as a constant, a consistent evaluation of somite number may be obtained.

1.6 RAT EMBRYOGENESIS

1.6.1 General Remarks

According to Schneider and Norton (1979), fertilization of the rat ova most frequently occurs in the ampulla tubae, the uppermost loop of the oviduct. From this anatomical position tubal passage to the uterus requires three days from the time of coitus. These three days are required for the blastocyst to begin its mitotic division and pass into the uterine horn. The fertilized ova enters the uterus at the late morula stage. A delay in the passage of the ova is apparent in the uterus at Day 5 of pregnancy. This delay is crucial to the final outcome of whether or not the blastula is to implant.

Implantation occurs on Day 6 of pregnancy, at which time the blastula stage is reached. This occurs approximately six days after mating for the rat (Witschi, 1956). The next two days from Day 7 to Day 9 are necessary to provide the blastula with an environment that will be conducive for supporting the growth of the embryo. After implantation on Day 6, a crucial event undertaken by the developing blastula is the establishment of a yolk sac placenta. This yolk sac placenta is formed on Day 7. The yolk sac placenta plays an important role in determining whether or not further development of the embryo will continue. It provides the mechanism by which the early developing embryo will be nourished.

Witschi (1956) reported that the primitive streak of the rat embryo began its formation around gestational Day 8½. This becomes the first indication of mesoderm and nervous system development. The time period from Day 9½ to Day 11½ is developmentally important and

has been called the period of organogenesis. This time period is important because most of the research done in experimental teratology is performed at this stage of embryonic development. Christie (1964) and Edwards (1968) have clearly elucidated specific embryological events that occur during the period of organogenesis.

1.6.2 Day 9½ - Day 9 3/4

Further development occurs in the nervous system. This is the time span in which the neural folds first develop. Also, this stage begins with the first appearance of somites, and ends just prior to the visible appearance of the post-otic sulcus. During this stage, the bilateral elevations of the newly developed neural folds become even further accentuated from the surrounding tissue. These neural folds become increasingly larger and begin to extend laterally. In the cranial direction, the neural folds protrude over the prochordal plate, while in the caudal direction, the neural folds flatten out. The flatness of the neural folds in the caudal direction is due to the placentation of the rat. The foregut invagination which appears on the ventral surface of the embryonic plate creates a sulcus which is deepest in the anterior aspect. The primitive developing limbs grow caudally to a level where the neural folds have begun to flatten out. The space between the primitive limbs possesses an elevation that corresponds to the neural sulcus, which is positioned dorsally.

Further along in development the neural folds attain prominence, and extend laterally in a direction anterior to the pre-otic sulcus. This pre-otic sulcus appears at two somites and is positioned midway between the anterior limit of the neural folds and the acute

bend of the embryo. Christie (1964) reported that this sulcus (pre-otic sulcus), which deepens progressively at this stage, marks the anterior limit of the otic or fourth rhombomere in the rat. The neural sulcus is also observed to extend in a caudal direction.

In the ventral aspect the migration caudally of the cardiac area is caused by the forward protrusion of the neural folds. This migration ultimately leads to the deepening of the anterior intestinal portal.

1.6.3 Day 9 3/4 - Day 10

The appearance of the post-otic sulcus, and consequent delimitation of the otic rhombomere, marks the commencement of this stage (Christie, 1964). Important features of this stage are: 1.) elevation of the neural folds; 2.) the caudal and cranial extension of the neural folds; 3.) the defined otic rhombomere.

Further along in this stage of development depressions are seen in the anterior surface of the neural folds. These depressions correspond to the optic foveae. These depressions are situated caudally at the level of the second somite and begin to approach each other as time progresses. The space between the neural folds and caudal to the region of the fifth somite narrows. The allantois which takes origin behind the caudal angle of this space increases in size, but does not yet fuse with the ectoplacenta (Christie, 1964).

On the lateral aspect of the developing embryo a bulge appears. This elevation represents the first branchial arch (Christie, 1964; Shepard, 1980). Situated beside the arch and forming the posterior aspect of the arch is the branchial cleft. At this stage the

cleft also becomes apparent.

Ventrally the developing heart-tube forms a C-shaped bend. The bend occurs towards the right, and emerges from the septum transversum. This orientation forms the cranial margin of the anterior intestinal portal. The heart tube continues towards the right side as the ventricle. The tube then turns dorso-cranially and runs medially as the bulbus. This structure disappears between the ventral aspect of the already established mandibular arches.

1.6.4 Day 10 - Day 10½

At this stage, the neural folds fuse from the second to the sixth somite. In the mesencephalic region the neural folds begin to approximate one another. This meeting of the neural folds coupled with the inward roll of the margins converts the embryonic plate into a tube. At this stage the primitive optic foveae and infundibulum become apparent. The mesencephalic segments become somewhat visible. Also, the otic placode is visible at the dorsal end of the first branchial cleft.

Midway in this stage reversal of the curvature of the developing embryo begins. While the embryo undergoes a reversal in curvature, the tail fold begins and hence the limits of the posterior intestinal portal become defined. Christie (1964) reported that the ventral edge of the posterior intestinal portal moves cranially, whilst the allantois now fuses with the ectoplacenta.

At this time little change is seen in the development of the heart. The heart tubes caudal end frees itself slightly from the septum transversum. The cranial end moves in a caudal direction,

between the ventral ends of the first and now developing second pair of branchial arches. As a result of all this movement the heart tube tends to lie in an orientation more dorso-ventrally than before (Christie, 1964).

1.6.5 Day 10½ - Day 10 3/4

Christie (1964) and Edwards (1968) both agreed that this stage is normally depicted by the fusion of the neural folds at the diencephalic-mesencephalic junction, and the first appearance of the otic pit. This fusion of the neural folds ultimately gives rise to the anterior neuropore.

The aperture of the rhombencephalic roof is narrowed by cranial extension from the level of the third rhombomere and the caudal progression from the diencephalic-mesencephalic junction. The narrowing of the midgut yolk sac communication occurred as a result of the rolling over of the embryo. The lateral body folds become very prominent, although the communication between the intra and extraembryonic coelom remains open (Christie, 1964).

The optic vesicles appeared towards the end of this stage and ventro-caudal to these optic vesicles are the prominent mandibular arches. The heart tube was seen through the thin-walled pericardium. Towards the end of this stage the rolling over process has shifted the orientation of the embryo. The dorsal surface of the embryo at the end of this stage is convex (Edwards, 1968).

1.6.6 Day 10 3/4 - Day 11½

Christie (1964) and Edwards (1968) agree that the beginning of this stage begins with the closure of the anterior neuropore

With respect to cardiac development, atrial dilatation of the heart tube is evident. Christie (1964) described the atrial dilatation as being visible on the left side, immediately above the septum transversum and below the atrial canal. Further along in development, once the atrial dilatation has undergone expansion, the atrial dilatation will appear visible on the right side of the embryo immediately behind the bulbus.

At this stage, as a result of the rolling over process that the embryo undergoes, the communication between the gut and yolk sac is sealed off. The attachment of the allantois now becomes more cranial. Also, another feature at the end of this stage is the growth of the mandibular arches toward the midline, but they do not fuse.

1.6.7 Day 11½ - Day 12

This stage begins with the closure of the posterior neuropore, followed by closure of the otic vesicles (Edwards, 1968). Rathke's pouch first appears and the thyroid diverticulum may also be seen.

In terms of heart development, at the beginning of this stage, the ventricle and bulbus have differentiated so as to give the heart the appearance of being four-chambered. The epi-pericardial ridge becomes prominent and the cervical sinus begins to form (Christie, 1964).

After the closure of the otic vesicle, a diverticulum appears that is dorso-medially directed. The diverticulum is the endolymphatic sac. Also during this time, the olfactory placode becomes visible. It lies at the bottom of a shallow pit, the edges

of which are formed by the medial and lateral nasal processes. These olfactory placodes are observed only once they have been accentuated by the nasal processes. These nasal processes are important for the scoring of the olfactory placodes according to Brown and Fabro (1981) Morphological Scoring System.

The liver anlage and posterior limb bud may be seen for the first time at this stage. The liver appears as a swelling above the anterior limb bud and later migrates caudally. The posterior limb bud also appears as a swelling that is positioned on the lateral surface of the embryo opposite somites number 23 to 28.

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Nulliparous albino rats (200 - 250g) of the Sprague-Dawley strain were used in this study. The rats were obtained from Central Animal Care Facility located in the Basic Medical Sciences Building of the University of Manitoba.

2.1.1 Environmental Conditions

All animals were kept in an environmentally controlled room. The female rats were separated into cages and remained relatively undisturbed for 72 hours in order that they become acclimatized to their new environment. The room in which the animals were housed was kept at a temperature of $22^{o}C \pm 1^{o}C$ and a relative humidity of $50\% \pm 10$.

A cycle of 12 hours of light from 0800 to 2000 hours, and darkness from 2000 to 0800 hours was maintained at all times throughout the study.

2.1.2 Animal Care

All animals received food and water ad libitum. The food diet consisted of (Wayne F6 Rodent Blox) pellets.

2.1.3 Randomizing

The virgin female rats were divided into two groups of twelve animals for control and treatment groups by the following randomizing technique. Twenty-four female rats were placed in separate cages which were numbered from 1 to 24. Papers on which the numbers 1 to 24 were written were folded and placed in a brown paper bag and

mixed. The first twelve numbers drawn represented those animals that were destined to become the treatment group. Consequently, the remaining twelve animals represented the control group. Within each group the animals were earclipped and catalogued. Ear clips were performed in a manner so as to preserve the individual identity of each rat.

2.1.4 Breeding of Animals

One male albino Sprague-Dawley rat (250 - 300g) was introduced into a cage that contained two virgin female rats. The male rat was placed in the cage at 1600 hours and separated from the females at 0900 $\pm$ 0030 hours the following morning. Vaginal smears were then taken.

2.1.5 Determination of Pregnancy

After removal of the male rats from their respective mating cages, the female rats were then inspected for the presence of vaginal plugs. The refuse pan situated beneath the cage was also inspected for vaginal plugs (yellowish crystalline formation). Pregnancy was confirmed by the presence of spermatozoa found in the vaginal smear.

2.1.6 Handling of Animals

All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care. The animals were given water and food ad libitum. Refuse pans and paper were cleaned every morning. Animals were contained in spacious cages in an environmentally controlled room. The animals were not subjected to fluctuations in room temperature or loud noises. All pregnant females were handled only by the investigators and a technician. The animals were handled

every morning for the purpose of obtaining their weight. Also, the animals were handled on the mornings of various treatment days. Finally, the animals were handled on gestational Day 12, the day on which the embryos were recovered. All handling of the animals took place without the use of gloves in order for the rat to feel the tactile sensation.

2.2 MATERIALS AND METHODS

The project was carried out in four stages. In the first part of the experiment, pregnant female rats were killed on Day 12 of gestation and embryos were recovered. These embryos were scored according to Brown and Fabro (1981) Morphological Scoring System. These embryos and their respective scores represented the group called Untreated Control. Maternal blood samples were also taken.

The second part of the experiment consisted of treating twelve pregnant female rats with an alcohol solution. The alcohol solution was prepared by diluting 100% pure ethyl alcohol (Canadian Industrial Alcohols and Chemicals Limited) with physiological saline to produce a 12.5%(^{v}/v) pure alcoholic solution. Twelve other pregnant female rats received physiological saline and served as a control group.

The third part of the experiment consisted of treating twelve pregnant females with a caffeine solution prepared from pure caffeine ($C_8H_{10}N_4O_2$, Molec. wt. 194.19, Eastman Kodak Co.). The injected volume was 0.5 ml and the dosage level was 25 mg/kg. The pregnant female rat was weighed on the morning of gestational Day 10 and on the basis of weight an appropriate caffeine solution was prepared. The animals were injected intravenously via the tail vein with the use of a 1cc tuberculin

syringe fitted with a 25 5/8 gauge needle. Twelve pregnant females representing the control group received physiological saline in a similar manner.

The fourth part of the experiment consisted of twelve experimental animals that were treated with both alcohol and caffeine. A corresponding control group received physiological saline.

2.3 TREATMENT

2.3.1 Part I - Untreated Control

Once mating was confirmed, the female rat was withdrawn from the mating cage and housed separately. The day on which spermatozoa were found in the vaginal smear was designated as gestational Day 1, which is equivalent to Embryonic Age Day 0.5 because of the assumption that mating had occurred at 12:00 P.M. the previous night. These animals remained relatively undisturbed for the remaining time of the study. Only contact that the researcher had with these pregnant females was that each morning these animals were weighed and the weight was recorded.

At 1400 hours on Day 12 of pregnancy, Embryonic Age Day 11.5, the animals were anaesthetized with ether. Following laparotomy the uterine horns were removed and placed in Hank's Balanced Salt Solution (Whittaker M.A. Bioproducts). In order to reveal the conceptuses, the uterine horns were excised along its antimesometrial border. With the aid of a dissecting microscope, the embryos were then scored, utilizing the Morphological Scoring System (Table 1) described by Brown and Fabro (1981). The embryos were stored in 10% formalin.

	0
YOLK SAC CIRCULATORY SYSTEM	no visible, or scattered, blood islands
ALLANTOIS	Allantois free in exocoelome
FLEXION	Ventrally convex
HEART	Endocardial rudiment no visible, or visible but not
CAUDAL NEURAL TUBE	Neural plate or neural folds
HIND BRAIN	Neural plate
MID BRAIN	Neural plate
FORE BRAIN	Neural plate or no visible prosencephalon
OTIC SYSTEM	No sign of otic development
OPTIC SYSTEM	No sign of optic development
OLFACTORY SYSTEM	No sign of olfactory development
BRANCHIAL BARS	None visible
MAXILLARY PROCESS	No sign of maxillary development
MANDIBULAR PROCESS	No sign of mandibular development from bar I
FORE LIMB	No sign of fore limb development
HIND LIMB	No sign of hind limb development
SOMITES	0 - 6

A blood sample was taken from the dorsal aorta. This was achieved by penetrating the dorsal aorta in the abdominal region with a 18½ gauge needle just 0.5 cm above the bifurcation of the blood vessel into the two common iliac arteries. The needle was inserted in such a manner so that the bevel of the needle faced the artery and hence blood entered the 20 ml syringe.

The blood was then placed into a B-D Microtainer Branch Capillary Blood Serum Separator Holder and allowed to stand for half an hour. Next, the sample was centrifuged at 6,000g for 90 seconds. The supernatant was then decanted and placed into another capillary tube. To this tube a 6% ($^{w}/v$) aqueous trichloroacetic acid (TCA) was added. The TCA was added in the proportion of 2.0 ml TCA/1.0 ml sample. This solution was mixed well and centrifuged again. The tube was then tightly stoppered. The concentration of ethyl alcohol in serum was determined according to ALC pack used in the DuPont Automatic Clinical Analyzer (aca). The ethyl alcohol estimations were carried out in the Clinical Chemistry Laboratory of the Health Sciences Center.

2.3.2 Part II - Alcohol Treated and Control Group

Twelve pregnant animals were treated from gestational Day 6 through Day 12 at 0900 hours. Each animal received a single intraperitoneal injection each morning of 12.5% ($^{v}/v$) ethyl alcohol solution in physiological saline. The volume of solution injected was .015 ml/gram body weight.

The control group, also consisting of twelve pregnant animals, was treated in the same manner, except that they received physiological saline instead of the alcohol solution.

At 1400 hours on Day 12 of pregnancy, Embryonic Age Day 11.5, the animals were anaesthetized and the uterine horns were excised and immediately placed in Hank's Balanced Salt Solution. Moments later, the uterine horns were opened along the antimesometrial border. The embryos were removed and morphologically scored as described previously (Brown and Fabro, 1981). The embryos were evaluated and placed in 10% formalin for storage and photography. Blood samples were obtained from both groups for alcohol estimation (Table 8).

2.3.3 Part III - Caffeine Treated and Control Group

Twelve pregnant animals were treated on the morning of gestational Day 10, Embryonic Age Day 9.5. Each pregnant female received one intravenous injection of caffeine dissolved in physiological saline. The injected volume was 0.5 ml and the dosage was 15 mg/kg. The intravenous injection was performed through the tail vein of the animal.

Twelve pregnant animals formed the control group. The control group was treated in the same manner, except that they received the intravenous injection consisting of physiological saline instead of the caffeine solution.

At 1400 hours, on Day 12 of pregnancy, Embryonic Age Day 11.5, the animals were anaesthetized with ether. Following laparotomy, the uterine horns were removed and placed in Hank's Balanced Salt Solution. In order to reveal the conceptuses, the uterine horns were excised along its antimesometrial border. With the aid of a dissecting microscope, the embryos were scored according to the Morphological Scoring System (Brown and Fabro, 1981). Embryos were evaluated and stored in 10% formalin.

2.3.4 Part IV - Alcohol and Caffeine Treated and Control Group

Twelve pregnant animals comprised the experimental group. Each of these rats was subjected to a single intraperitoneal injection of the 12.5%(^{v}/v) alcohol solution at 0900 hours on gestational Days 6, 7, 8, 9, 10, 11 and 12. The injected volume was in accordance with the ratio of .015 ml solution/gram body weight. In addition to the alcohol injection, each of these pregnant females received on gestational Day 10 a single intravenous injection of the caffeine solution (0.5 ml, dosage: 25 mg/kg).

Twelve pregnant animals comprised the control group. The control group was treated in the same manner as the treatment group, except that they received physiological saline.

At 1400 hours, on Day 12 of pregnancy, Embryonic Age Day 11.5, the animals were anaesthetized with ether. Following laparotomy, the uterine horns were removed and placed in Hank's Balanced Salt Solution. The uterine horns were excised along its antimesometrial border in order to reveal the conceptuses. With the aid of a dissecting microscope, the embryos were evaluated and scored in accordance with the Morphological Scoring System (Brown and Fabro, 1981). Embryos were stored in 10% formalin.

Blood samples were obtained as previously described and sent to the Health Sciences Center for alcohol estimation (Table 8).

2.4 ISOLATION OF IMPLANTATION SITES

Following hysterotomy of the anesthetized animals, the uterine horns were removed and placed immediately in a petri dish containing Hank's Balanced Salt Solution. The uterine horns were then excised along its entire antimesometrial border. Once this excision was completed, the tissue on either side of the incision was grasped with forceps and reflected backwards. Reflection of the tissue ultimately brought the pear-shaped implantation sites to the surface. In order to facilitate the identification of each individual implantation site, pressure was carefully applied to the area that lies between adjacent pear-shaped swellings in order to accentuate the borders of the individual implantation sites. Once isolated, the individual implantation sites were excised and stored in Hank's Balanced Salt Solution for subsequent studies. (Figures 1 and 2)

2.5 DIVESTMENT OF MEMBRANES (EMBRYO RETRIEVAL)

Each pear-shaped decidual swelling was transferred to a petri dish containing Hank's Balanced Salt Solution. The petri dish was placed on the stage of a dissecting microscope (Wild of Canada) equipped with a fibre optic light source. The entire procedure of the removal of the embryo from its surrounding membranes is performed under the dissecting microscope.

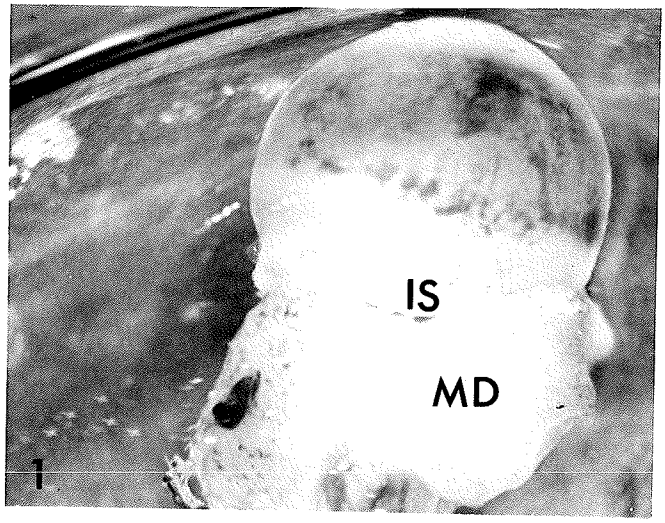
Dealing with each decidual swelling individually, the investigator must patiently and precisely debride the membranes and tissue surrounding the embryo. One begins by carefully grasping the decidual swelling at its broad end or base with the aid of very fine Type 5 Dumont forceps. After acquiring a secure grasp with both

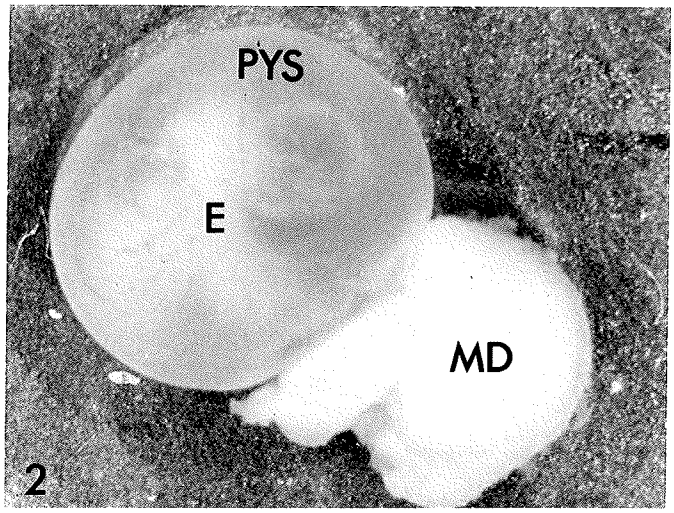
Figure: 1 Conceptus explanted from the uterus, showing implantation site (IS) and maternal decidua (MD), (Embryonic Age Day 11.5).

(x 32)

Figure: 2 Parietal yolk sac (PYS) and maternal decidua (MD). Embryo is discernible through the yolk sac. Note the blood vessels of the visceral yolk sac. (Embryonic Age Day 11.5).

(x 32)


IS
MD
1


PYS
E
MD
2

forceps, one begins to tear away the tissue. This tearing procedure is accomplished by virtue of moving the forceps away from one another while still maintaining hold of the tissue. One observes through the dissecting microscope a groove on the decidual swelling that ultimately represents the separation between maternal tissue and embryonic tissue. This groove aids in the further removal of tissue in that once the forceps are aligned with the groove and are allowed to cut through the groove, the maternal tissue may then be discarded. Hence one is now left with strictly embryonic tissue. Once this separation has been achieved, all that remains in terms of the embryonic half is an embryo enveloped by membranes and covered by a cotton-like decidua. After the debridement of the decidua, adherent endoderm and trophoblastic cells, the yolk sac circulatory system becomes very apparent. At this point it is evaluated and classified according to the Morphological Scoring System (Brown and Fabro, 1981). Also at this stage a measurement of the yolk sac diameter is made with the aid of a micrometer located in the the eyepiece of the dissecting microscope. Yolk sac diameter is measured at the mid-line, horizontal to the placenta (Brown and Fabro, 1981). (Figures 2, 3 and 4)

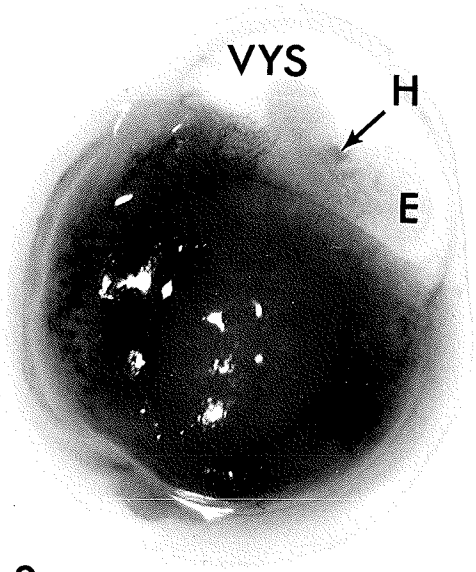
The allantois may be evaluated next. In order to evaluate the allantois, the yolk sac is punctured at the furthest point away from the entry and exit of the separate aortic origins of umbilical and vitelline vessels. After this procedure, the yolk sac is removed and the embryo is visualized. The embryo is encased in a glistening transparent body stocking-like covering called the amnion. By careful manipulation of the forceps, one is able to remove the amniotic covering that ultimately covers the embryo.

Figure: 3 Parietal yolk sac and maternal decidua removed, showing embryo in visceral yolk sac (VYS), primitive circulatory system and developing heart (H) (Embryonic Age Day 11.5).

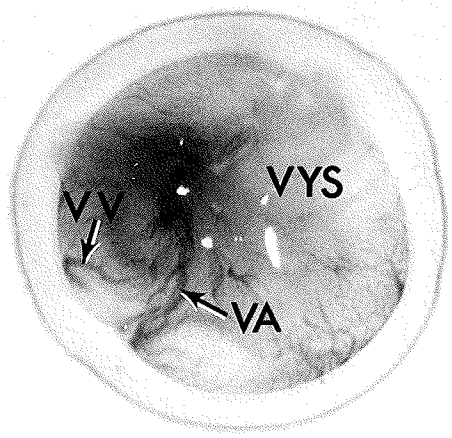
(x 42)

Figure: 4 Note the dense vascularity of the visceral yolk sac (VYS), the vitelline artery (VA) and the vitelline vein (VV).

(x 42)



3



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2.6 EXAMINATION OF THE EMBRYO

For embryos to be included in the evaluation, the following criteria should be satisfied: 1.) a detectable heartbeat; 2.) an intact embryo (no damage having occurred to the embryo); 3.) amniotic covering removed; 4.) ability to visualize individual somite segments.

2.7 EVALUATION OF EMBRYOS

2.7.1 Measurement of Embryo

After removal of the amniotic covering the embryo is aligned in the petri dish and the following measurements are made by use of a micrometer situated in the eyepiece of the dissecting microscope: 1.) crown-rump length; 2.) head length. The crown-rump length is measured according to the maximum length of the embryo in its natural position, whereas the head length is measured as the maximum length from frontal tip of prosencephelon to most dorsal portion of mesencephalon (Brown and Fabro, 1981). After these measurements have been taken and recorded, the embryo is then placed upon the stage of a Zeiss Inverted Microscope and morphologically scored. (Figures 5 - 10)

2.7.2 Morphological Scoring of Embryo

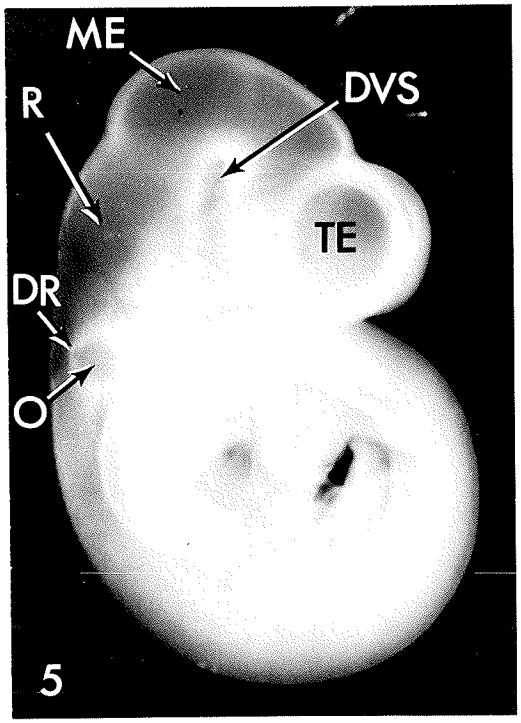
A Morphological Scoring System, developed by Brown and Fabro (1981) was used to evaluate the embryos. The scoring sheet consists of 17 parameters that are defined and separated into specific developmental events. For each developmental event a score of a value between 0 to 5 exists. The morphological score of an individual embryo was calculated using the following technique: each feature that is mentioned on the score sheet was evaluated for individual embryos.

Figure: 5 Profile of normal embryo (Embryonic Age Day 11.5). Prominent features seen: telencephalic evaginations (TE), mesencephalon (ME), rhombencephalon (R), developing ventricular system (DVS), otocyst (O) and its dorsal recess (DR).

(x 68.4)

Figure: 6 Profile of normal embryo (Embryonic Age Day 11.5). Prominent features seen: optic primordium (OP), somites (S), cardiac primordia (CP) and forelimb bud (F).

(x 68.4)


ME
DVS
R
TE
DR
O
5

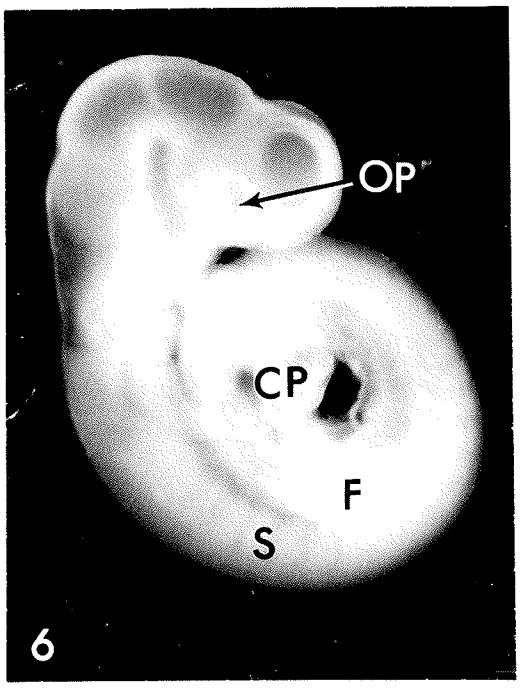
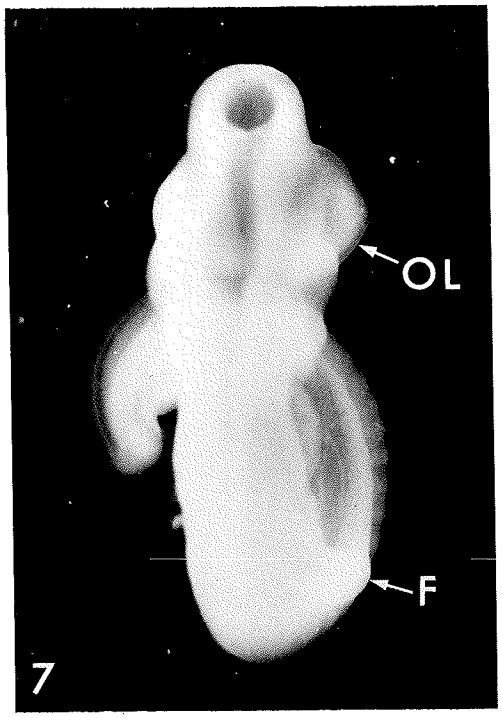

OP
CP
F
S
6

Figure: 7 Dorsal view of normal embryo (Embryonic Age Day 11.5). Prominent features include: tube-like appearance of central nervous system, olfactory plate (OL) and forelimb bud (F).

(x 68.4)

Figure: 8 Frontal view of normal embryo (Embryonic Age Day 11.5). Prominent embryonic features include: spiral torsion orientation, cardiac primordium (CP), forelimb bud (F) and first branchial bar (B1).

(x 68.4)


OL
F
7

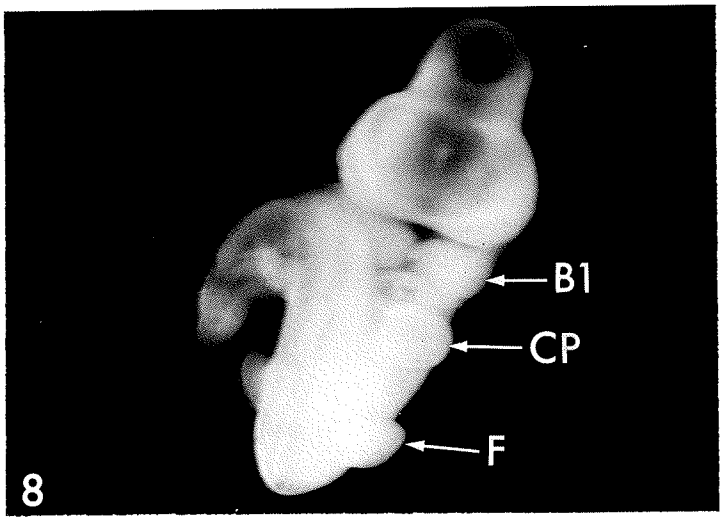
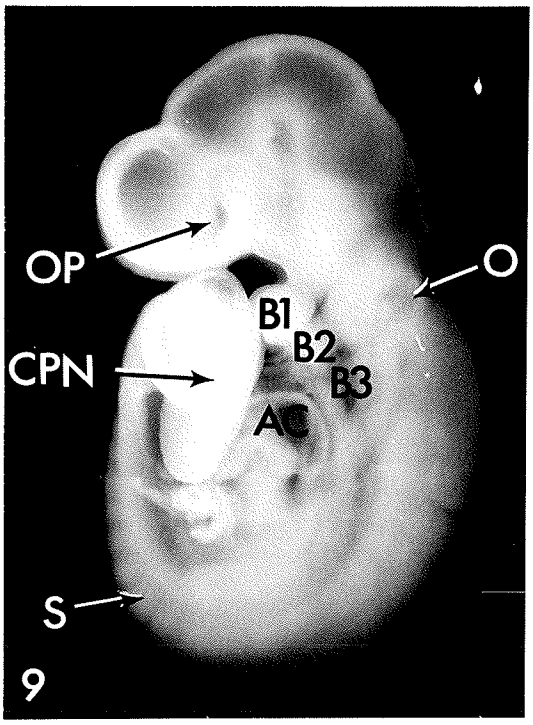

B1
CP
F
8

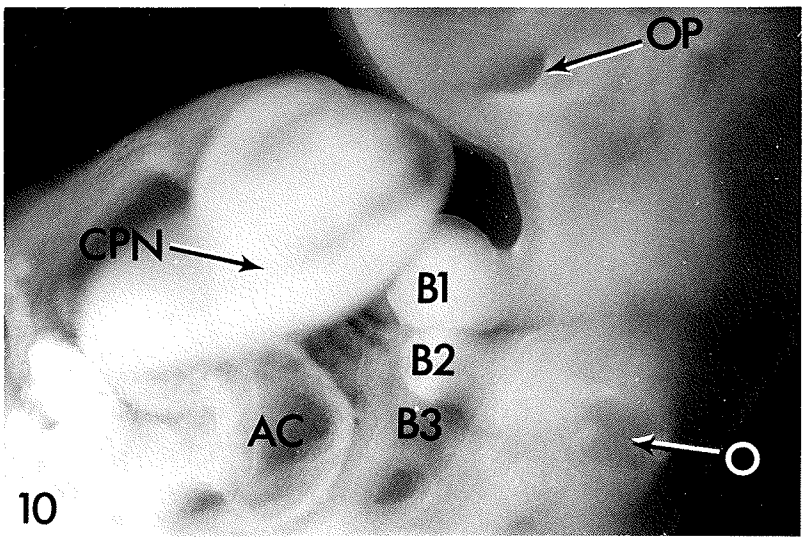
Figure: 9 Control embryo (Embryonic Age Day 11.5). Prominent features: optic primordium (OP), otocyst (O), branchial bars 1, 2 and 3 (B1, B2, B3), somites (S), atrium commune of heart primordia (AC) and closed posterior neuropore (CPN).

(x 75.6)

Figure: 10 Control embryo (Embryonic Age Day 11.5), showing optic primordium (OP), otocyst (O), branchial bars 1, 2 and 3 (B1, B2, B3), closed posterior neuropore (CPN) and atrium commune (AC).

(x 192)


OP
O
B1
B2
B3
CPN
AC
S
9


OP
CPN
B1
B2
AC
B3
O
10

The feature was examined, scored and recorded on a scoring sheet.

In some cases the stage of development was observed to be between two defined stages, and hence received a half mark value. Ultimately, all scores for the 17 parameters were added together and formed the overall morphological score for that particular embryo.

2.7.3 Fixation and Preservation of Embryo

After the evaluation and morphological scoring of the embryo, the embryos were fixed in 10% formalin.

2.8 STATISTICAL ANALYSIS

Data corresponding to the parameters listed in the Morphological Scoring System (Brown and Fabro, 1981) was collected for 1,051 embryos. These embryos represented the offspring of the seven respective groups (1 untreated control, 3 experimental, 3 control) of this study.

A designed computer program (Rollwagen, 1973) was used to perform a one-way analysis of variance and Multiple Range Test. Inherent in program (ST41) are limitations and restrictions. One restriction that was crucial to the analysis of the above data was that the maximum number per group was set at 100, and hence the researcher had to select ultimately 700 embryos from the 1,051.

Seven hundred embryos were selected with the aid of a random table of numbers. The researcher blindly selected 100 numbers from this table per group. Those numbers selected represented the embroys, of which their respective morphological scores would be utilized. This selection procedure occurred seven times, each time corresponding to another group. Ultimately, 700 embryos were chosen.

Files were created for each individual parameter and a mean, standard deviation and standard deviation of the mean (standard error) were obtained for each group. Also the number of observations, mean, standard deviation and standard deviation of the mean (standard error) were attained as a composite of the seven groups. Finally, an analysis of variance and multiple comparison were performed on the data. Duncan's test of significant studentized ranges for 5% and 1% level new multiple-range test was used in describing the data.

3. RESULTS

3. RESULTS

3.1 ASSESSMENT OF DATA

3.1.1 Statistical Analysis of Developmental Characteristics

The following twenty-one parameters listed in the Morphological Scoring System of Brown and Fabro (1981) were studied: yolk sac circulatory system, allantois, flexion, heart, caudal neural tube, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, branchial bars, maxillary process, mandibular process, forelimb, hindlimb, somites, crown-rump length, head length, yolk sac diameter and absolute somite number. The results were statistically analyzed using Duncan's New Multiple Range Test.

In order to determine whether the incidence of resorption sites and abnormal offspring between treatment and control groups was significantly different, a chi-square (X^2) test was performed (Table 2).

3.2 ALCOHOL TREATED

3.2.1 Embryonic Growth

In the alcohol-treated group of animals, embryonic growth was affected. The embryos displayed a significant decrease in yolk sac diameter ($\rho<0.01$) crown-rump length ($\rho<0.01$) and head length ($\rho<0.01$), when compared to their corresponding control group and the untreated control group (Table 3). Two embryos were grossly underdeveloped for Embryonic Age Day 11.5, lagging approximately twenty-four hours behind, and hence were of a developmental stage characteristic of Embryonic Age Day 10.5. (Figure 11)

In terms of flexion, one embryo failed to rotate. Another embryo was noticeably malrotated and did not achieve a dorsally convex orientation, but rather remained concave and twisted, giving it a characteristic "sea-horse" type of appearance.

3.2.2 Central Nervous System

Six embryos displayed alterations in normal central nervous system development. Failure of the anterior neuropore to close by Embryonic Age Day 11.5 was apparent in three embryos, while in another embryo both the anterior and posterior neuropores failed to close. In all of these six embryos, head formation was affected, being smaller in size and distorted in orientation. In two of the six embryos in which the development of the central nervous system was affected, the head region was so severely malformed that measurement of head length was difficult to achieve. This difficulty in the measurement of head length arose due to the fact that the tip of the prosencephalon was indiscernible. No developmental disturbances were noted in the otic, optic or olfactory systems. (Figures 14 and 15)

3.2.3 Cardiovascular System

With regards to the cardiovascular system, four embryos displayed a relative delay in the development of the cardiac primordia. Instead of the heart tube differentiating into its various component parts (bulbus cordis, atrium commune and ventriculus communis), it persisted as a beating "S" shaped cardiac tube. Three of these embryos that displayed a delay in cardiac development also revealed a distorted head shape. One embryo with both central nervous system and cardiac

anomalies was also grossly malrotated. In all embryos of this group that were scored, no alterations occurred in either the yolk sac circulatory system or the allantois. All embryos showed a full yolk sac plexus of vessels and separate origins of the umbilical and vitelline vessels.

3.2.4 Musculoskeletal System

Forelimb development was affected in the alcohol-treated group. Of the ten maldeveloped embryos, five displayed a delay in forelimb development. One of these embryos showed no sign of forelimb development, while the four others displayed only the distinct evaginations of Wolffian crest at levels of somite 9 - 13 (characteristic of Embryonic Age Day 10 3/4). No evidence of hindlimb development was seen which is in accordance with developmental horizons at this stage. Hindlimb development begins at Embryonic Age Day 11 3/4 (Christie, 1964).

In terms of somite number, no significant differences were seen between the alcohol-treated group and the corresponding control group. In Brown and Fabro's (1981) Morphological Scoring System, somite numbers are placed in ranges. The range of somites (28 - 34) was recorded, and hence according to their scoring system, received a morphological score equal to four.

3.2.5 Craniofacial Region

In both the alcohol-treated and control groups, the embryos displayed no signs of maxillary process and mandibular process development. This is to be expected because these two facial primordia

do not begin to form until Embryonic Age Day 12. Hence, the observations made were in accordance with the documented stage characterization of Christie (1964) and Edwards (1968). (Figures 11 and 12)

With regards to branchial bar development, it was found that as a group the alcohol-treated embryos consistently showed only two readily recognizable branchial bars (Branchial bars I and II). This observation was significantly different ($\rho<0.01$) from the corresponding control group (Table 7). In most cases control group embryos displayed three discernible branchial bars (Branchial bars I, II and III). (Figures 9 and 10)

3.3 CAFFEINE TREATED

3.3.1 Embryonic Growth

In the caffeine-treated group of animals overall embryonic growth was impaired. The embryos showed significant decreases ($\rho<0.01$) in yolk sac diameter, crown-rump length and head length when compared to the corresponding control and the untreated control groups (Table 3). One embryo was grossly underdeveloped for Embryonic Age Day 11.5. This embryo appeared as a saccule-like configuration within the visceral yolk sac. According to normal developmental charts, this embryo appeared to be at Embryonic Age Day 9.5, and hence was morphologically scored as such. (Figure 12)

Malrotation was evident in three embryos. It appeared as if these embryos were at the "turning stage" (morphological score = 2), a stage between being originally convex ventrally and definitively convex dorsally. This malrotation was quite obvious and easily recognizable.

The number of somites in one embryo was substantially decreased. Instead of having a range of 28-34 somites, the embryo displayed only 24 somites. This reduction in the number of somites was responsible for a lower morphological score in this treatment group.

3.3.2 Central Nervous System

Several of the caffeine-treated embryos were grossly malformed. Two embryos had completely unfused neural tubes, while four others displayed failure of closure of the anterior and posterior neuropores, respectively.

Similar to the alcohol-treated group, the head shape was abnormal. Three caffeine-treated embryos displayed distortions in head shape. The outline of the head of these embryos appeared sac-like and triangular, giving these embryos a disproportionate look and significantly affecting the head length values ($\rho<0.01$). (Table 3 and Figures 12 and 16)

3.3.3 Cardiovascular System

Cardiovascular abnormalities were not detected in any of the caffeine-treated embryos. No significant differences were found between the caffeine-treated and control embryos with respect to yolk sac circulatory system, allantois and heart development.

3.3.4 Musculoskeletal System

Forelimb buds did not develop in one of the caffeine-treated embryos. The anterior neuropore of this embryo also failed to close.

The total number of somites was reduced in one embryo. Instead of displaying a number of somites that fell within the range for Embryonic Age Day 11.5 (28 - 34 somites), this embryo possessed 24 somites, and hence was morphologically scored lower than the others.

3.3.5 Craniofacial Region

In both the caffeine-treated and corresponding control group, the embryos revealed no sign of maxillary or mandibular processes development. This observation is in agreement with the developmental characteristics described by Christie (1964) and Edwards (1968).

No significant difference was seen between the caffeine-treated group and its corresponding control group with respect to branchial bar development. (Figure 12)

3.4 ALCOHOL AND CAFFEINE TREATED

3.4.1 Embryonic Growth

Embryonic growth was significantly affected ($\rho<0.01$) in those animals treated with both alcohol and caffeine compared to the corresponding control group (Table 3). The measurements of yolk sac diameter, crown-rump length and head length in the alcohol and caffeine-treated group were reduced further than if the alcohol or caffeine had been administered alone. These three developmental parameters were found to be significantly different ($\rho<0.01$) when compared to all the control groups. Eight embryos were growth retarded and their development did not correspond to the gestational age. Of these embryos, three were estimated to be at a stage of development equivalent to Embryonic Age Day 9.5, while the other five displayed developmental features

characteristic of a stage equivalent to Embryonic Age Day 10.5.

Flexion of the embryo was affected. Six embryos were grossly malrotated, as well as being malformed. Four of the malrotated embryos showed central nervous system developmental defects coupled with a significant reduction in the absolute number of somites. As a group, the alcohol and caffeine-treated embryos were significantly different ($\rho<0.01$) as compared to the control groups with respect to the parameter of absolute number of somites (Table 3 and Figure 13).

3.4.2 Central Nervous System

Embryos treated with alcohol and caffeine revealed abnormalities in the development of the central nervous system. A significant difference ($\rho<0.01$) was seen in the development of the caudal neural tube, hindbrain, midbrain and forebrain between the treated and control groups (Table 4). Each of these developmental disturbances varied in severity. Three embryos displayed open anterior neuropores at Embryonic Age Day 11.5, while two others displayed an open posterior neuropore as its sole developmental defect.

With respect to neural tube closure, three embryos presented with completely open neural tubes, while one had a fused neural tube bounded by both the open anterior and posterior neuropores. Of the embryos with central nervous system anomalies, five also displayed severe distortion of the head shape. The extent of head shape distortion was greater in this group than in embryos exposed to either alcohol or caffeine. Because of the distorted head shapes, head length measurements were reduced. In those embryos with distortions in head shape, midbrain

formation was greatly affected. The mesencephalic folds which were swollen in appearance and in close opposition to each other had not yet fused, and hence received a morphological score of 2.5, because of this intermediate state.

3.4.3 Cardiovascular System

Specific defects were induced in the embryonic heart following treatment with alcohol and caffeine. Cardiac development was significantly affected ($\rho<0.01$) when compared to the corresponding control group (Table 5). Two types of defects were observed in the seven embryos showing developmental disturbances of the cardiovascular system. The yolk sac circulatory system was underdeveloped in three embryos, while four others demonstrated primitive heart development for a stage equivalent to Embryonic Age Day 11.5. The three embryos with disturbances in the development of the yolk sac circulatory system had a rudimentary blood circulation. Because of its stage of development, it was given a morphological score of 1.5. It showed abundant blood islands and anastomoses with only a beginning of blood vessel development.

Of the remaining four embryos with disturbances in heart development, two had a beating "S" shaped cardiac tube, while the others displayed cardiac tubes that had not yet differentiated into the component parts of the heart (bulbis cordis, atrium commune and ventriculus communis), but rather remained at the convoluted cardiac tube stage. Three of the embryos that showed cardiac maldevelopment also had central nervous system anomalies with head shape affected. (Figure 13)

3.4.4 Musculoskeletal System

Forelimb development was affected in the alcohol and caffeine treatment group. Significant differences ($\rho<0.01$) were found between the treatment group and its corresponding controls (Table 6). Numerous embryos displayed poorly developed forelimbs. Notably in two embryos forelimb buds did not appear, and hence were assigned a morphological score of 0. Six other embryos showed only a distinct evagination of Wolffian crest at somite levels 9 - 13, and consequently were assigned a morphological score equal to 1. (Figure 13)

There was no evidence of hindlimb development at Embryonic Age Day 11.5, which is in accordance with the stage characterization of Christie (1964) and Edwards (1968).

In terms of absolute somite number, there was a significant difference ($\rho<0.01$) between the treatment group and the corresponding control groups (Table 3). Absolute number of somites were reduced, but still remained within the range that is associated with a morphological score of 3. Most of these embryos showing a decrease in the absolute number of somites also displayed central nervous system anomalies.

3.4.5 Craniofacial Region

In both the alcohol and caffeine-treated group and in the corresponding control group, neither the maxillary nor mandibular process was evident. This observation is in agreement with the developmental characteristics described by Christie (1964) and Edwards (1968) for this stage. (Figure 17)

With respect to branchial bar development, a significant difference ($\rho<0.01$) was found between the alcohol and caffeine-treated group and its corresponding control group (Table 7). In some of these embryos only branchial bar I was distinguishable and some evidence of branchial bar II was seen. In these cases a morphological score of 1.5 was assigned. Normally for this stage of development branchial bars I and II are readily identifiable, while branchial bar III is also frequently evident.

Figure: 11 Embryonic growth differences between alcohol treated embryo (left) and an embryo from the corresponding control group (right). Observe marked reduction in crown-rump length and head length of alcohol treated embryo. (Embryonic Age Day 11.5).

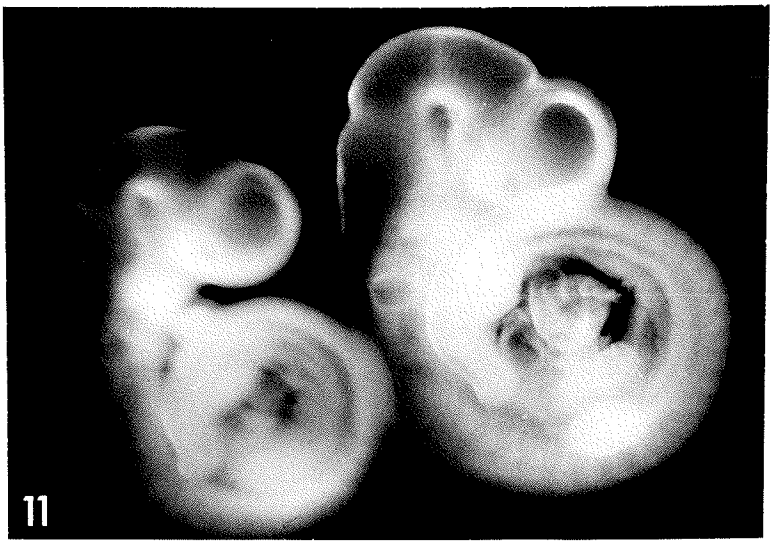
(x 70)

Figure: 12 Embryonic growth differences between caffeine treated embryo (left) and an embryo from the corresponding control group (right). Observe the reduction in crown-rump length, head length and flattened head shape of the caffeine treated embryo (Embryonic Age Day 11.5).

(x 78)

Figure: 13 Embryonic growth differences between alcohol and caffeine treated embryo (right) and an embryo from a corresponding control group (left). Observe the differences in crown-rump length, head length as well as head shape differentiation in the alcohol and caffeine treated embryo (Embryonic Age Day 11.5).

(x 72)



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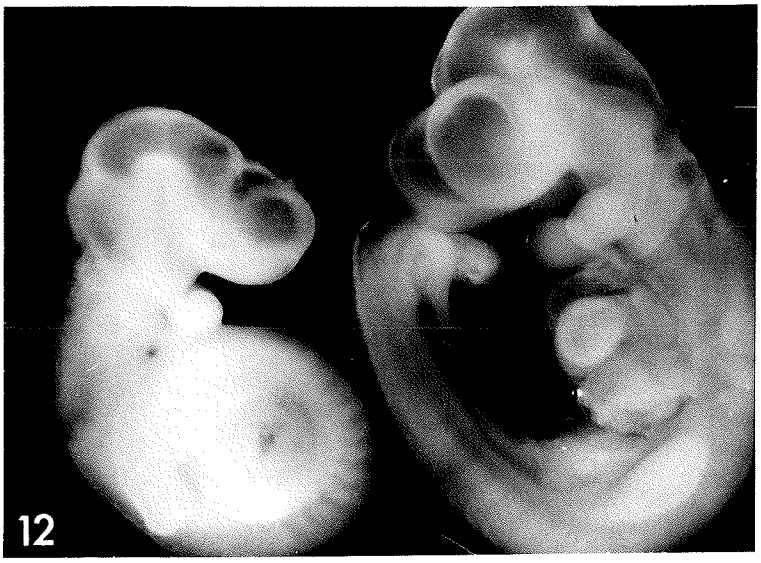

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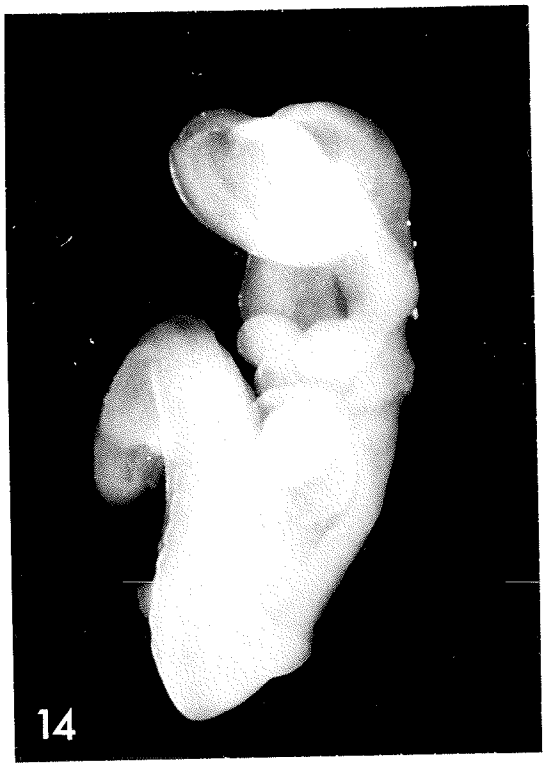
Figure: 14 Alcohol treated embryo (Embryonic Age Day 11.5), showing abnormal head shape.

(x 75)

Figure: 15 Failure of closure of anterior neuropore in alcohol treated embryo (Embryonic Age Day 11.5).

(x 100)



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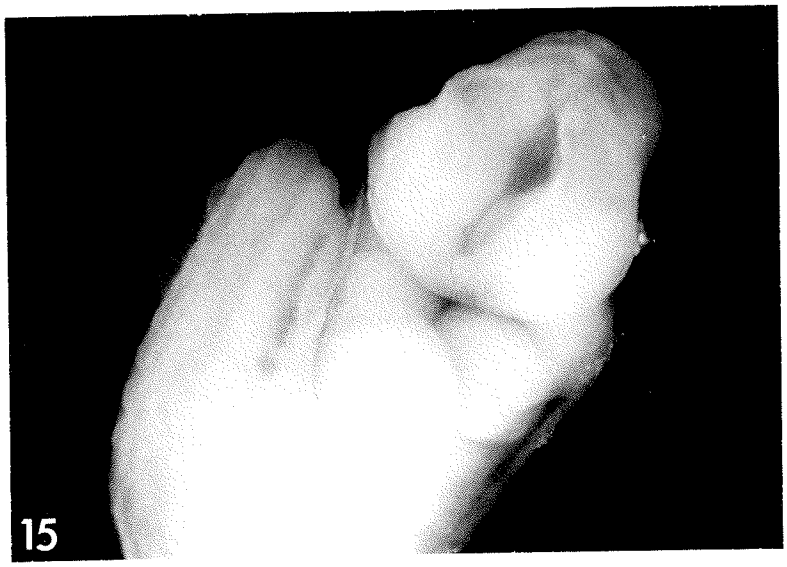

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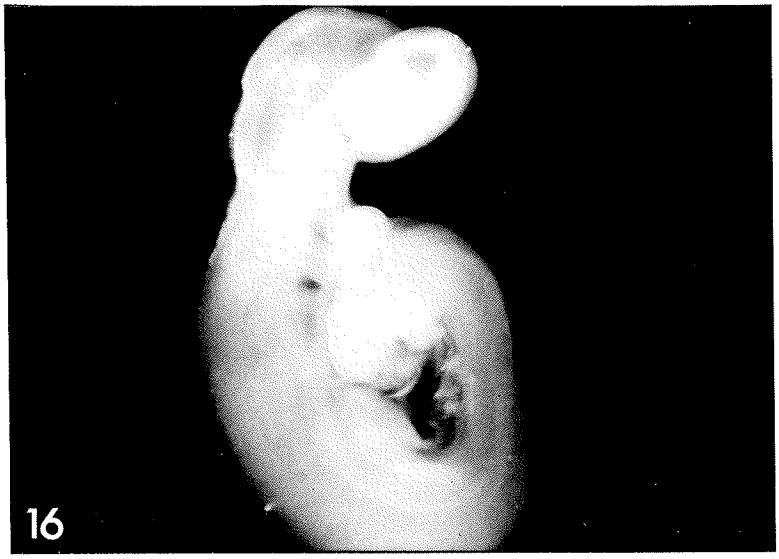
Figure: 16 Caffeine treated embryo (Embryonic Age Day 11.5) with abnormal head shape.

(x 68.4)

Figure: 17 Alcohol and caffeine treated embryo (left) compared to a corresponding control embryo (right). Note the peculiar head shape of the alcohol and caffeine treated embryo (Embryonic Age Day 11.5).

(x 80)



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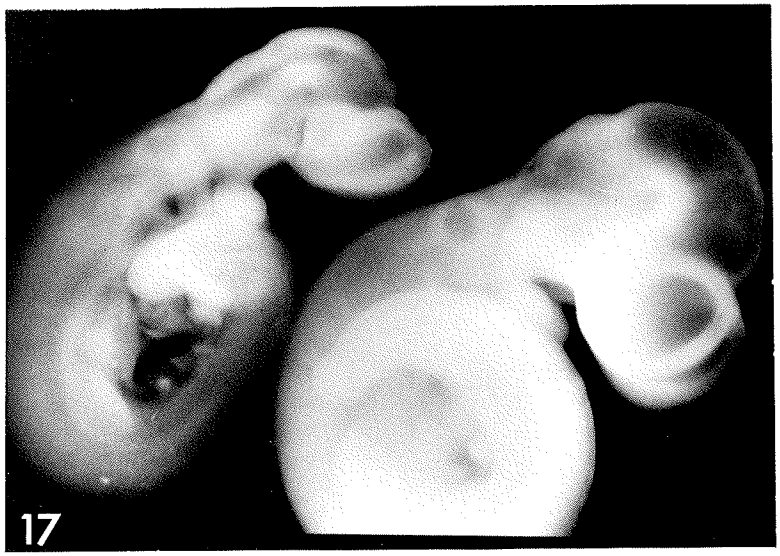

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Table 2: Rat Embryonic Development Following Maternal Treatment with Alcohol and Caffeine *

Treatment Group	No. of Pregnant Animals	No. of Embryos**	Resorptions (%)***	Abnormal Embryos (%)
Untreated Control	22	226	6 (2.6)	1 (0.44) (malrotation)
Alcohol Treated	12	150	15 (9.1) ($\rho<0.05$)	10 (6.7) ($\rho<0.01$)
Control to Alcohol Treated	12	121	2 (1.6)	0
Caffeine Treated	12	143	23 (13.9) ($\rho<0.01$)	14 (9.8) ($\rho<0.01$)
Control to Caffeine Treated	12	130	4 (3.0)	0
Alcohol and Caffeine Treated	12	137	26 (16.0)**** ($\rho<0.01$)	21 (15.3) ($\rho<0.01$)
Control to Alcohol and Caffeine	12	144	3 (2.0)	0

* Data subjected to Chi-square (X^2) test, ρ values reflect significant differences between treatment and corresponding control groups.

** All embryos were morphologically scored, but for statistical analysis, 100 embryos were randomly selected from each group.

*** Resorption rates in all groups are expressed as a percentage of total implantation sites observed on gestational Day 12 (Embryonic Age Day 11.5).

**** One animal confirmed pregnant - resorbed all fetuses.

Table 3: Rat Embryonic Growth Following Maternal Treatment with Alcohol and Caffeine *

Treatment Group	Yolk Sac Diameter(mm) $\bar{X}\pm$S.D.M.	Crown-Rump Length(mm) $\bar{X}\pm$S.D.M.	Head Length (mm) $\bar{X}\pm$S.D.M.	Flexion (0-3)*** $\bar{X}\pm$S.D.M.	Somite Number (0-41)*** $\bar{X}\pm$S.D.M.
Untreated Control	4.59±.007	3.57±.007	1.90±.008	2.98±.020	28.2±.071
Alcohol Treated	4.35±.018**	3.43±.011**	1.71±.010**	2.84±.033**	27.9±.080**
Control to Alcohol Treated	4.58±.009	3.54±.008	1.81±.080	3.00±0	28.7±.073
Caffeine Treated	4.28±.018**	3.38±.011**	1.73±.008**	2.88±.027**	28.0±.096**
Control to Caffeine Treated	4.57±.006	3.55±.006	1.85±.005	3.00±0	28.8±.067
Alcohol and Caffeine Treated	4.09±.022**	3.17±.018**	1.60±.014**	2.78±.040**	27.5±.099**
Control to Alcohol and Caffeine Treated	4.55±.008	3.54±.008	1.82±.008	2.98±.010	28.6±.084

* Statistical differences between treated and control groups analyzed by Duncan's New Multiple Range Test
** $\rho<0.01$
*** See Table 1

Table 4: Rat Embryonic Central Nervous System Development Following Maternal Treatment with Alcohol and Caffeine *

Treatment Group	CNT (0-4) $\bar{X}$±S.D.M.	HDB (0-4) $\bar{X}$±S.D.M.	MDB (0-4) $\bar{X}$±S.D.M.	FRB (0-4) $\bar{X}$±S.D.M.	OTIC SYS(0-5) $\bar{X}$±S.D.M.	OPT SYS(0-5) $\bar{X}$±S.D.M.	OLF SYS(0-4) $\bar{X}$±S.D.M.
Untreated Control	3.99±.021	3.00±0	3.00±0	3.00±0	3.48±.040	2.99±.009	1.00±0
Alcohol Treated	3.83±.029**	3.00±0	3.00±.005	2.98±.010	3.42±.038	2.99±.009	1.00±0
Control to Alcohol Treated	4.00±0	3.00±0	3.00±0	3.00±0	3.47±.047	3.00±0	1.00±0
Caffeine Treated	3.91±.021**	2.99±.011	3.00±0	2.99±.007	3.47±.036	3.00±0	1.00±0
Control to Caffeine Treated	3.99±.005	3.00±0	3.00±0	3.00±.005	3.45±.044	3.00±.005	1.00±0
Alcohol and Caffeine Treated	3.72±.034**	2.89±.030**	2.91±.026**	2.74±.031**	3.44±.040	2.98±.023	0.98±.022
Control to Alcohol and Caffeine Treated	3.99±.007	3.00±0	3.00±.005	2.98±.011	3.47±.043	2.99±.007	1.00±0

* Statistical differences between treated and control groups analyzed by Duncan's New Multiple Range Test.
** $\rho<0.01$

Legend
CNT = Caudal Neural Tube
HDB = Hindbrain
MDB = Midbrain
FRB = Forebrain
OTIC SYS = Otic System
OPT SYS = Optic System
OLF SYS = Olfactory System

Table 5: Rat Embryonic Cardiovascular System Development Following Maternal Treatment with Alcohol and Caffeine *

Treatment Group	Yolk Sac Circulatory System (0-4)*** $\bar{X}$ ± S.D.M.	Allantois (0-3)*** $\bar{X}$ ± S.D.M.	Heart (0-4)*** $\bar{X}$ ± S.D.M.
Untreated Control	2.99±.009	2.99±.009	3.00±.005
Alcohol Treated	3.00±0	3.00±0	2.90±.027**
Control to Alcohol Treated	3.00±0	3.00±0	2.99±.007
Caffeine Treated	2.99±.007	2.99±.007	2.99±.011
Control to Caffeine Treated	3.00±0	3.00±0	3.00±0
Alcohol and Caffeine Treated	2.99±.009	2.99±.009	2.93±.024**
Control to Alcohol and Caffeine Treated	3.00±0	3.00±0	2.99±.007

* Statistical differences between treated and control groups analyzed by Duncan's New Multiple Range Test

** $\rho<0.01$

*** See Table 1

Table 6: Rat Embryonic Musculoskeletal System Development Following Maternal Treatment with Alcohol and Caffeine *

Treatment Group	Forelimb (0-4) *** $\bar{X}$ ± S.D.M.	Hindlimb (0-3) *** $\bar{X}$ ± S.D.M.	Somites (0-5) *** $\bar{X}$ ± S.D.M.
Untreated Control	1.93±.029	0 ± 0	3.84±.039
Alcohol Treated	1.74±.033**	0 ± 0	3.79±.043
Control to Alcohol Treated	1.94±.041	0 ± 0	3.88±.033
Caffeine Treated	1.86±.023	0 ± 0	3.81±.039
Control to Caffeine Treated	1.92±.019	0 ± 0	3.86±.035
Alcohol and Caffeine Treated	1.70±.034**	0 ± 0	3.48±.050**
Control to Alcohol and Caffeine Treated	1.90±.020	0 ± 0	3.87±.034

* Statistical differences between treated and control groups analyzed by Duncan's New Multiple Range Test

** $\rho<0.01$

*** See Table 1

Table 7: Rat Embryonic Craniofacial Region Development Following Maternal Treatment with Alcohol and Caffeine*

Treatment Group	Branchial Bars (0-3)*** $\bar{X}$ ± S.D.M.	Maxillary Process (0-2)*** $\bar{X}$ ± S.D.M.	Mandibular Process (0-1)*** $\bar{X}$ ± S.D.M.
Untreated Control	2.57±.040	0 ± 0	0 ± 0
Alcohol Treated	2.17±.036**	0 ± 0	0 ± 0
Control to Alcohol Treated	2.56±.041	0 ± 0	0 ± 0
Caffeine Treated	2.07±.024**	0 ± 0	0 ± 0
Control to Caffeine Treated	2.59±.027	0 ± 0	0 ± 0
Alcohol and Caffeine Treated	1.98±.020**	0 ± 0	0 ± 0
Control to Alcohol and Caffeine Treated	2.55±.036	0 ± 0	0 ± 0

* Statistical differences between treated and control groups analyzed by Duncan's New Multiple Range Test

** $\rho<0.01$

*** See Table 1

Table 8: Serum Ethanol Levels in Pregnant Rats

Treatment Group	Serum Ethanol Concentration (mmol/L) $\bar{X}$ ± S.D.M.
Alcohol Treated (N=4)*	12.25 ± 1.41
Control to Alcohol Treated (N=5)*	0 ± 0
Alcohol and Caffeine Treated (N=8)*	10.36 ± 2.84
Control to Alcohol and Caffeine Treated (N=9)*	0 ± 0

* Denotes the number of pregnant rats tested for serum ethanol.

4. DISCUSSION

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4.1 GENERAL CONCEPTS

It has been known for years that the response of the developing embryo to various chemical agents and their metabolic by-products occurs via complex mechanisms and is influenced by several related factors. These factors are interdependent and include: 1.) the species being studied; 2.) the stage(s) of development during which effective exposure to the chemical agent occurs; 3.) the route of exposure; and 4.) the degree of reactivity of sensitive cell populations within the maternal embryo-placental unit (Skalko, 1985). In addition to these factors, the influence of genotypes, dietary factors, maternal metabolism and placental transport (Wilson, 1974) play an important role in the development of the embryo.

In today's highly industrialized society, it is difficult to attribute the deleterious effects on the development of the conceptus to any one agent, because of the wide variety of chemical agents that the pregnant mother is exposed to.

Hence, it is quite rare that the maldevelopment of a fetus may be attributed to the effects of any one particular substance as exposure in humans to various teratogenic agents is rarely an isolated event, (Skalko, 1985). Pregnant mothers are constantly exposed to a number of embryotoxic agents in the form of pharmaceutical formulations, over-the-counter drugs, and quite frequently to a number of socially abused drugs. Skalko and Kwasigroch (1983) found that exposure of pregnant mothers to these various agents is usually the rule than the exception. Alcohol and caffeine are two common examples of pharmacologically active

substances that pregnant mothers are often exposed to prior to and during their course of pregnancy.

Today, in the research literature, acceptable animal models have been found describing Fetal Alcohol Syndrome (Sandor and Elias, 1968; Randall and Taylor, 1969; Streissguth et al., 1980; Sulik et al., 1981). Also, various experiments on a number of animal species have been performed, with a wide myriad of results looking into the effects of caffeine on the developing fetus (Nishimura and Nakai, 1960; Fujii et al., 1969; Fujii and Nishimura, 1972; Lee et al., 1982; Gilani et al., 1983; Scott, 1983). Although all these studies have contributed immensely to our knowledge of teratology, they can only be applied in isolated cases on account of varying exposure time and the species of animal used. Studies involving the combination of both alcohol and caffeine have been few in number. It is this potentially deleterious combination of alcohol and caffeine during pregnancy that has caused great concern.

4.2 ALCOHOL

The most common features in children born with the Fetal Alcohol Syndrome are growth deficiency, microcephaly and developmental delay (Jones et al., 1973; Clarren and Smith, 1978). It is estimated that approximately one-third to one-half of the infants born to alcoholic mothers exhibit characteristics of the Fetal Alcohol Syndrome to some degree (Hanson et al., 1976; Corrigan, 1976; Smith et al., 1976). Also, it has been proposed that varying incidence rates and the expression of characteristics are determined by the amount and length

of alcohol exposure in utero, (Mulvihill et al., 1976; Rosett and Weiner, 1980; Chernoff, 1980).

In order to assess the in utero effects of ethanol and its metabolic by-products, animal models were developed. The rat has served as a useful experimental model with many of the characteristics of the Fetal Alcohol Syndrome being successfully induced. Prenatal growth retardation (Abel and Greizerstein, 1979; Tze and Lee, 1975; Henderson and Schenker, 1977), impaired protein synthesis in fetal tissues (Rawat, 1975; Wunderlich et al., 1979), and anomalies of the central nervous system, cardiovascular system and skeleton (O'Shea and Kaufman, 1979) have been observed in the offspring of alcohol treated mothers. Although many of these characteristics have been reported, the majority of animal studies have not revealed evidence of any gross teratogenicity associated with in utero alcohol exposure.

There has been much difficulty in interpreting the research literature pertaining to ethanol's embryotoxicity, particularly in regard to its direct effects upon the developing embryo. In many experiments problems arise as to the dosage level that was administered to the maternal rat. It has been established that the lethal blood alcohol level varied with the species. According to Maling (1970), blood alcohol levels approximating 800 mg/100 ml is lethal in rats. Hence, it is important when designing any toxicological study to take into account the dosage and blood level. Throughout the literature, there appear studies in which dosages administered to the pregnant animals have been close to the lethal dose, which might have accounted for the high incidence of increased mortality, morbidity and

developmental defects.

In this study, ethanol was injected intraperitoneally at a dose that was below the approximated LD50 for the rat. These injections began on gestational Day 6 (day of implantation) and proceeded through gestational Day 12. This treatment period included the critical stages of organogenesis (Embryonic Day 9½ - Embryonic Day 11½) and, therefore, any deleterious effect of the tested substance should have been evident. The effects of such a short term exposure to alcohol during pregnancy was then studied using a strict morphological protocol which lends itself to quantitative assessment.

In humans Harlap and Shiono (1980) discovered that women who regularly consume one to two drinks daily in the first trimester were twice as likely as non-drinkers to have spontaneous abortions in the second trimester. Intraperitoneal treatment of the pregnant rats with ethanol caused a significant increase in the number of resorptions when compared to its corresponding control group. This observation is in agreement with the findings of others (Blakley and Scott, 1984; Persaud, 1983; Kronick, 1976). An increase in the incidence of resorptions has been described in other species, including non-human primates. Scott and Fradkin (1984), upon administering ethanol to cynomolgus monkeys, observed an increased pregnancy wastage in the form of abortions and stillbirths.

The intraperitoneal injections of ethanol induced a number of quite visible morphological alterations in the embryo. Using the Morphological Scoring System (Brown and Fabro, 1981), these differences were quantitatively evaluated. Smaller offspring born to alcoholic

animals have been consistently reported in the literature (Tze and Lee, 1975; Marquis et al., 1984; Scott and Fradkin, 1984; Uphoff et al., 1984). In the present study, measurement of crown-rump length was used to assess the growth of the embryo. It was found that those embryos whose mothers were treated with the ethanol solution displayed a significant shortened overall crown-rump length.

Head length, another measurement of intrauterine growth, was also affected in the ethanol treated embryos. A large number of embryos displayed a reduction in the overall head length as well as distortions in head shape. These two phenomena, head length reduction and head shape distortion, may account for some of the variations seen in craniofacial and central nervous system development. Other researchers have noted exencephaly (Fernandez et al., 1983) and disturbances in gastrulation and neurulation (Nakatsuji and Johnson, 1984). In monkeys Scott and Fradkin (1984) discovered a peculiar head shape in one of the monkeys treated with 5g/kg/day of ethanol. X-ray of the skull revealed that the cranial bones were not orientated properly and that the peculiar head shape was due to a modelling effect caused by autolysis and intrauterine pressure.

The reduction in the measurements of the embryonic growth developmental characteristics (crown-rump length, head length and yolk sac diameter) displayed by the ethanol-treated embryos serve to further support the important phenomenon of intrauterine growth retardation seen in children born to alcoholic mothers. Jones et al. (1981) believed that the intrauterine growth retardation is caused by a reduction of blood flow from the maternal circulation through the

placenta. Such a reduction in placental circulation was thought to severely reduce the level of nutrients in the fetal circulation. Marquis et al. (1984) have reported that maternal alcohol consumption produces a significant reduction in fetal plasma glucose levels coupled with changes in fetal and maternal plasma amino acid concentrations. These changes ultimately limit the growth of the embryo.

The state of flexion achieved by the ethanol treated embryos was affected in many cases. These changes ranged from failure to turn to mild alterations in flexion. It is ultimately through the state of flexion that one could predict the growth pattern of that particular embryo. Normally proper flexion is reflected by orderly embryonic growth.

With respect to the central nervous system, the caudal neural tube differed significantly in the alcohol treated embryos from the controls. Disturbances in neurulation induced by the alcohol treatment are in agreement with the results from other studies (Bannigan and Burke, 1982; Nakatsuji, 1983; Nakatsuji and Johnson, 1984). It is believed that ethanol inhibits the migration of mesodermal cells toward the animal pole during gastrulation, thus causing a smaller neural plate to be formed (Nakatsuji, 1983).

The development of the heart was affected in several alcohol treated embryos. At Embryonic Age Day 11.5, the heart displays differentiating compartments (bulbis cordis, atrium commune and ventriculus communis), but in a number of alcohol exposed embryos, it remained as a convoluted cardiac tube. In a few extreme cases, the embryos had only a beating "s" shaped cardiac tube. These alterations

in the embryogenesis of the heart reflect an overall developmental delay. Chernoff (1975) had previously reported an increased rate of cardiac anomalies in the offspring of mice that had consumed a liquid alcohol diet prior to and during pregnancy. However, these cardiac defects were more severe in that developmental delay was not the embryological basis for the anomalies, but rather a derangement in the actual formation of the heart tube and its subsequent subdivision.

Anomalies of the skeletal system have been reported in the Fetal Alcohol Syndrome (Herrmann et al., 1980). Major malformations such as tetraectrodactyly, cleft palate and clubfeet, as well as shortness of metatarsals and metacarpals, have been described. In rats micromelia and impaired skeletogenesis have also been observed (Sreenathan et al., 1983). Failure of the skeletal system to develop normally reflects the overall growth deficiency of the embryo. Marked decrease in fetal plasma glucose concentrations may be partly responsible for the growth retardation of the alcohol exposed fetuses (Marquis et al., 1984). One manifestation of this overall depressed intrauterine growth may be the delayed growth of the forelimb bud. In the present study the most common musculoskeletal delay was reduction of the forelimb, or in some cases failure of the forelimb bud to develop.

One major pattern of defects that ultimately group the children suffering from Fetal Alcohol Syndrome into a discernible clinical entity are the anomalies present in the craniofacial region. Alterations in the development of the craniofacial region have been observed in a number of animal species, as well as in man (Kronick, 1976; Fernandez et al., 1983; Nakatsuji, 1983; Nakatsuji and Johnson,

1984; Scott and Fradkin, 1984; Herrmann et al., 1980). In the present study intraperitoneal injections of ethanol did not cause any gross morphological changes in craniofacial development because of the fact that those primordia that are commonly associated with the craniofacial complex are not present at the time of treatment. However, the branchial bars, which will give rise to some of the definitive structures of the craniofacial region, show some changes. At Embryonic Age Day 11.5, three branchial bars are usually seen. Alterations in the number of branchial bars present reflect developmental delay and may eventually result in a distortion of the facial appearance. Intimately associated with the decrease in branchial bars and their appearances is the peculiar shape of the head region. Usually these two alterations occurred together, which would further accentuate the facial dysmorphology of the embryo.

Thus, intraperitoneal injections of ethanol to pregnant rats caused disturbances in embryonic growth, caudal neural tube development, heart development, forelimb development and the appearance of branchial bars. Because the embryos were scored morphologically at Embryonic Age Day 11.5, it is difficult to predict what the final outcome or extent of these developmental disturbances would be at term. Theories have advanced to explain the role of compensatory repair and growth in the embryo (Anders and Persaud, 1980). These processes may explain why only a few embryos from each litter of ethanol treated mothers reveal fetal alcohol effects.

4.3 CAFFEINE

Caffeine, a methyl-xanthine derivative, can act as a mitotic poison in mammalian embryos. Eichler and Mugge (1932) found no deleterious effects on embryonic development when pregnant rats were injected subcutaneously with large doses of caffeine. However, continuous treatment of pregnant rabbits with high doses of caffeine resulted in occasional early embryonic death or retarded development.

One important consideration in studying the teratogenicity of caffeine is its rapid metabolic rate. The rapid metabolic rate of caffeine with no apparent accumulation of this compound in the body has been established in both man and animal (Stolman and Stewart, 1960; Elmazar et al., 1981). With this in mind, it is then advantageous to administer caffeine to the pregnant animals at an appropriate embryonic developmental stage.

In the present study, caffeine was administered intravenously at a moderate dose of 25 mg/kg on gestational Day 10 (Embryonic Age Day 9.5). This time period was selected for the treatment because it coincided with the critical phase of organogenesis.

Caffeine not only acts as a mitotic poison, but it also affects blood flow. During pregnancy, placental circulation plays a crucial role in the development of the fetus. In third trimester pregnant women, it has been reported that maternal serum caffeine and epinephrine levels were significantly elevated, compared with fasting values, thirty minutes after the consumption of two cups of coffee. The intervillous placental blood flow was also significantly decreased (Kirkinen et al., 1983). Such a decrease in placental circulation

would increase the risk of fetal damage and actually be a contributing factor to the intrauterine growth retardation.

The number of resorptions found in the caffeine treated group significantly differed from the corresponding control group. Kimmel et al. (1984) also reported a significant amount of resorptions following oral administration of caffeine to pregnant rats at a dose level of 120 mg/kg on Day 12 of gestation.

The developmental characteristics of embryonic growth following exposure to caffeine were all significantly affected in the present study. It is believed that maternal treatment with caffeine has altered utero-placental circulation to such an extent that normal embryonic development is impaired (Adamsons et al., 1971; Chernoff and Grabowski, 1971).

A striking feature in fetuses recovered from pregnant mice treated with caffeine was their edematous appearance (Fujii and Nishimura, 1972). Fujii and Nishimura (1972) induced hydrocephaly in several offspring of pregnant mice following treatment with caffeine (0.25% or 0.50%), mixed with the diet, throughout selected periods of gestation. In the present study, peculiar head shapes were observed in embryos that were exposed to caffeine. Some of these cranial alterations were accompanied by disturbances in the development of the caudal neural tube. Edematous features were not evident in any of the embryos that were morphologically scored. However, in agreement with the report of Fujii and Nishimura (1972), caudal neural tube development was significantly different from that of the corresponding control group.

With reference to the musculoskeletal system, the most common defects seen following treatment of pregnant mice with caffeine are cleft palate, digital defects and the occurrence of clubfoot (Nishimura and Nakai, 1960; Fujii et al., 1969). In the present study, disturbances in the development of the forelimb were observed. This would imply that caffeine produces its initial teratogenic effects already at the limb bud stage with more major consequences becoming manifested at birth.

As well as the musculoskeletal system, the craniofacial region was also affected. The number of branchial bars observed in the caffeine treated embryos was significantly fewer than in the corresponding control group. Absence of the second and third branchial bars could lead to conspicuous facial defects, and in particular to the well-documented defects of cleft palate and micrognathia (Fujii et al., 1969).

In summary, the administration of caffeine at a dose level of 25 mg/kg caused an increased incidence of resorptions, abnormal offspring and developmental delay. Alterations of several important developmental characteristics of the embryo was also prevalent. Even though epidemiological studies have found no real association between coffee consumption during pregnancy and adverse fetal outcome (Linn et al., 1982), the United States Food and Drug Administration still advises pregnant women to avoid caffeine-containing foods and drugs. The rationale for this is that caffeine is a methylxanthine which resembles the purines found in genetic material. Thus, caffeine possesses the potential to intercept the processes involved in cell proliferation. Because it has been known for some time that caffeine readily crosses the placenta and reaches the fetus (Goldstein and

Warren, 1962), the warning of the Food and Drug Administration merits serious consideration.

4.4 ALCOHOL AND CAFFEINE

The action of a chemical agent upon the developing embryo is rarely an isolated event (Skalko, 1985). Quite often the pregnant woman may consume several prescribed, as well as non-proprietary, drugs during the course of her pregnancy. Alcohol and caffeine are examples of commonly and causally consumed vasoactive agents. Alcohol and caffeine are potent vasoactive substances in that they both have the ability to markedly increase the levels of catecholamines in the body. In the pregnant woman this causes an alteration in blood flow. In particular, these hormones tend to alter both uterine and placental blood flow patterns in that a decreased perfusion of blood occurs throughout the entire reproductive tract. This leads to a reduced blood flow to the developing embryo and consequently developmental delay ensues.

Intraperitoneal administration of ethanol to pregnant rats on gestational Days 6 through 12, coupled with an intravenous injection of caffeine on gestational Day 10, caused severe disturbances in embryonic growth. Developmental characteristics describing embryonic growth (yolk sac diameter, crown-rump length, head length, flexion and absolute somite number) were all significantly reduced compared to the corresponding control group as well as the untreated control group. Weatherbee and Lodge (1979) found that alcohol and caffeine both possess the biochemical ability to affect fetal levels of cAMP,

and in doing so potentially alter the course of fetal development. They noted that the complex and diverse cellular mechanisms which apparently operate through the various cyclic nucleotides are able to become modified upon the administration of alcohol and caffeine. In the fetus this is of considerable importance, because at this developmental stage cellular division and differentiation are the major events occurring. Hence, it is through these indirect effects in the mother, coupled with the direct effects of the agent on the embryo that jeopardize the normal completion of the intricate developmental processes.

Short-term ingestion of ethanol impairs the elimination of caffeine in both human subjects and dogs (Mitchell et al., 1983), as well as in rodents (Mitchell et al., 1982). The ability of caffeine to remain non-metabolized increases its transfer across the placenta and its accumulation in fetal tissues. Because caffeine is a mitotic poison, it may alter the development of the embryo by inhibiting or even arresting cell division. Cell division and cell proliferation contribute to the growth of the embryo; therefore, impairment of these crucial cellular processes could account for the lack of development or developmental delay observed in the alcohol and caffeine treated embryos.

In the present investigation, developmental defects occurred in the caudal neural tube, hindbrain, midbrain and forebrain. The neural folds failed to fuse in a number of embryos with caudal neural tube defects; hence, these embryos possessed open posterior neuropores. In the case of hindbrain development, some embryos displayed open

anterior neuropores. Midbrain maldevelopment was represented in the form of failure of the mesencephalic folds to fuse whilst the forebrain often showed developmental delay with the telencephalic evaginations often absent. These neural developmental disturbances coincided with what was seen when alcohol and caffeine were administered alone. The peculiar head shapes that were evident following administration of either alcohol or caffeine were further accentuated when both were administered together.

Cardiovascular development was altered in the alcohol and caffeine treated embryos, in the sense that the cardiac tube or heart was not as yet fully differentiated into the bulbis cordis, atrium commune and ventriculus communis. In the previous treatment groups the effect occurred only in animals treated with alcohol. Hence, derangement of heart embryogenesis might still be due solely to the effects of the alcohol, and not caffeine (Blakley and Scott, 1984).

With respect to the craniofacial region, maternal treatment with both alcohol and caffeine affected the number of branchial bars that were observed. The branchial bars were less discernible in embryos of mothers treated with alcohol and caffeine. Of interest also is that the alcohol and caffeine treated group displayed fewer branchial bars than either the alcohol treated group or the caffeine treated group alone. Even though facial morphology cannot be observed in embryos at Embryonic Age Day 11.5, the branchial bars are good indicators of the ultimate facial shape. Because the branchial bars give rise to the facial primordia, any alteration in number or developmental delay may lead to facial dysmorphology at birth, a characteristic commonly seen

in infants with Fetal Alcohol Syndrome. Alcohol-induced facial dysmorphology may be potentiated by caffeine, because caffeine has been observed to cause developmental delay in skeletogenesis (Elmazar et al., 1981; Fujii and Nishimura, 1972; Scott, 1983). Thus caffeine acts as a coteratogen when administered together with alcohol to pregnant rats (Skalko, 1985).

BIBLIOGRAPHY

BIBLIOGRAPHY

Abel, E.L. (1981). Fetal Alcohol Syndrome. Vol. 1 CRC Press Inc. Florida.

Abel, E.L., Dintcheff, B.A. and Bush, R. (1981). Effects of beer, wine, whisky and ethanol on pregnant rats and their offspring. Teratology 23: 217-222.

Abel, E.L. and Greizerstein, H.B. (1979). Ethanol-induced prenatal growth deficiency: Changes in foetal body composition. J Pharmacol Exp Ther 211: 668-671.

Adamsons, K., Mueller-Heuback, E. and Myers, R. (1971). Production of fetal asphyxia in the rhesus monkey by administration of catecholamines to the mother. Am J Obstet Gynecol 109: 248-262.

Anders, K. and Persaud, T.V.N. (1980). Compensatory embryonic development in the rat following maternal treatment with ethanol. Anat Anz Jena 148: 375-383.

Axelrod, J. and Reichenthal, J. (1953). The fate of caffeine in man and a method for its estimation in biological material. J Pharmacol Exp Ther 107: 519-523.

Bannigan, J. and Burke, P. (1982). Ethanol teratogenicity in mice: A light microscopic study. Teratology 26: 247-254.

Beaudoin, A.R. (1976). NAD precursors as antiteratogens against aminothiazole. Teratology 13: 95-100.

Berggren, S.M. and Goldberg, L. (1940). Absorption of ethyl alcohol from gastro-intestinal tract as diffusion process. Acta Physiol Scandinav 1: 246-270.

Bertrand, M., Schwam, E., Frandon, A. and Alary, J. (1965). Systematic and specific teratogenic effects of caffeine in rodents. Cr Scan Soc Biol 159: 2199.

Blakley, P.M. and Scott, W.J. (1984). Determination of the proximate teratogen of the Mouse Fetal Alcohol Syndrome. Toxicol Appl Pharmacol 72: 355-363.

Boggan, W.O. and Randall, C.M. (1979). Renal anomalies in mice prenataly exposed to ethanol. Res Comm Chem Path Pharmacol 23: 127-142.

Brien, J.F., Loomis, C.W., Tranmer, J. and McGrath, A. (1983). Disposition of ethanol in human maternal venous blood and amniotic fluid. Am J Obstet Gynecol 146: 181-186.

Brock, N.V. and Kreybig, T.V. (1964). Teratogenese als pharmakolgisch-toxikologisches Problem. Arzneimittel-Forshung 6: 655.

Brockman, R.W. (1974). Biochemical aspects of drug combinations. Cancer Chemother Rep (Supp) 4: 115-129.

Brown, N.A. and Fabro, S. (1981). Quantitation of rat embryonic development in vitro: A Morphological Scoring System. Teratology 24: 65-78.

Brown, N.A., Goulding, E.H. and Fabro, S. (1979). Ethanol embryotoxicity: direct effects on mammalian embryos in vitro. Science 206: 573-575.

Burlingame, P.L. and Long, J.A. (1939). The development of the external form of the rat, with some observations on the origin of the extraembryonic coelom and foetal membranes. Calif Univ Publ Zool 43: 143-183.

Burton, R. (1621). The Anatomy of Melancholy. William Tegg, London, England.

Butcher, E.O. (1929). Development of somites in the white rat. Amer J Anat 44: 381-439.

Cadwallader, D.B. (1983). Biopharmaceutics and Drug Interactions. 3rd edition, Raven Press, New York.

Carpenter, T.M. (1929). Ethyl alcohol in fowls after exposure to alcohol vapor. J Pharmacol Exp Ther 37: 217-259.

Chernoff, G.F. (1975). A mouse model of the fetal alcohol syndrome. Teratology 11: 14A.

Chernoff, G.F. (1977). The fetal alcohol syndrome in mice: An animal model. Teratology 15: 223-230.

Chernoff, G.F. (1980). The fetal alcohol syndrome in mice: Maternal variables. Teratology 22: 71-75.

Chernoff, N. and Grabowski, C.T. (1971). Responses of the rat foetus to maternal injections of adrenaline and vasopressin. Brit J Pharmacol 43: 270-278.

Christie, G. (1964). Developmental stages in somite and post-somite rat embryo, based on external appearance, and including some features of the macroscopic development of oral cavity. J Morphol 114: 263-286.

Clarren, S.K. and Smith, D.W. (1978). The fetal alcohol syndrome. New Eng J Med 298: 1063-1067.

Collins, T.F.X., Welsh, J.J., Black, T.N. and Collins, E.V. (1980). A comprehensive study of the teratogenic potential of caffeine in rats when given oral intubation. In Report on Caffeine. Washington, D.C.

Collins, T.F.X., Welsh, J.J., Black, T.N. and Collins, E.V. (1981). A study of the teratogenic potential of caffeine given by oral intubation to rats. Regul Toxic Pharmac 1: 355.

Corrigan, G.E. (1976). The fetal alcohol syndrome. Tex Med 72: 72-74.

Crone, C. (1965). The permeability of brain capillaries to non-electrolytes. Acta Physiol Scand 64: 407-417.

Dehaene, P.H., Titran, M. and Samaille-Villete, C. (1977). Frequence du syndrome d'alcoolisme foetal. Nouv Presse Med 6: 1763.

Edwards, J.A. (1968). The external development of the rabbit and rat embryo. Advances In Teratology 3: 239-262.

Eichler and Mugee. (1932). Zur Frage der Schädlichkeit des Kaffeins bei chronischer Zuführ. Arch f Exp Path u Pharmakol Vol 168: 89.

Elmazar, M.M.A., McElhatton, P.R. and Sullivan, F.M. (1981). Acute studies to investigate the mechanism of action of caffeine as a teratogen in mice. Hum Toxic 1: 53-63.

Fernandez, K., Caul, W.F., Boyd, J.E., Henderson, G.I. and Michealis, R.C. (1983). Malformations and growth of rat fetuses exposed to brief periods of alcohol in utero. Terat Carcino and Mutag 3: 457-460.

Fujii, T. and Nakatsuka, T. (1983). Potentiating effects of caffeine on teratogenicity of alkylating agents in mice. Teratology 28: 29-33.

Fujii, T. and Nishimura, H. (1969). Teratogenic actions of some methylated xanthines in mice. Okajimas Folia Anat Jap 46: 167-175.

Fujii, T. and Nishimura, H. (1972). Adverse effects of prolonged administration of caffeine on rat fetus. Toxicol Appl Pharmacol 22: 449-457.

Fujii, T. and Nishimura, H. (1974). Reduction in frequency of fetopathic effects of caffeine in mice by pretreatment with propanolol. Teratology 10: 149-152.

Fujii, T., Sasaki, H. and Nishimura, H. (1969). Teratogenicity of caffeine in mice related to its mode of administration. Jap J Pharmacol 19: 134-138.

Gilani, S.H., Giovinazzo, J.J., Persaud, T.V.N. (1983). Embryopathic effects of caffeine in the chick. Exp Path 23: 79-83.

Gilbert, E.F. and Pistey, W.R. (1973). Effect on the offspring of repeated caffeine administration to pregnant rats. J Reprod Fertil 34: 495-499.

Goldstein, A. and Warren, R. (1962). Passage of caffeine into human gonadal and fetal tissue. Biochem Pharmac 11: 166-168.

Groisser, D.S., Rosso, P. and Winick, M. (1982). Coffee consumption during pregnancy: subsequent behavioral abnormalities of the offspring. J Nutr 112: 829-832.

Groupe D'estudes des Risques Tératogènes. (1960). Tératogènes expérimentale etude de la caféine chez la souris. Thérapie 24: 575-580.

Gruneberg, H. (1943). Normal staging of the mouse embryo. J Hered 34: 89-92.

Haggard, H.W. and Jellinek, E.M. (1942). Alcohol Explored. Doubleday, Doran and Co., New York.

Hamburger, V. and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. J Morph 88: 49-92.

Hanson, J.W., Jones, K.L. and Smith, D.W. (1976). Fetal alcohol syndrome: experience with 41 patients. JAMA 235: 1458-1460.

Harlap, S. and Shiono, P.H. (1980). Alcohol, smoking and incidence of spontaneous abortions in the first and second trimester. Lancet ii: 173-176.

Hartshorn, E.A. (1976). Handbook of Drug Interactions. 3rd edition, Hamilton Press, New York.

Henderson, G.I., Hoyumpa, A.M., McClain, C. and Schenker, S. (1979). The effects of chronic and acute alcohol administration on fetal development in the rat. Alcoholism clin exp Res 3: 99-106.

Henderson, G.I., Hoyumpa, A.M., Rothschild, M.A. and Schenker, S. (1980). Effect of ethanol and ethanol-induced hypothermia on protein synthesis in pregnant and fetal rats. Alcoholism clin exp Res 4: 165-177.

Henderson, G.I. and Schenker, S. (1977). The effect of maternal alcohol consumption on the viability and visceral development of the newborn rat. Res Comm Chem Path Pharmacol 16: 15-32.

Henderson, G.I. and Schenker, S. (1984). Effects of ethanol and/or caffeine on fetal development and placental amino acid uptake in rats. Dev Pharmacol Ther 7: 177-187.

Henderson, G.I., Turner, D., Patwardhan, R.V., Lumeng, L., Hoyumpa, A.M. and Schenker, S. (1981). Inhibition of placental valine uptake after acute and chronic maternal ethanol consumption. J Pharmac exp Ther 216: 465-472.

Henneberg, B. (1937). Normentafeln zur Entwicklungsgeschichte der Wanderratte. Vol. 15 of Normentafeln zur Entwicklungsgeschichte/ Entwicklungsgeschichte der Wilbertiere. Gustav Fischer, Jena.

Herrmann, J., Pallister, P.D. and Opitz, J.M. (1980). Tetraectrodactyly and other skeletal manifestations in the Fetal Alcohol Syndrome. Eur J Pediatr 133: 221-226.

Hodgman, C.D. (1959). Ed. Handbook of Chemistry and Physics. Chemical Rubber Publ Co., Cleveland.

Horning, M.G., Stratton, C., Nowlin, J., Wilson, A., Horning, E.C. and Hill, R.M. (1973). in Fetal Pharmacology. L. Boreus Ed., Raven Press, New York.

Huber, G.C. (1915). The development of the albino rat, Mus norvegicus albinis. Amer Anat Mem 5: 3-142.

Jones, P.J.H., Leichter, H.J. and Lee, M. (1981). Placental blood flow in rats fed alcohol before and during gestation. Life Sciences 29: 1153-1159.

Jones, K.L. and Smith, D.W. (1973). Recognition of the fetal alcohol syndrome in early infancy. Lancet 2: 999-1001.

Jones, K.L., Smith, D.W., Streissguth, A.P. and Myrianthopoulos, N.C. (1974). Outcome in offspring of chronic alcoholic women. Lancet 1: 1076-1078.

Jones, K.L., Smith, D.W., Ulleland, C.N. and Streissguth, A.P. (1973). Pattern of malformations in offspring of chronic alcoholic mothers. Lancet 1: 1267-1271.

Kaufman, M.H. and Woollam, D.H.M. (1981). The passage to the foetus and liquor amnii of ethanol administered orally to the pregnant mouse. Brit J Exper Path 62: 357-361.

Kimmel, C.A., Kimmel, G.L., White, C.G., Grafton, T.F., Young, J.F. and Nelson, C.J. (1984). Blood flow changes and conceptal development in pregnant rats in response to caffeine. Fund and Appl Toxicol 4: 240-247.

Kimmel, C.A., Laborde, J.B. and Trammel, C.T. (1982). Evaluation of cartilage and bone formation in fetal skeletons following prenatal insult reveals abnormalities not apparent in alizarin-stained specimens. Teratology 25: 54A.

Kirkinen, P., Jouppila, P., Koivula, A., Vuori, J. and Puukka, M. (1983). The effect of caffeine on placental and fetal blood flow in human pregnancy. Am J Obstet Gynecol 147: 939-942.

Kling, O.R. and Christensen, H.D. (1979). Caffeine elimination in late pregnancy. Fed Proc 38: 266.

Knoche, C. and Konig, J. (1964). Zur pränatalen Toxizität von Diphenylpyralin - 8 - Chlortheophyllinat unter Berücksichtigung von Erfahrungen mit Thalidomid und Kaffein. Arzneim-Forsch 14: 415-424.

Kronick, J.B. (1976). Teratogenic effects of ethyl alcohol administered to pregnant mice. Am J Obstet Gynecol 124: 676-680.

Landauer, W. (1979). Antiteratogens as analytical tools. In Advances in the Study of Birth Defects, Persaud, T.V.N. (ed), University Park Press, Baltimore, vol. 1 pp. 19-35.

Lee, H., Nagele, R.G. and Pietrolungo, J.F. (1982). Toxic and teratologic effects of caffeine on explanted early chick embryos. Teratology 25: 19-25.

Lemoine, P., Harosseau, H., Borteyru, J.P. and Menuet, J.C. (1968). Children of alcoholic parents: anomalies observed in 127 cases. Quest Medical 25: 476-482.

Leuschner, F. and Schwerdtfeger, W. (1968). The influence of caffeine and other methylxanthines on the reproduction of Wistar rats. Caffeine Andere Methylxanthine Int. Symp: 209-215.

Linn, S., Schoenbaum, S.C., Monson, R.R., Rosner, B., Stubblefield, P.G. and Ryan, K.J. (1982). No association between coffee consumption and adverse outcomes of pregnancy. New Eng J Med 306: 141-144.

Maling, H.M. (1970). Toxicity of single doses of ethyl alcohol. In, Trèmolieres (Ed), Alcohol and Derivatives, vol. 2, Pergammon Press, Oxford, pp. 277-295.

Majewski, F. and Bierich, J.R. (1978). Auxological and clinical findings in the alcohol embryopathy: experience with 76 patients: proceedings of the first International Congress of Auxology. Acta Med Auxolog 1: 1-4.

Marquis, S.M., Leichter, J. and Lee, M. (1984). Plasma amino acids and glucose levels in the rat fetus and dam after chronic maternal alcohol consumption. Biol Neonate 46: 36-43.

Martin, J.C. (1982). An overview: maternal nicotine and caffeine consumption and offspring outcome. Neurobehav Toxicol Teratol 4: 421-427.

Maugh, T.H. (1981). Fetal alcohol advisory debated. Science 214: 642-644.

Mellanby, E. (1919). Alcohol: Its absorption into and disappearance from the blood under different conditions. Special Report Series No. 31, Medical Research Council, London.

Minot, C. (1905). The development of the rabbit. Normentafeln zur Entwicklungsgeschichte der Wilbertiere. Keibel, Jena.

Mitchell, M.C., Hoyumpa, A.M., Schenker, S., Johnson, R.F., Nichols, S. and Patwardhan, R.V. (1983). Inhibition of caffeine elimination by short-term ethanol administration. J Lab clin Med 101: 826-834.

Mitchell, M.C., Hoyumpa, A.M., Schenker, S. and Patwardhan, R.V. (1982). Differential effects of chronic ethanol feeding on cytochrome P-448 and P-450 - mediated drug metabolism in the rat. Biochem Pharmacol 31: 695.

Mulvihill, J.J., Klimas, J.T., Stokes, D.C. and Risemberg, H.M. (1976). Fetal alcohol syndrome: seven new cases. Am J Obstet Gynecol 125: 937-941.

Nakatsuji, N. (1983). Craniofacial malformation in Xenopus laevis tadpoles caused by the exposure of early embryos to ethanol. Teratology 28: 299-305.

Nakatsuji, N. and Johnson, K.E. (1984). Effects of ethanol on the primitive streak of mouse embryo. Teratology 29: 369-375.

Nakatsuka, T., Hanada, S. and Fujii, T. (1983). Potentiating effects of methylxanthines on teratogenicity of mitomycin C in mice. Teratology 28: 243-247.

Neims, A.H., Bailey, J. and Aldridge, A. (1979). Disposition of caffeine during and after pregnancy. Clin Res 27: 236A.

Nishimura, H. and Nakai, K. (1960). Congenital malformations in offspring of mice treated with caffeine. Proc Soc Exp Biol Med 104: 140-142.

Nolen, G.A. (1981). The effect of brewed and instant coffee on reproduction and teratogenesis in the rat. Toxic Appl Pharmac 58: 171-183.

Nomura, T., Enomoto, T., Shibata, K., Kanzaki, T., Tanaka, H., Hata, S., Kimura, S., Kusafuka, T., Sobue, K., Miyamoto, S., Nakano, H. and Gotch, H. (1983). Antiteratogenic effects of tumor inhibitors, caffeine, antipain and retinoic acid in mice. Cancer Res 43: 5156-5163.

Øisund, J.F., Fjorden, A. and Mørlund, J. (1978). Is moderate ethanol consumption teratogenic in the rat? Acta Pharmacol et Toxicol 43: 145-155.

Olegard, R., Sable, K.G. and Aronsson, M. (1975). Effects on the child of alcohol abuse during pregnancy. Retrospective and prospective studies. Acta Pediatr Scand 279: 112-121.

O'Shea, K.S. and Kaufman, M.H. (1979). The teratogenic effect of acetaldehyde: implications for the study of the fetal alcohol syndrome. J Anat., London 128: 65-76.

Oullette, E.M., Rosett, H.L., Rosman, N.P. and Werner, L. (1977). Adverse effects on offspring of maternal alcohol abuse during pregnancy. New Eng J Med 297: 528-530.

Palm, P.E., Arnold, E.P., Rachwall, P.C., Leyczek, J.C., Teague, K.W. and Kensler, C.J. (1978). Evaluation of the teratogenic potential of fresh-brewed coffee and caffeine in the rat. Toxicol Appl Pharmacol 44: 1-16.

Patwardhan, R.V., Schenker, S., Henderson, G.I., Abov-Mourad, N.W. and Hoyumpa, A.M. (1981). Short-term and long-term ethanol administration inhibits the placental uptake and transport of valine in rats. J Lab Clin Med 98: 251-262.

Persaud, T.V.N. (1983). Prenatal loss in the rat following moderate consumption of alcohol incorporated in a liquid diet. Anat Anz., Jena 153: 169-174.

Pilstrøm, L. and Kiessling, K.H. (1967). Effect of ethanol on growth and on the liver and brain mitochondrial functions of the offspring of rats. Acta pharm et tox 25: 225-232.

Randall, C.L. and Taylor, W.J. (1979). Prenatal ethanol exposure in mice: Teratogenic effects. Teratology 19: 305-312.

Randall, C.L., Taylor, J. and Walker, D.W. (1977). Ethanol-induced malformations in mice. Alc Clin Exp Res 1: 219-224.

Rasmussen, B.B. and Christensen, N. (1980). Teratogenic effect of maternal alcohol consumption on the mouse. Acta Path Microbiol Scand Sect A., Køhenhaun 88: 285-289.

Rawat, A.K. (1975). Ribosomal protein synthesis in the fetal and neonatal rat brain as influenced by maternal ethanol consumption. Res Comm Chem Path Pharmacol 12: 723-732.

Reinhold, L., Hütterotth, H. and Schulte-Wissermann, H. (1975). Das fetale Alkohol-Syndrom: Fallbericht über zwei Geschwister. Münch Med Wochenschr 117: 1731-1734.

Ritter, E.J., Scott, W.J., Wilson, J.G., Manthinos, P.R. and Randall, J.L. (1982). Potentiative interactions between caffeine and various teratogenic agents. Teratology 25: 95-100.

Rollwagen, R.I. (1973). Statistics on Line for Health Sciences. U. of Manitoba Press, Winnipeg, Canada.

Root, A.W., Reiter, E.O., Andriola, M. and Duckett, G. (1975). Hypothalamic-pituitary function in the fetal alcohol syndrome. J Pediatr 87: 585-588.

Rosett, H.L. and Weiner, L. (1980). Clinical and experimental perspectives on prevention of the fetal alcohol syndrome. Neurobehav Toxicol 2: 267-270.

Sandor, S. (1968). The influence of ethyl alcohol on the developing chick embryo. Rev Roum Embryol 5: 167-171.

Sandor, S. and Amels, D. (1971). The action of aethanol on the prenatal development of albino rats. Rev Roum Embryol Cytol Ser Embryol 8: 105-118.

Sandor, S. and Elias, S. (1968). The influence of aethyl alcohol on the development of the chick embryo. Rev Roum Embryol Cytol Ser Embryol 5: 1-26.

Schneider, B.F. and Norton, S. (1979). Equivalent ages in rat, mouse and chick embryos. Teratology 19: 273-278.

Scott, W.J. (1983). Caffeine-induced limb malformations: description of malformations and quantitation of placental transfer. Teratology 28: 427-435.

Scott, W.J. and Fradkin, R. (1984). The effects of prenatal ethanol in cynomolgus monkeys. Teratology 29: 49-56.

Shepard, T.H. (1980). Catalog of Teratogenic Agents, 3rd edition, Johns Hopkins University Press, Baltimore.

Shurygin, G.I. (1974). Ob osobennustiiakh psikhickeskogo razvitiia detei ot materei stradaivshschikh kronicheskim alkogolizmom. Pediatriia 11: 71-73.

Sieber, S.M. and Fabro, S. (1970). Identification of drugs in the preimplantation blastocyst and in the plasma, uterine secretion, and urine of the pregnant rabbit. J Pharmacol Exptl Ther 176: 65-75.

Skalko, R.G. (1985). In Basic Concepts in Teratology. Chapter six (ed), Persaud, T.V.N., Chudley, A.E. and Skalko, R.G., Alan R. Liss Publ Co., New York. pp. 103-118.

Skalko, R.G. and Kwasigroch, T.E. (1983). The interaction of chemicals in pregnancy: an update. Biol Res Preg Perinatol 4: 26-35.

Skalko, R.G., Poche, P.D. and Kwasigroch, T.E. (1984). The toxicology of chemical interactions during pregnancy in the mouse: caffeine and phenytoin. Toxicology 30: 7-16.

Smith, D.W., Jones, K.L. and Hanson, J.W. (1976). Perspectives on the cause and frequency of the fetal alcohol syndrome. Ann NY Acad Sci 273: 138-139.

Sreenathan, R.M., Padmanabhan, R. and Singh, S. (1983). Effect of low doses of ethanol on fetus and fetal milieu in rat. Indian J Exp Biol 21: 108-112.

Stolman, A. (1967). Combined action of drugs with toxicological implications - Part I. In Stolman, A. (ed). Progress in Chemical Toxicology. Academic Press. New York. vol. 3: 305-361.

Stolman, A. and Stewart, C.P. (1960). Toxicology, edited by Stewart, C.P. and Stolman, A., vol. 1, p. 120, Academic Press, New York.

Streissguth, A.P., Landesman-Dwyer, S., Martin, J.C. and Smith, D.W. (1980). Teratogenic effects of alcohol in humans and laboratory animals. Science 209: 353-361.

Sulik, K.K., Johnston, M.C. and Webb, M.A. (1981). Fetal alcohol syndrome: Embryogenesis in a mouse model. Science 214: 936-938.

Terada, M. and Nishimura, H. (1975). Mitigation of caffeine-induced teratogenicity in mice by prior chronic caffeine ingestion. Teratology 12: 79-82.

Thayer, P.S. and Kensler, C.J. (1973). Exposure of four generations of mice to caffeine in drinking water. Toxic appl Pharmac 25: 169.

Tze, W.J. and Lee, M. (1975). Adverse effects of maternal alcohol consumption on pregnancy and foetal growth in rats. Nature vol. 257: 479-480.

Uphoff, C., Nyquist-Battie, C. and Toth, R. (1984). Cardiac muscle development in mice exposed to ethanol in utero. Teratology 30: 119-129.

Valeriote, F.A. (1979) The use of cell kinetics in the development of drug combinations. Pharmac Ther 4: 1-33.

Van Beneden, E. and C. Julin (1884). Recherches sur la formation des annexes foetales chez les mammiferes (Lapin et Cheiropteres). Arch. Biol. 5: 369-434.

Warren, R.D. and Bender, R.A. (1977). Drug interactions with antineoplastic agents. Cancer Treat 61: 1231-1241.

Waterman, A. (1943). Normal development of the New Zealand White strain of rabbit. Amer J Anat 72: 473-515.

Weathersbee, P.S. and Lodge, J.R. (1979). Alcohol, caffeine and nicotine as factors in pregnancy. Post Grad Med vol. 66 No. 3: 165-171.

Widmark, E.M.P. (1932). Possible errors in blood tests for determination of alcohol. Svenska lak-tidning 29: 344-348.

Wilson, J.G. (1964). Teratogenic interaction of chemical agents in the rat. J Pharmacol Exp Ther 144: 429-436.

Wilson, J.G. (1974). Factors determining the teratogenicity of drugs. Ann Rev Pharmacol 14: 205-217.

Witschi, E. (1956). The Development of Vertebrates. W. B. Saunders, Philadelphia.

Wright, J.T., Barrison, I., Toplis, P.J. and Waterson, J. (1983). Alcohol and the fetus. Br J Hosp Med 29: 260-266.

Wunderlich, S.M., Baliga, B.S. and Munro, H.N. (1979). Rat placental protein synthesis and peptide hormone secretion in relation to malnutrition from protein deficiency or alcohol administration. J Nutr 109: 1534.