INFLUENCE OF NICOTINE AND CAFFEINE ON PRENATAL DEVELOPMENT IN THE RAT

A Thesis

Presented to the Faculty of Graduate Studies,

University of Manitoba, in Partial Fulfillment

of the Requirements for the Degree of

Master of Sciences

by

Joan Elizabeth Nash

February 1987 

ISBN 0-315-37458-6

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Dedicated with love, to Richard.

ACKNOWLEDGEMENTS

I wish to express my thanks to all the members of the academic and technical staff for their helpful advice and assistance throughout this investigation.

In particular, I would like to thank Mr. B. Sam for his guidance in animal care and treatment procedures, Linda Snell for teaching me histological processing techniques, Mr. R. Simpson for his expertise in photography and Carolyn Rempel for diligently typing this thesis.

Special thanks to Dr. J. Vriend for his assistance in working with the computers, and Dr. F. Bertalanffy for his advice in assessing histological data.

I would also like to thank the Department of Anatomy for awarding me two Anatomical Research Fund Graduate Student Scholarships during my course of study.

Finally, to my advisor, Dr. T.V.N. Persaud, I express my sincere appreciation and thanks for his expert guidance in introducing me to a new realm of knowledge. I am truly grateful for his endless patience and encouragement which instilled in me the confidence to see this project to completion.

ABSTRACT

The teratogenicity of nicotine and caffeine at dose levels approximating human consumption was investigated in Sprague-Dawley rats. One group of animals received nicotine administered subcutaneously by an Alzet mini-osmotic pump from gestational days 6 through 12 (25 mg over 7 days; rate 149 micrograms/hr). Control animals received physiological saline in a similar manner. A second group received a single intravenous injection of caffeine (25 mg/kg) on gestational day 6. Control animals were treated with physiological saline. A further group received both nicotine and caffeine on gestational day 6 as described for the two previous groups.

There were no significant differences among any of the groups with respect to maternal weight gain, litter size, embryolethality, fetal weight, or crown-rump length. The offspring of nicotine treated animals showed a significantly higher incidence of hydrocephaly when compared to the controls, but in the combined treatment group no malformed fetuses were observed. Bifid sternal and vertebral ossification centers occurred only in the combined treatment group.

Light microscopic examination of maternal liver, kidney and placentas revealed changes in the hepatic sinusoids, glomeruli and intervillous spaces after nicotine and combined treatment. In addition, the decidua basalis was

poorly developed compared to the controls. Chorionic villi and fetal kidney appeared normal in all groups.

A coteratogenic effect was evident between nicotine and caffeine on account of the increased incidence of delayed and abnormal skeletal development.

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1. REVIEW OF THE LITERATURE

1.1 Nicotine

1.1.1 Chemistry

Nicotine (C10 H14 N2; MW 162.24) is a natural alkaloid found in the leaves of the tobacco plant (nicotiana tabacum). In its pure form it is colourless and odorless, acquiring these characteristics only on exposure to air. This base is highly water and lipid soluble and readily forms water soluble salts (Abel, 1980; Taylor, 1980).

Though nicotine has no therapeutic applications, its medical significance lies in the fact that it is extensively consumed through the use of tobacco products.

1.1.2 Statistics

The adverse effects of cigarette smoking during pregnancy were first reported by Simpson in 1957. Since that time numerous studies, while further documenting these findings, have also identified a broad spectrum of additional adverse consequences (Werler et al., 1985; Nieburg et al., 1985; ACOG Bulletin 1979). Despite warnings that smoking is harmful to the fetus, cigarette smoking during pregnancy is still common.

Based on 1983 statistics from the United States, of the estimated 31% of women who smoke before pregnancy, 25.5% continued the habit during their pregnancies (Werler et al., 1985).

Though the overall trend towards smoking has shown steady decline since 1965, this is due primarily to a reduction of cigarette consumption in adult male smokers. Unfortunately, in young white women, aged 18 to 24 years, the rate of cigarette smoking has actually increased, and in fact is greater than that among women aged 25 to 34 years, being approximately 40% and 27% respectively (Remington et al., 1985).

Thus, not only are women of child bearing age subjecting themselves to the health risks associated with smoking, but during pregnancy, their unborn children as well.

1.1.3 Nicotine and Cigarette Smoking

Cigarette smoke contains from two to four thousand different compounds (Abel, 1980; Jaffe, 1980). Investigators agree that although all of these substances affect the smoker to some degree, nicotine is primarily responsible for the pharmacological response to smoking (Kelley et al., 1984; Phillip et al., 1984).

The particulate matter of smoke, constituting approximately 10%, is composed mainly of three substances, nicotine, water and tar. The nicotine component is inhaled, suspended on a tiny particle of tar. Tar is the general term for polycyclic aromatic hydrocarbon products, many of which are proven carcinogens. The remaining 90% of smoke is gaseous, containing carbon monoxide, carbon dioxide,

nitrogen oxides, cyanides and numerous others (Abel, 1980; Jaffe, 1980; Taylor, 1980).

Among the many toxins identified as producing undesirable effects, carbon monoxide, nicotine and hydrogen cyanide form the highest concentrations (Luck et al. 1985). Furthermore, it is believed that these three substances are the primary agents responsible for the various effects of cigarette smoking during pregnancy (Luck et al., 1985; Suzuki et al., 1974 and 1980; Harrison et al., 1983; Mosier and Jansons, 1973; Bottoms et al., 1982).

1.1.4 Absorption

Nicotine is absorbed from the respiratory tract, buccal mucous membranes, skin and intestines. Due to the alkaline nature of the drug, however, little absorption occurs in the stomach (Jaffe, 1980). That nicotine is rapidly distributed throughout all body and tissue fluids is not surprising, considering its high lipid and aqueous solubility.

1.1.5 Pharmacological Effects

The ultimate systemic responses resulting from the administration of nicotine are complex, and represent the summation of several different and sometimes opposing effects of the drug. Though described in detail elsewhere (Goodman and Gillman, 1980), the following represents a summary of these effects.

Nicotine is a central nervous system stimulant, exerting its major action on all autonomic ganglion cells. Though considered a stimulant nicotine in fact has both stimulant and depressive phases of action (Abel, 1980).

During the stimulant phase, nervous stimulation occurs as a result of an acetylcholine-like depolarization of cholinergic receptors. This is followed by a rapid blockade of these primary cholinergic receptor sites, constituting the depressive phase (Volle, 1969). Though the mechanisms are not fully understood, it appears that nicotine in some manner desensitizes these receptor sites causing impairment of action (Taylor, 1980).

In addition, nicotine also causes the release of catecholamines, primarily from the adrenal medulla and nerve cells, but also from chromaffin tissues of various organs.

The systemic responses to ganglion cell stimulation and catecholamine release are elevation of heart rate, blood pressure, oxygen consumption and vasoconstriction. The activation of chemoreceptors of the carotid and aortic bodies also contributes to this sympathomimetic response.

In the gut, parasympathetic stimulation in combination with the activation of cholinergic nerve endings results in increased tone and motor activity, sometimes causing nausea, vomiting and diarrhea.

Finally, nicotine also has an antidiuretic action, causing the release of antidiuretic hormone through

stimulation of the hypothalamo-hypophyseal system (Jaffe, 1980).

1.1.6 Transplacental Passage

There is currently no doubt that the developing conceptus is exposed to nicotine administered to the mother, and that this transplacental passage occurs rapidly. Numerous investigations on a variety of animal models have made similar observations (Hansson and Schmiterlow, 1962; Suzuki et al., 1974; Mosier and Jansons, 1973, Luck et al., 1985).

Nicotine and its metabolites can be identified in rat fetal tissues as early as five minutes following maternal injection (Mosier and Jansons, 1973). In experiments with pregnant rhesus monkeys, Suzuki et al. (1974) examined fetal and maternal plasma concentrations of nicotine, and found that not only does nicotine equilibrate rapidly between mother and fetus, but that fetal plasma concentrations actually exceeded those of the mother, and remained in the fetal circulation for longer periods of time. A similar phenomenon was also observed by Mosier and Jansons (1973).

In recent human studies performed by Luck et al. (1985), nicotine and cotinine concentrations were measured in placental tissue, amniotic fluid, and fetal serum and term placenta during the first and second trimesters of pregnancy, and at birth respectively. Comparisons with maternal plasma concentrations of nicotine revealed higher

fetal values in all areas. Fetal cotinine concentrations on the other hand, were lower than maternal values.

An interesting report by Fabro and Sieber (1969), revealed that nicotine actually penetrates the preimplantation blastocysts in rabbits. Furthermore, levels of nicotine in these blastocysts were found to be at least twice as high as those in the maternal plasma, indicating an ability of these early embryonic tissues to accumulate or concentrate nicotine. Thus, nicotine is transferred from mother to conceptus throughout pregnancy, and embryonic and fetal tissues are exposed to higher nicotine concentrations than the mothers.

1.1.7 Distribution

Once administered, nicotine is absorbed very rapidly and tends to concentrate itself in certain target organs, these being the brain, pituitary gland, and adrenal medulla (Hansson and Schmiterlow, 1962; Suzuki et al., 1974; Luck et al., 1985). High nicotine levels have also been observed in the liver, kidney, stomach wall and bone marrow (Hansson and Schmiterlow, 1962).

In the fetus, exposure to nicotine results in a similar pattern of distribution. Distribution to brain, adrenal glands, heart, kidneys, stomach wall and spleen occurs via fetal circulatory conduits (Suzuki et al., 1974; Mosier and Jansons, 1973).

During pregnancy the placenta, and in particular the decidua basalis, and the amniotic fluid have also been identified as having higher nicotine concentrations than in maternal plasma (Luck et al., 1985; Mosier and Jansons, 1973).

1.1.8 Metabolism

Eighty to 90% of maternal nicotine is metabolized by the liver, kidneys and lungs (in that order) into two main metabolites: a) Cotinine, the most essential quantitative metabolite, and b) nicotine-1-n-oxide (Abel, 1980; Luck et al., 1985).

The main route of elimination of both nicotine and its metabolites is through excretion in the urine. In humans nicotine administered either through inhalation or parenterally, has an average half-life of between 30 and 140 minutes (Jaffe, 1980). Cotinine, on the other hand, has a much longer half-life, ranging between six and 30 hours (Luck et al., 1985).

While the maternal metabolism and elimination of nicotine is quite rapid, the same is not true for the fetus. Because fetal tissues are relatively incapable of metabolizing nicotine, it is excreted unmetabolized into the amniotic fluid. Ultimately, it re-enters the maternal circulation by way of the umbilical arteries and is eliminated through maternal excretory mechanisms (Suzuki et al., 1974; Mosier and Jansons, 1973; Hansson and

Schmiterlow, 1962). This explains the longer fetal exposure time and higher nicotine concentrations found in fetal compartments, as opposed to maternal serum concentrations.

1.1.9 Effects of Smoking on Fetal Development

The cause-effect relationship between smoking during pregnancy and adverse fetal effects is a difficult one to establish. Numerous confounding factors, such as maternal age, parity pre-pregnant status, socioeconomic status, race and geographic location all affect the ultimate outcome of pregnancy to varying degrees. Though new and sophisticated techniques exist to evaluate human fetal development and biological mechanisms, the complexities involved in these processes make interpretation of investigative data very difficult.

1.1.10 Intrauterine Growth Retardation

Intrauterine growth retardation (I.U.G.R.) is probably the best documented effect of maternal smoking. The association between smoking and decreased birth weight was first reported by Simpson in 1957. Since then, virtually all studies have confirmed that smoking 10 to 20 cigarettes per day results in babies that are, on average, 200 to 250 grams below normal weight, (normal being defined as >2500 grams) and small for gestational age (Appendix 1). The degree to which I.U.G.R. occurs is directly proportional to the number of cigarettes smoked (Meyer et al., 1976; Nieburg et al., 1985; Lubs, 1973; Cnattingius et al., 1985;

Phillip et al., 1984; Mochizuki et al., 1984; Harrison et al., 1983; Spira et al., 1975).

Multiple regression analyses of data from large population studies have enabled researchers to control at least to some degree, confounding factors, such as maternal age and weight gain, parity, gestational age, socioeconomic status and race (Meyer, 1978; Meyer et al., 1976; Spira et al., 1975). Despite these controls, the relationship between smoking and low birthweights remains persistent. Light and heavy smokers (Appendix 1) have increased risk factors of 53% and 130%, respectively, of giving birth to babies weighing less than 2500 grams, compared to non-smokers. Of practical importance is the observation that women who stopped smoking during pregnancy gave birth to babies of normal weight and length (Meyer et al., 1976).

The mechanisms by which cigarette smoking causes fetal growth retardation are not fully understood. However, two hypotheses have been postulated.

It is believed that retarded fetal growth in smokers is due to impairment of uteroplacental circulation, caused by the vasoconstricting effect of nicotine. This in turn, results in fetal tissue hypoxia, and reduced nutritional elements which may interfere with normal growth (Mochizuki et al., 1984; Suzuki et al., 1980).

In the rhesus monkey, Suzuki et al. (1980) demonstrated an acute decrease in uterine blood flow (up to 38%)

immediately following maternal infusion of nicotine. This effect has been subsequently demonstrated to occur in the human uteroplacental circulation as well (Mochizuki et al., 1984). Further placental flow studies performed by Phillip et al. (1984) revealed that uteroplacental blood flow in smokers is in fact chronically diminished when compared to that in non-smokers. In addition, smoking two cigarettes results in a further decrease in placental perfusion. In all three studies , I.U.G.R. was observed, and the authors concluded that nicotine, as a vasoconstrictor, causes I.U.G.R. through decreased perfusion of fetal tissues.

Fetal hypoxia due to elevated carboxyhemoglobin levels secondary to smoking has also been strongly implicated as an etiological factor of I.U.G.R. (Suzuki et al., 1980; Bureau et al., 1982 and 1983).

Rapid transplacental passage of carbon monoxide results in fetal carboxyhemoglobin levels that are approximately twice those of the mother. Of additional note, is that the half-life of carboxyhemoglobin is three times longer in fetal than in maternal blood (Bureau et al., 1982). Though compensatory mechanisms occur in the mother, the fetus does not possess the biologic capacity to accommodate to these high levels. The result is chronic fetal hypoxia, due to impaired oxygen transport mechanisms, which may in turn impair fetal growth and development (Bureau et al., 1983).

The theory that nicotine inhibits prostacyclin (a known endogenous vasodilator) production by fetal endothelial cells has not been substantiated (Werler et al., 1985). Investigations into this mechanism have produced conflicting results.

Ylikorkala et al. (1985), reported that nicotine has no effect on prostacyclin and thromboxane A2 (its endogenous antagonist) synthesis in the umbilical arteries, and that thromboxane A2 production by fetal platelets was actually inhibited by nicotine. On the other hand, studies have also been reported suggesting the converse is true; that inhibition of prostacyclin synthesis does occur and that this may represent a further mechanism contributing to a reduction in uteroplacental blood flow (Dadak et al., 1981; Busacca et al., 1984).

The direct effect of nicotine on fetal biological systems may also contribute to in-utero growth retardation.

The effect of maternal smoking on fetal blood flow was investigated by Eriksen and Marsal (1984). By combining real-time ultrasonography with the pulsed Doppler technique they were able to measure blood flow in the fetal aorta and umbilical vein. These investigators demonstrated that maternal smoking during the third trimester of pregnancy induced acute circulatory changes in the fetus similar to those seen in adults; ie. elevated heart rate and blood

pressure. These direct fetal effects were also observed by Suzuki et al. (1980) in the rhesus monkey.

It is generally accepted that maternal catecholamines do not reach the fetal compartments via the placenta. However, increased catecholamine concentrations have been measured in the amniotic fluid of smoking mothers during the second and third trimesters, indicating activation of fetal adrenergic systems secondary to nicotine exposure (Luck et al., 1985).

As mentioned previously, cyanide is also a major constituent of cigarette smoke. Metabolic detoxification of cyanide to thiocyanate requires among other things, vitamin B12 as well as sulfur containing amino acids (Bottoms et al., 1982). Cyanide itself is known to be toxic in high concentrations, and is believed to be a contributing factor to intrauterine growth retardation, through depletion of essential vitamins and amino acids (Bottoms et al., 1982).

With final reference to I.U.G.R., there is no evidence to suggest that it is due to nutritional deficits, even assuming that smokers eat less (Meyer, 1978).

Harrison et al. (1983) correlated maternal smoking with decreased infant weight, length, and arm, leg and head circumference. Of particular interest is that maternal smoking did not appear to affect the deposition of subcutaneous fat, suggesting that decreased birth weight was due to a reduction in lean body mass, and not fat.

Conversely, low birthweight infants suffering from in-utero malnutrition tend to be of normal length, with substantial decreases in subcutaneous fat as opposed to lean body mass (Werler et al., 1985).

1.1.11 Early Fetal Loss

Nasarat et al. (1986) have shown that in mice, nicotine administered at dose equivalents of 10, 20 and 30 cigarettes per day, resulted in an increase in early perinatal mortality. Moderate to high doses of nicotine administered during the second and third trimesters also caused a significantly higher incidence of premature deliveries and stillborns.

The use of nicotine during pregnancy has been implicated to cause spontaneous abortions in humans. A multivariate statistical analysis performed by Hemminki et al. (1983) demonstrated a weak link between smoking 10 or more cigarettes per day and a higher incidence of spontaneous abortion. In this instance, maternal age, parity, coffee and alcohol consumption were statistically controlled. Kline et al. (1977) demonstrated that women who smoked during pregnancy were twice as likely to abort spontaneously as non-smokers.

Further reports by Kline et al. (1983) revealed an interaction between smoking and maternal age in relation to chromosomally abnormal fetuses. Women over the age of thirty who smoke are more likely to have a chromosomally

abnormal abortus (trisomy) than non-smokers, while smoking women under age 30, are less likely to have a trisomic abortus than non-smokers.

1.1.12 Perinatal Mortality

Perinatal mortality is defined as "reproductive loss after the 20th week of gestation and before the 7th day of life" (Werler et al., 1985). Though investigators have correlated maternal smoking with increased stillbirth rates (Nasarat et al., 1986), and perinatal mortality (Meyer et al., 1976), this relationship remains controversial. Other factors known to be important predictors of perinatal mortality must be taken into account. These include social class, previous history of embryonic loss, and maternal age and parity (Werler et al., 1985). Despite difficulties in interpreting data due to lack of control of these factors, certain trends can be indentified.

All women are considered to have a higher risk of perinatal mortality if they are in a low socioeconomic bracket, have a history of perinatal mortality or are of advanced age (Abel, 1980). This risk increases significantly if they smoke during pregnancy. Interestingly, it appears that among women who smoke, their risk for perinatal mortality is lower if they have a higher socioeconomic status (Comstock et al., 1971).

White et al. (1986) investigated the relationship between smoking and infant respiratory distress syndrome in

premature infants (Appendix 1). Following adjustments for gestational age and method of delivery, they found the probability of infant respiratory distress syndrome to be higher in babies born to non-smokers than to smokers (38% in non-smokers and 25% in smokers). These results were considered to be in support of the theory that pulmonary maturity and development are accelerated as a result of adverse pregnancy conditions.

1.1.13 Placental Function and Morphology

Not only does smoking increase the risks of fetal death, but it also increases the risk of potentially lethal complications in the mother, specifically abruptio placenta and placenta previa. As with other effects, these complications are dose related.

Meyer et al. (1976) revealed that the increase in incidence of placenta previa and abruptio placenta for light smokers (<1 package per day) was 25% and 23% respectively, while that for heavy smokers (>1 package per day) was 92% and 86% respectively for these two complications. Abruptio placenta, when severe, carries a 10% maternal mortality rate (A.C.O.G. Bulletin, 1979). It is hypothesized that the mechanisms responsible for these dramatic effects is fibrosis of the uterine artery, with resultant decrease in uteroplacental blood flow due to the cummulative effect of smoking (Naeye et al., 1979).

Structural abnormalities observed to occur in the placentas of smokers include large and small infarcts, fibrinoid changes in arterial supply, fibrotic peripheral villi, marginal placental necrosis (Naeye et al., 1979), and hypotrophic and avascular stem villi (Mochizuki et al., 1984; Naeye et al., 1979).

Spira et al. (1975), reported a higher placental:infant weight ratio (PW/IW) to occur in smokers. In this study, placental weights showed no significant difference between smokers and non-smokers. However, the birth weights of infants born to smokers was, as expected, lower than normal. Using a regression analysis model, this was translated to show that placental weights were approximately 18 grams heavier in smokers than in non-smokers. Thus, it becomes apparent that smoking is associated with a compensatory hypertrophy occurring in the placentas of smoking women, believed to be due to hypoxia.

1.1.14 Teratological Effects

Whether or not cigarette smoking during pregnancy represents significant teratologic risk to the fetus remains a controversial topic, currently under intensive investigation. Numerous complicating factors are encountered when attempts are made to identify an association between smoking and various congenital malformations. Human development involves complex biologic, chemical and physiological mechanisms, many of which are not

fully understood. Similarly, the etiology of most malformations remains unknown. The wide variety of malformations reported in the literature makes the definition and classification of these defects very difficult, and indeed these vary in the literature. Lack of control for potential risk factors in the human population represents another confounding factor.

Ethical considerations prohibit the use of humans for teratological studies. Though animal models allow researchers to control for specific variables, there exist major disadvantages as well. Probably the most important is that due to species variation. Observations made in animal models cannot be directly extrapolated to the human. Negative results in animal models may not necessarily indicate that no effect will occur in man. This fact is best exemplified by the thalidomide disaster of the 1960's (Persaud, 1979, and 1985). Finally, due to the overall low incidence of specific malformations in the population, extremely large sample sizes are required to detect statistical significance.

Epidemiological studies have not been able to establish a clear relationship between smoking and the overall occurrence of congenital malformations. Christianson (1980) reported that there was no significant difference in the incidence of overall congenital malformations between smokers (all dose levels) and non-smokers. However, the

male infants of women who smoked more than 20 cigarettes a day had higher incidences of inguinal hernia and strabismus. This study reviewed only live births, however, and did not include stillbirths. Hemminki et al. (1983) found that though central nervous system defects, orofacial clefts and musculoskeletal defects were slightly more common in the infants of smokers, these differences were not statistically significant. On the other hand, Kelsey et al. (1978) reported that a slight correlation does exist between heavy cigarette smoking (>20 cigarettes per day) and a greater incidence of congenital defects in general, as well as specific malformations, including neural tube defects and orofacial clefts. Shiono et al. (1986) analyzed and compared prospective data from two large studies to determine the relationship between smoking and congenital malformations. These authors concluded that smoking during pregnancy is unlikely to be responsible for increases in malformations such as ventricular septal defects, hydrocoeles, hemangiomas, clubfoot, neural tube defects and Down syndrome at birth. Also, the increased risk for gut abnormalities and strabismus reported by Christianson (1980) could not be confirmed by these investigators.

Since Evans et al. (1979) published data finding a weak association between smoking and anencephaly and spina bifida, central nervous system defects have received more attention in the current literature. Golding and Bulter

(1983) reviewed this problem and found no association between maternal smoking and the incidence of central nervous system defects.

Nicotine has been reported to affect protein metabolism and synthesis in both developing and adult brain tissue (Lajtha and Sershen, 1986). In addition, prenatal nicotine injections have been shown by Seidler et al. (1986) to inhibit DNA synthesis and subsequent cell replication, differentiation and growth of central nervous system neurons in rats. It has been postulated that nicotine exposure during early fetal development interferes with early biochemical events which regulate growth and development of the central nervous system, eventually translating into various functional and structural abnormalities (Al-Hachim and Mahmoud, 1985; Lajtha and Sershen, 1986; Seidler et al., 1986).

Animal studies using variable doses of nicotine administered to pregnant rats, revealed no significant teratological effects (Persaud, 1982; Lindenschmidt and Persaud, 1980).

In-utero exposure to nicotine may have long lasting effects on children. Postnatal effects believed to be related to maternal smoking during pregnancy include behavioral problems and decreased physical growth (Lichtensteiger and Schlumpf, 1985; Abel, 1980), increased incidence of childhood respiratory illnesses (Werler et al.,

1985), poor cognitive performance on achievement tests (low I.Q. scores, reading disorders) and hyperactivity (Martin, 1982; A.G.O.G. Bulletin 1979). Evidence to support these statements remains inconclusive.

1.1.15 Smoking and Fertility

Baird and Wilcox (1985) reported that female smoking is detrimental to fertility, and as with other effects, this is dose dependant. Fertility of smokers (1 or more cigarettes per day) was estimated to be only 72% that of non-smokers. Heavy smokers demonstrated even lower fertility rates, when compared to light and non-smokers. Thus, reduced fertility can be added as another reproductive hazard associated with cigarette smoking.

1.2 CAFFEINE

1.2.1 Chemistry and Physical Properties

Caffeine, or 1,3,7 trimethylxanthine is a natural alkaloid that is found in a variety of plants. It has a low aqueous solubility, but it is lipid soluble, and at a physiological pH caffeine is surprisingly capable of permeating biological membranes (Dews et al., 1984; Rall, 1980).

1.2.2 Sources of Caffeine

Caffeine is considered to be the most popular drug in North America. Though coffee is the primary source of caffeine (containing from 85 to 110 mg per cup), caffeine

is found in numerous other food products as well. These include tea (about 50 mg/cup), cola beverages (30-45 mg/12 oz serving), cocoa (5 mg/cup), chocolate (25 mg/small bar), as well as food preservatives and many pharmaceutical preparations (Rall, 1980; Soyka, 1981; Lelo et al., 1986).

1.2.3 Statistics

It has been estimated that greater than 90% of adults consume at least one caffeine containing beverage each day (Soyka, 1981). In a recent study on the assessment of caffeine exposure, Lelo et al. (1986), reported the average daily caffeine intake of moderate to heavy consumers to be 462.8 +/- 172.8 mg/day. These figures correspond to 5.8 cups of brewed coffee, or 17.3 cups of tea per day, and can further be translated to a dose level of 6.8 mg/kg day.

In general, women tend to decrease their caffeine intake during pregnancy; however few discontinue its use altogether. According to a report by Watkinson and Fried (1985), 90-95% of pregnant women consume caffeine from various sources throughout their pregnancies, and up to 18% consume amounts equivalent to 4 or more cups of coffee daily.

1.2.4 Absorption and Distribution

Caffeine is absorbed primarily from the gastrointenstinal tract (Rall, 1980), and rapidly equilibrates between blood plasma and tissue fluids. Its disposition within body tissues is uniform, and in direct

proportion to the water content of different tissues (Soyka, 1981; Dews et al., 1984).

During pregnancy, caffeine readily crosses the placenta in both experimental animals (Kimmel et al., 1984) and in humans (Yaffe and Juchau, 1974; Goldstein and Warren, 1962; Brazier and Salle, 1981). Accumulation of caffeine in fetal tissues, amniotic fluid, and umbilical cord vessels has been shown to be at least equal to maternal plasma concentrations (Kimmel et al., 1984; Goldstein and Warren, 1962; Yaffe and Juchau, 1974; Soyka, 1981). Brazier and Salle (1981), found that at birth, caffeine levels in the fetal umbilical cord were actually greater than maternal plasma levels. Furthermore, they believe that due to the slow fetal elimination of caffeine, excretion of caffeine into amniotic fluid, and fetal conversion of theophylline to caffeine, fetal caffeine levels are always higher than in the mother.

In addition, Fabro and Sieber (1969), revealed that in rabbits caffeine, like nicotine, penetrates the preimplantation blastocyst. Concentrations of caffeine were approximately equal to those in maternal plasma. A second interesting discovery by these investigators was that pregnancy could modify the degree to which caffeine passes from maternal circulation into uterine fluid. Caffeine concentrations in uterine secretions of pregnant animals were observed to be 50% greater than in maternal plasma.

Also, this phenomenon did not occur in non-pregnant animals under the same experimental conditions.

1.2.5 Metabolism

The major route of elimination of caffeine is by metabolism in the liver (Rall, 1980). Renal excretion of unmetabolized caffeine is relatively inefficient. Approximately 99% of the caffeine filtered through the glomeruli is reabsorbed in the renal tubules, only 1% being excreted in the urine unchanged (Dews et al., 1984).

The rate of biotransformation of caffeine is highly species specific, and varies a great deal from individual to individual as well. In man, the average half-life of caffeine ranges between 3.5 and 4 hours (Rall, 1980; Dews et al., 1984). Several factors have been shown to significantly affect the rate of caffeine metabolism in adults.

People suffering from hepatic disease or pulmonary edema demonstrate a much slower rate of elimination (Aldridge et al., 1981). Kling and Christensen (1979), found that in non-human primates the rate of maternal caffeine metabolism diminishes significantly as pregnancy progresses, particularly in the last trimester, being only about 1/3 the prepregnant rate. Metabolic rates of elimination return to normal several days after parturition. These results were later confirmed in human studies by Aldridge et al. (1981). During the first trimester,

caffeine clearance was approximately equal to that in nonpregnant women. However, during the 2nd and 3rd trimesters, caffeine half-life increased from 5.3 to 18.1 hours, and returned to normal levels one week post partum.

Cigarette smoking on the other hand nearly doubles the clearance rate of caffeine (Rall, 1980; Dews et al., 1984).

The liver of the human fetus and neonate is relatively incapable of metabolizing caffeine (Yaffe and Juchau, 1974; Soyka, 1981; Rall, 1980), the half life in fetal plasma being 150 hours on average (Rall, 1980). As the liver matures, caffeine metabolism improves and actually surpasses adult rates after the first year of life. Older children in fact, metabolize caffeine two to three times faster than do adults (Dews et al., 1984; Aldridge et al., 1979).

Several investigators have reported that fetal liver is capable of converting maternally ingested theophylline to caffeine (Aranda et al., 1979; Brazier and Salle, 1981), and propose that this mechanism may contribute to the excessively slow rate of caffeine elimination from fetal tissues.

1.2.6 Pharmacological Effects

In view of the wide distribution of caffeine throughout the body, it follows that somatic responses are equally as diverse. As with nicotine, systemic responses to caffeine occur as the result of several different and sometimes opposing effects. A broad overview of these

effects is presented in Table (1). Caffeine is generally considered to be a stimulant. Though it influences almost all organ systems, the most prominant effects involve the central nervous and cardiovascular systems.

The exact mechanisms underlying these varied responses to caffeine are not fully understood. However, recent evidence suggests that they are mediated by the blockade of adenosine receptors by caffeine (Rall, 1980; Dews et al., 1984).

These receptors are located on both pre- and postsynaptic membranes and have a wide distribution within the central nervous system, as well as systemically. Furthermore, the sensitivity of these receptors to caffeine blockade varies, depending on both the tissue in which they are located and the extent to which they are occupied by adenosine (Rall, 1980; Dews et al., 1984). Though it appears that adenosine blockade is at least partially responsible for the pharmacological effects of caffeine, it seems likely that other mechanisms are also involved. These include phosphodiesterase inhibition, resulting in increased cAMP levels (Ward and Maisels, 1981), translocation of intracellular calcium, and the possibility that caffeine may decrease the uptake and metabolism of catecholamines in non-neural tissues (Rall, 1980).

1.2.7 Reproductive Effects of Caffeine

1.2.7.1 Embryotoxicity and Teratogenesis

TABLE I

PHARMACOLOGICAL EFFECTS OF CAFFEINE

SYSTEM	RESPONSE
1) Cardiac stimulation	- ↑ heart rate* - ↑ cardiac output*
2) Stimulation of the central nervous system at all levels	- ↑ transmission of impulses in nerves, across synapses, and at motor end plates* - antisoporific effect/improved mental acuity (low dose) - restlessness/hyperreflexia/ insomnia/tremors (moderate dose) - focal or generalized convulsions (high dose) - ↑ rate and depth of respiration*
3) Vascular effects	- vasodilation with decreased peripheral vascular resistance in systemic vessels (including coronary arteries) - vasoconstriction in cerebral vessels with ↑ cerebral vascular resistence/reduced blood flow and oxygen tension*
4) Muscular system	- relaxation of smooth muscle in locations other than vascular system, particularly the tracheobronchial tree - stimulation of skeletal muscle
5) Stimulation of the gastrointestinal system	- ↑ gastric HCL and fluid secretion*
6) Kidney	- ↑ renal blood flow* - inhibited Na reabsorption from proximal convoluted tubules, causing diuresis and ↑ urine output*

7) Multiple metabolic and endocrine effects	- promotes lipolysis, resulting in ↑ plasma free fatty acid levels* - stimulates catecholamine release from the sympatho-adrenal system (epinephrine/norepinephrine) - ↑ serum glucose levels* - augmentation of the basal metabolic rate - ? inhibition of insulin output
8) Hematologic function	- shortened coagulation time, with mild ↑ in clotting*

Adapted from (Rall, 1980; Howell et al., 1981; Ward and Maisels, 1981; Dews et al., 1984)

* ↑ indicates an increase.

Caffeine and other xanthine derived substances have been shown to have teratological effects in experimental animals. Though evidence regarding such an action in humans remains inconclusive, women are cautioned to refrain from consuming caffeine-containing beverages during pregnancy.

1.2.7.2 Animal Studies

Caffeine, at near toxic maternal levels was found to cause significant embryotoxicity and fetal abnormalities (Fujii and Nishimura, 1969). These investigators administered a single intraperitoneal injection of caffeine to ICR-JCL mice at dose levels of 200 and 250 mg/kg, on the 12th day of gestation. Fetuses were examined for any abnormalities on the 18th gestational day. Significant increases in embryotoxicity (evidenced by the number of resorptions) were observed in both treatment groups. Fetal malformations involved primarily the skeletal system and included cleft palate, micrognathia, and digital defects, in that order. With reference to the skeletal defects, of particular interest is that subcutaneous hematomas were observed in regions adjacent to the malformations, and that digital deformities tended to occur more in the left fore- and hindlimbs than in the right. Also, these effects were shown to be dose related. Fetal weight was significantly decreased in both treatment groups.

Similar observations were made by Scott (1983). In these experiments, CD-1 mice were subjected to intraperitoneal injection of caffeine at doses ranging from 80 to 250 mg/kg on day 11 and 12 of pregnancy. A clear dose response relationship was observed with regards to both embryolethality and skeletal malformations. While resorption rates at 80 to 150 mg/kg were essentially the same as in water injected controls, doses of 175-225 mg/kg, and 250 mg/kg were associated with significant resorption rates of 21-23%, and 52% respectively. Skeletal malformations were increased at all dose levels. These included mainly cleft palate, subcutaneous hematoma and digital malformations, specifically ectrodactyly, synarthrosis and a variety of other fusions. Limb malformations displayed the same asymmetric response reported by Fujii and Nishimura (1969).

In other studies, Sprague-Dawley rats were given an oral dose of caffeine equivalent to 180 and 330 mg/kg throughout pregnancy and the offspring examined near term (day 21) for evidence of adverse effects (Fujii and Nishimura, 1972). Embryotoxic effects, including fetal resorptions and decreased fetal weight, were found in both treatment groups. Animals receiving 330 mg/kg/day also revealed significant delays in skeletal ossification, generalized fetal edema as well as various visceral malformations, including hydrocephaly, intravenous

hemorrhage, misplaced esophagus and ventricular septal defect. Placental examination showed a marked congestion of maternal capillaries in these animals.

An oral dose of 30 mg/kg/day of caffeine administered throughout pregnancy was also found by Palm et al. (1978) to produce developmental anomalies in the offspring. Among the observed defects were poor development of the renal pelvis, as well as significant reductions in fetal organ weights, especially the brain, lungs and liver.

Nolen (1981) found no teratogenic or embryolethal effects in the offspring of Sprague-Dawley rats given oral caffeine doses of 20, 40 or 80 mg/kg/day. Fetal weights were not significantly reduced, and the only evidence of fetal toxicity was a delay in sternal ossification.

Kimmel et al., (1982) reported abnormal cartilage development, as well as delayed ossification patterns as a result of a single intraperitoneal injection (dose level, 38 mg/kg) given on day 11 of gestation.

Tanaka et al. (1984) found a decrease in mean fetal cerebral and body weights related to daily maternal caffeine ingestion during pregnancy. An interesting aspect of these studies is that some of the female rats were subjected to caffeine in their drinking water for 134 days prior to mating, as well as for the duration of their pregnancies. Based on experimental results from these and additional studies, these authors propose that prolonged, relatively

low oral doses of caffeine prior to pregnancy may contribute to reduced fetal growth, and that reduction in cerebral weight in combination with low body weight occurs as a result of an acute teratogenic effect of caffeine given throughout the gestational period.

In studies by Fujii et al. (1969) the adverse effects of caffeine were shown to vary according to the mode of administration. Caffeine was administered to pregnant mice on day 12 of gestation by four different methods. A dose of 200 mg/kg was injected once, intraperitoneally, and once subcutaneously. In 2 additional groups, this dose was divided into two injections (100 mg/kg each) and given intraperitoneally at 2 or 4 hour intervals. The single subcutaneous injection had a more severe embryolethal effect than that of a single intraperitoneal injection, whereas the teratogenic effects were the same in both groups.

In comparison among the intraperitoneally injected groups, a single high dose of caffeine caused a greater incidence of malformations, but lower embryolethality than two lower doses given 2 or 4 hours apart. Subcutaneous administration of caffeine results in a slower absorption rate than with intraperitoneal injection (Fujii et al., 1969). These results indicate that in the mouse embryo, whereas lethality is related to the duration of caffeine exposure, teratologic effects are more dependant on sufficiently high concentrations of the drug. The fetal

malformations observed were a high incidence of cleft palate and micrognathia, with the occasional occurrence of clubfoot. As in other studies, subcutaneous hematoma was usually found in close approximation to skeletal malformations.

In vitro experimentation on mouse blastocysts was conducted by Spindle and Wu (1985), to study the effects of caffeine on early embryonic development. Parameters evaluated were hatching, trophoblast outgrowth, inner cell mass growth and differentiation of the inner cell mass to form primary endoderm and ectoderm.

Caffeine interfered with blastocyst development in a dose related manner. The highest concentrations (4 mM) interfered with all 4 parameters of differentiation. Inner cell mass growth and differentiation were reduced at 2 mM, but hatching and trophoblast outgrowth remained unaffected. At 1 mM, only inner cell mass differentiation was reduced.

Cell proliferation was also evaluated in terms of cell numbers and was shown to be relatively insensitive to caffeine at concentrations below 2 mM. Thus, in addition to having teratogenic effects, caffeine may cause developmental failure at much earlier stages of development.

While the teratogenicity of caffeine is well established in experimental rodents, high doses of caffeine have also produced malformations in non-mammalian systems.

In the chick, doses of 400 and 1100 micrograms injected into the air sac at 48 h incubation resulted in a significantly high number of abnormal embryos. The most common embryonic defects seen included reduced body size, micropthalmia, exencephaly, everted viscera and short neck (Gilani et al., 1983).

The toxic and teratologic effects of caffeine on chick embryos explanted at stages four to seven, and cultured for 19-22 hours were investigated by Lee et al. (1982). Major findings of this study were that dose levels of 200 to 300 micrograms/ml significantly increased the incidence of neural tube defects, and that concentrations of 500 micrograms/ml or higher inhibited morphogenesis of nearly all organ primordia.

The developing neuroepithelium appears to be the most sensitive tissue, with caffeine concentrations of 400 micrograms/ml causing selective inhibition in the formation of the neural folds. Caffeine has an inhibitory action on the contractile activity of apical microfilament bundles in developing neuroepithelial cells. The inability of these cells to contract, could cause the neural tube to remain open, resulting in a defect (Lee et al., 1982).

In summary, caffeine ingestion during various stages of pregnancy has been found to cause skeletal abnormalities including cleft palate, various digital defects, delayed ossification and subcutaneous hematoma, decreased fetal size

and organ weights, various visceral anomalies and neural tube defects in experimental animals.

1.2.7.3 Human Studies

There is no conclusive evidence to associate caffeine consumption with adverse outcome of human pregnancy.

As part of the Ottawa Prenatal Prospective Study, Watkinson and Fried (1985) investigated the relationship between some pregnancy outcome variables and heavy maternal caffeine consumption during pregnancy. Heavy caffeine use was defined as consumption in excess of 300 mg/day. The most significant finding reported was that when caffeine intake was more than 300 mg/day, infants were of lower birthweight and had a smaller head circumference than those in the remaining sample. Also, this effect was persistent even when other factors such as cigarette smoking and alcohol consumption were statistically controlled. No relationship was found between the incidence of Caesarian sections, breech births, miscarriage or premature births (Watkinson and Fried, 1985).

Caffeine has a vasoconstrictive effect on placental blood flow (Kirkinen et al., 1983). Whereas the fetal umbilical vein flow was unchanged, an acute decrease was observed in intervillous placental flow in response to ingestion of two cups of coffee. In addition, maternal serum epinephrine levels were significantly elevated 30

minutes following caffeine ingestion. It is hypothesized that serum epinephrine levels and reduced placental blood flow may represent some risk potential to the fetus. These effects were confirmed in an animal model by Kimmel et al. (1984).

In a case control study of 2,030 malformed infants, Rosenberg et al. (1982) evaluated the relationship between maternal caffeine consumption and six selected birth defects. Following statistical correction for potential confounding factors, no correlation was found to exist between caffeine and inguinal hernia, cleft lip (with or without cleft palate), neural tube defects, cardiac malformations, pyloric stenosis or isolated cleft palate. These investigators concluded that caffeine is not teratogenic with regard to these six defects.

1.3 CAFFEINE AND NICOTINE

Nicotine and caffeine are two clear examples of physiologically addictive substances that are frequently abused, both in the general population and during pregnancy.

Individuals who consume excessive amounts of caffeine (especially from coffee), tend to smoke cigarettes as well (Martin, 1982; Cagguila et al., 1986). Watkinson and Fried (1985) demonstrated that this correlation persists during pregnancy. While in general, pregnant women decreased their

coffee consumption, heavy use was significantly correlated with heavier use of nicotine during pregnancy.

The adverse effects of cigarette smoking during pregnancy are well documented. Though there are no reports attributing caffeine to human birth defects, the teratogenic effects of this drug in experimental animals have led to increasing concern for the human conceptus.

Surprisingly, only a single study investigating the combined effects of caffeine and nicotine on the developing conceptus has been reported in the literature (Gilani and Persaud, 1986). Caffeine (1 mg) and nicotine (1 mg) dissolved in physiological saline was injected into the air sacs of fertile White Leghorn chick eggs, separately, or in combination at 48 or 72 h incubation. On day 13, the live embryos were removed from the eggs and examined for the presence of gross malformations.

At 48 h incubation, the combined treatment of caffeine and nicotine produced a higher incidence of embryonic death when compared to either of these substances administered alone. At 72 h incubation, the combined treatment resulted in significant increases in both teratogenicity and embryolethality compared to any other group.

These results indicate that a coteratogenic activity between caffeine and nicotine is evident at 72 h incubation. Whereas older embryos are less susceptible to damage following exposure to either caffeine or nicotine alone,

exposure to these two substances in combination resulted in a potentiation of their teratogenic effects. Malformations observed included edema, everted viscera, twisted limbs, reduced body size, short neck and abnormal tails.

1.3.1 Factors Determining the Teratogenicity of Drugs

The ability of a teratogenic agent to affect the developing embryo is influenced by several interdependant factors. The species being studied, the stage(s) of development during which effective exposure occurs, the route of administration and the degree to which sensitive cell populations within the maternal-embryo-placental unit react are all important variables that must be considered (Wilson, 1974). According to Wilson (1964), all chemicals are capable of producing some embryotoxic effect if given in adequate dosages during critical stages of development, to appropriately sensitive species.

Of particular importance with regards to mammalian development is the influence of maternal biological mechanisms in determining the ultimate level and duration of exposure. These include maternal metabolism, placental transport and in the later stages of gestation, the ability of fetal liver to metabolize a transported drug.

Any given chemical may have multiple sites of action in both maternal and embryonic tissues, for example, in body organs such as liver and kidney, in the gut, placenta, at

plasma protein binding sites, or at various effective receptor sites (Skalko, 1985; Skalko and Kwasigroch, 1983).

1.3.2 Chemical Interactions

In humans, exposure is rarely an isolated event, and the conceptus is frequently exposed to a variety of potentially toxic agents in the form of prescription drugs, over-the-counter pharmaceutical preparations, environmental pollutants and socially abused drugs (Ross and Persaud, 1986; Skalko et al., 1984).

Though the mechanisms related to the pathogenesis of congenital malformations are poorly understood, continued interest has focused on the role of chemical interactions. This refers to the ability of one chemical or agent to alter the biological effects of another by either prior or simultaneous administration (Skalko, 1985).

Based on research done by Hartshorn (1976) and Cadwallader (1983), the types of interactions were summarized by Skalko (1985) as follows:

1) Homergic - two chemicals produce the same effect.
   a) summation
   b) additive
2) Heterergic - only one pair of chemicals produces an effect.
   a) synergism
   b) antagonism

1.3.3 Classification of Teratogens

Concurrent administration of various chemical agents, normal metabolites or altered physiological states can all alter or modify embryotoxic effects. The ability of one agent to modify the response of another is further dependant upon the dosages employed, the developmental stage and duration of exposure, as well as the time sequence between treatments (Skalko, 1985). Attempts to describe this effect have resulted in some confusion in terminology among researchers.

Skalko and Kwasigroch (1983) proposed that one chemical under study be designated as the "primary teratogen", and should be given at minimally embryotoxic doses. The other agent, or "secondary teratogen", should be administered at essentially noneffective doses. In this way, these researchers were able to gauge the ability of how one substance (the secondary teratogen) could amplify the effects of the other (primary teratogen).

Some compounds, when co-administered with a known teratogen, have been shown to antagonize the action of that primary teratogen, and either reduce, or prevent the occurrence of malformations. These substances have been defined by Salko (1985) as antiteratogens. Fujii (1976) demonstrated that the beta-adrenergic blocking agent propranolol significantly reduced caffeine induced fetopathy when administered 60 minutes prior to caffeine treatment.

Some normal metabolites are also antiteratogenic (Beaudoin, 1976; Landauer, 1979).

1.3.4 Potentiation

Whereas in some cases caffeine can be described as an antiteratogen (Skalko, 1985; Fujii, 1976), it is also a known primary teratogen (Fujii and Nishimura, 1969 and 1972; Fujii et al., 1969; Scott, 1983; Lee et al., 1982; Gilani et al., 1983; Gilani and Persaud, 1986; Nolen, 1981).

Potentiation is defined as "the enhanced effect occurring when the combined action of two agents exceeds the sum of their separate actions" (Ritter et al., 1982). Wilson (1964) has suggested that potentiative interactions between low level teratogens may be responsible for some birth defects of unknown etiology. Yielding et al. (1976) reported that low doses of caffeine significantly enhanced the incidence of cleft palate in mice following exposure to x-rays.

In other experiments, caffeine was shown to potentiate the embryotoxic and teratogenic effects of various inhibitors of DNA, RNA and protein synthesis in rats (Ritter et al., 1982), and 5-bromodeoxyuridine (Kwasigroch and Skalko, 1985), mitomycin C (Nakatsuka et al., 1983; Fujii and Nakatsuka, 1983), phenytoin (Skalko et al., 1984), and chlorambucil (Fujii and Nakatsuka, 1983) in mice.

Ross and Persaud (1986) have implicated that caffeine may potentiate the effect of alcohol, resulting in impaired cardiovascular morphogenesis.

The fetopathic effects of caffeine (as a primary teratogen) were shown by Hayasaka and Fujii (1977) to be significantly enhanced by combined treatment with cocaine or pargyline.

Thus, it can be seen that caffeine will display different abilities depending on the chemical environment in which it is found. According to definitions outlined by Skalko (1985), caffeine displays both heterergic effects of synergism and antagonism, and therefore may be designated as a "coteratogen". Positive coteratogen refers to the potentiative (or synergistic) effects of caffeine, while negative coteratogen refers to its antagonistic (or antiteratogenic) properties. The ultimate coteratogenic effect of caffeine, positive or negative, is determined by the nature of the primary teratogen with which it is combined (Skalko, 1985).

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Throughout this investigation, virgin albino rats (weighing between 200 and 300 grams) of the Sprague-Dawley strain were used. These animals were obtained from "Charle's River Canada Incorporated", Saint Constant, Quebec.

Prior to being utilized for experimental purposes, the animals were left undisturbed for a minimal period of seven days, to allow them to acclimatize to their new environment.

2.1.1 Environment

All animals were housed in an environmentally controlled room, in spacious wire mesh cages.

Temperature was kept constant at 22 degrees Centigrade, (+/- 1 degree C.) with a relative humidity of 50% (+/- 10%). A cycle of twelve hours of light, from 0800 to 2000 hours, and twelve hours of darkness, from 2000 to 0800 hours was also maintained throughout this study.

Refuse pans beneath the cages were cleaned daily.

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

Treatment and control animals were pair-fed by the investigator from day 6 through 12 of gestation, inclusive. For the remainder of the experimental period, food pellets were available ad libitum.

2.1.2 Breeding

One male albino rat (Sprague-Dawley strain) was introduced into a cage with two female rats at 1530 hours (+/- 30 min.), and remained there overnight. At 0900 hours (+/- 30 min.) the following morning, the males were returned to their separate cages. Vaginal smears were then taken.

2.1.3 Determination of Pregnancy

Pregnancy was determined by the presence of a vaginal plug in the refuse pan below the cages, and confirmed by the presence of spermatozoa in the vaginal smear.

2.2 EXPERIMENTAL DESIGN

Once pregnancy was confirmed, animals were randomly assigned to one of six treatment groups, summarized as follows:

Group 1 Untreated controls

Group 2 Nicotine treated

Group 3 Control to nicotine

Group 4 Caffeine treated

Group 5 Control to caffeine

Group 6 Caffeine and nicotine treated

Each treatment group consisted of 10 dams, for an overall total of 60 experimental animals.

In all cases, treatment was administered at 0900 hours (+/- 30 minutes), on gestational day 6.

The animals were killed between 0800 and 1000 hours on gestational day 20.

2.2.1 Animal Handling

All treatment animals were exposed to constant environmental conditions. They were handled only by this investigator and one technician for the purposes of obtaining weights, administration of treatment and autopsy.

Animals were weighed on day 1 of pregnancy and left undisturbed until the sixth day. On gestational day 6, the animals were weighed again, anaesthetized with ether, and their respective treatments administered. Post anaesthetically, they were observed for a period of approximately 30 minutes to determine any possible deleterious effects.

Further handling was restricted to obtaining maternal weights on the ninth, twelfth and twentieth days of gestation.

2.2.2 Nicotine Treatment

Nicotine was administered to the pregnant animals over a 7 day period, from gestational day 6 through 12.

The mode of administration was via an "ALZET mini-osmotic pump" (Alza Corporation), implanted subcutaneously.

The osmotic mini-pump is a miniature self-powered pump designed to deliver test agents to experimental animals at a constant controlled rate, over a fixed period of time.

Though several models exist, the one utilized in this investigation was the "2001 Model", delivering a volume of 1 microlitre/hour, (standard deviation: 0.06 microlitres/hr), over a period of 7 days.

The mean fill volume of the mini-pump was 225 microlitres, with a standard deviation of 14 microlitres. The dosage was calculated to ensure that animals would receive a total dose of 25 mg nicotine over the seven day treatment period. The mini-pump delivery rate of nicotine was 149 micrograms/hour.

A stock solution was prepared mixing 1.0 ml of pure nicotine (C10 H14 N2; Eastman Kodak Co.) with 3.5 ml of sterile physiological saline (0.9% NaCl injection). This solution was then refridgerated for subsequent use in the mini-pumps. Each mini-pump utilized was filled on the same day it was to be implanted. At that time, the stock solution was further diluted 1:1 with physiological saline to achieve the above mentioned doses.

The pumps were filled according to the guidelines outlined by the Alza Corporation, and then subcutaneously implanted between the scapulae of the animals. After surgical implantation, further handling of the experimental

animals was unnecessary due to the unique design of the mini-pump.

2.2.3 Caffeine Treatment

Caffeine was given to the experimental animals as a single, intravenous injection, on the morning of the 6th day of pregnancy. The injected volume varied according to the weight of the animal on the day of treatment. Dose level was 25 mg/kg of body weight.

As with nicotine, a stock solution was made, utilizing pure caffeine (C8 H10 N4 O2, Eastman Kodak Co.), and physiological saline. This was stored at room temperature in a light shielded sterile injection bottle.

Caffeine, is known to have a low solubility (Goodman and Gilman, 1980). To determine an appropriate concentration of stock solution to be used, a small pilot study was undertaken.

Four separate solutions were made, mixing 1 ml of physiological saline with 100 mg, 50 mg, 25 mg, and 12.5 mg of pure caffeine, respectively. These were then vigorously agitated for a period of approximately 10 minutes. Each solution was examined to determine the degree to which the caffeine dissolved.

With 100 and 50 mg caffeine, much of the substance remained in crystalline form. Twenty five and 12.5 mg caffeine dissolved completely in the solvent. The stock solution was made up corresponding to 25 mg of caffeine per

ml of saline, as this concentration correlated well with the proposed doses to be utilized for treament purposes.

On day 6 of pregnancy, the animals were weighed and the appropriate volume of solution to be injected was calculated. The animals were then anaesthetized with ether and a single injection was given intravenously into the dorsal tail vein using a 1 cc tuberculin syringe and a 30 1/2 guage sterile needle.

2.3 MATERIALS AND METHODS

2.3.1 Untreated Controls

The project was carried out in four stages. The first stage constituted the untreated control group.

Confirmed pregnant females were weighed and housed in separate cages. Food and water were available ad libitum. These animals remained relatively undisturbed throughout the experimental period.

At 0900 hours (+/- 30 min.) on the 20th day of gestation the animals were weighed and anaesthetized with ether. Laparotomy was done to expose the uterine horns. Total litter size and condition of the fetuses were assessed in situ.

At this time tissue samples of maternal liver and kidney were taken and fixed in 70% ethyl alcohol for histological studies.

The uterine horns were excised along the antimesometrial border to reveal the fetuses, embryonic

membranes and placentas. These were gently removed in totality from the uterus utilizing the blunt end of a pair of forceps.

Once removal of conceptuses was completed, the walls of the uterus were reflected back to clearly reveal the maternal decidua. Resorption sites were evaluated in situ and recorded. A single placenta was excised from the umbilical cord and preserved in 70% ethyl alcohol for histological studies. An incision along the dorsal surface of the membranes revealed the fetus. General fetal morphology was observed and recorded at this time. This procedure was repeated for each fetus of the litter.

Once recovered, fetuses and placentas were placed in Bouins fixative for subsequent teratological evaluation.

Depending on the size of the litter two fetuses were processed by the method of Dawson (1926) in order to study the skeletal system. Each fetus was anaesthetized with ether, and totally eviscerated of all internal organs through a small midline incision in the anterior abdominal wall. Following this procedure they were placed in 95% ethyl alcohol.

2.3.2 Nicotine Treated and Control Groups

Ten pregnant females were treated from gestational day 6 through 12. Nicotine was administered at a constant rate of 149 micrograms/hour using the Alzet mini-osmotic pump.

2.3.2.1 Implantation of the Pump

At 0900 hours on the sixth day of pregnancy, treatment animals were weighed and anaesthetized with ether. A small incision was made in the area between the scapulae. A subcutaneous pocket was created by inserting a pair of forceps into one end of the wound in order to insert the filled pump. The wound was closed with 2 stainless steel wound clips. Animals were closely observed for appoximately 30 minutes to establish any immediate adverse effects from the anaesthesia, and for postsurgical complications.

The control group, also consisting of ten animals, was treated in the same manner, except that the mini-osmotic pump was filled with physiological saline. Treated and control animals were pair-fed for the 7 day treatment period. Control animals were paired as closely as possible with treatment animals on the basis of maternal weight on the first day of pregnancy. The mean difference in maternal weight between nicotine treated and paired control was 7.2 grams, with a range from 0 to 25 grams.

Following implantation of the pump the animals were left undisturbed, except for feeding and when determining their weight on the 9th, 12th and 20th day of gestation.

2.3.3 Caffeine Treated and Control Groups

Ten pregnant animals were treated on the morning of gestational day 6. Each animal received a single,

intravenous injection of the prepared stock solution of caffeine. The animal was weighed and an appropriate volume of the solution was calculated corresponding to a dose of 25 mg/kg body weight.

Once the volume was established, the animal was anaesthetized with ether and the substance injected into the dorsal tail vein, using a tuberculin syringe fitted with a 30 1/2 gauge needle. Again, animals were closely observed immediately following treatment. This same procedure was followed for the control animals, except that they received an injection of physiological saline instead of caffeine.

Though caffeine treatment was given as a single injection, these animals and their controls were also pair-fed for a seven day period in the same manner as the nicotine and control treated groups. This was done to maintain experimental consistency and to allow more accurate comparison between various treatment groups.

Control animals were paired with treatment animals according to their weight on gestational day one. The mean weight differences between caffeine treated and control animals was 8.5 grams, with a range from 1 to 19 grams.

Animals were weighed in the morning on the 9th, 12th and 20th gestational days and killed on day 20 between 0800 and 1000 hours. Surgical procedure, tissue collection and recovery of the fetuses remained consistant for all experiments.

2.3.4 Combined Caffeine and Nicotine Treated Groups

One group of 10 animals received a combined treatment of caffeine and nicotine. Both drugs were administered in the morning of the 6th day of pregnancy. The animals received a single injection of caffeine intravenously into the dorsal tail vein (25 mg/kg of body weight). Nicotine (25 mg) was administered by means of an osmotic mini-pump as previously described.

Once the animals were weighed and their respective volume of caffeine calculated, they were anaesthetized with ether. The mini-osmotic pump was implanted first to ensure full anaesthesia throughout the procedure, after which the caffeine injections were given.

Post-surgical observation was carried out as with previous treatment groups. Maternal weights were recorded as previously described, and on the morning of the 20th day of pregnancy, the animals were killed.

Though control experiments for the caffeine and nicotine combined treatment groups were not undertaken, the daily food intake of these animals was recorded for future comparison.

2.3.5 Filling the Pumps

This was accomplished in accordance with the guidelines established by the Alza Corporation (1983). Surgical gloves were worn throughout this procedure. Nicotine treated animals received the "2001 model" pumps.

Following dilution of the nicotine stock solution, a volume of 0.3 ml was drawn into a 1 cc tuberculin syringe, using a 25 5/8 gauge sterile needle. All air bubbles were carefully eliminated from the syringe.

Next, the needle was replaced with the blunt tipped 25 gauge filling tube. With the pump held in an upright position the filling tube was inserted and the nicotine solution delivered into the pump. With the final insertion of the flow modulator, the pump was ready for implantation.

2.3.6 Pair Feeding

For the duration of the treatment period, experimental animals were pair-fed. On the morning of treatment, the nicotine treated animals received 50 grams of rodent pellets. The following morning, the amount of feed remaining in the trough was weighed again to calculate the amount of food eaten by the animal over a 24 hour period. Fifty grams of feed was then administered for the next 24 hour period. This procedure was repeated for the 7 day treatment period.

After implantation of the mini-pump or the saline injection, control animals received a 24 hour food allocation equal to the amount eaten by the treatment animal with which it was paired.

Because each treatment animal was matched with a specific control, it should become apparent that for all

experimental procedures, a lag time of at least 24 hours was necessary between treatment and control experiments.

2.4 TERATOLOGICAL EVALUATION

In all cases, the animals were killed on gestational day 20 to prevent the mothers from devouring any damaged offspring. At the time of hysterectomy, uterine horns were examined in situ for resorption sites. Fetal position within the uterine horns, as well as the number of live and dead fetuses, was also recorded.

2.4.1 External Evaluation

At the time of recovery, each fetus was scrutinized for external anomalies. Parameters assessed included:

a) Head size and shape

b) Orofacial development - eyes, ears, palate

c) Limb development

d) Vertebral column and tail. The fetuses were then fixed in Bouins solution for a minimal period of 2 weeks. After this period any remaining membranes were debrided, and the umbilical cords removed. Following this, individual crown-rump measurements and fetal weights were recorded. The placentas were also weighed.

2.4.2 Visceral Examination

Internal organs were studied using the Wilson razor blade technique (Wilson, 1964). Whole fetuses were sectioned (3-5 mm) in a cranio-caudal direction and the

slices sequentially examined under the dissecting microscope for any gross visceral abnormalities. Each organ was systematically visualized, and the findings recorded.

Samples of fetal liver and kidney were also collected for examination by light microscopy. Three litters per treatment group were randomly chosen to represent each group. From each of these litters, 2 fetuses were further chosen at random as being representative of each litter. Fetal tissues were transferred from the Bouins solution to 70% ethyl alcohol prior to histological processing.

2.4.3 Histological Preparation for Light Microscopy

Collected tissue samples were processed for routine light microscopy, blocked in paraffin, and cut into sections 7 micrometers thick using a Sorval JB-4 microtome. Once transferred to glass slides, the tissue was stained with hematoxylin and eosin, and cover slips applied.

2.4.4 Microscopic Examination

The sections were examined using a Nikon, binocular light microscope. All tissues were inspected for evidence indicating functional disturbances, vascular changes and degeneration.

Parameters evaluated in the maternal and fetal kidney included:

a) glomerular structure and vascularity, and

b) integrity of the nephron tubular system.

General structural characteristics were recorded, as well as abnormalities, such as glomerular disorganization, fibrinoid deposits, vacuolar degeneration in tubular epithelial linings, vascular congestion, and precipitates obstructing tubular lumen.

Evaluation of maternal and fetal liver focused on:

a) general organization of hepatic cords,

b) cellular integrity,

c) vascularity, and

d) appearance and structure of hepatic sinusoids.

Parameters utilized for placental evaluation are as follows:

a) decidua basalis and decidual plates,

b) integrity and function of intervillous spaces,

c) appearance and structure of chorionic villi (anchoring and branch villi) and

d) functional appearance of the chorionic plate.

The evaluation of each tissue sample was based on the comparative analysis between similar tissue types of different treatment groups. Results, however, are based on the subjective interpretation of the investigator.

2.4.5 Skeletal Staining

Two fetuses per litter (when available) were eviscerated and preserved in 95% ethyl alcohol at the time of recovery. In order to evaluate the skeletal system, the

staining methods described by Dawson were employed (Dawson, 1926).

The specimens were cleared in a solution of 1% KOH until the bones were clearly visible through the surrounding tissues. They were then transferred to a fresh solution of 1% KOH and the alizarin red stain added. Overstaining was corrected by storing the specimens in Mall's solution (79 parts distilled water, 20 parts glycerine, and 1 part KOH). The specimens were then transferred to solutions containing 30%, 50% and 70% glycerine respectively, and stored in pure glycerine. For storage in 100% glycerine, a small thymol crystal was added to prevent fungal contamination.

2.4.6 Skeletal Evaluation

Based on the literature, (Aliverti et al., 1979; Fritz and Hess, 1970; Walker and Wirtschafter, 1957) a skeletal scoring chart was devised (see Table 2), indicating the various ossification centers expected to be present in a 20 day rat fetus. Each specimen was examined with the aid of a dissecting microscope and scored according to the norms on the chart.

Ossification was scored as being either complete or delayed. Delayed centers were further classified as partial, or incomplete. Bony elements showing partial ossification were very pale staining, with only a faint transparent outline visible. Though poorly visualized these centers showed bony symmetry indicative of a normal

TABLE 2: SKELETAL SCORING CHART FOR COUNTING OSSIFICATION CENTERS IN THE RAT FETUS.

Lateral View	Right	Left
SKULL:		
Frontal		
Parietal		
Interparietal		
Supraoccipital		
Exoccipital		
Basoccipital		
Nasal		
Premaxilla		
Maxilla		
Ethmoid		
Presphenoid		
Alispehnoid		
Zygoma		
Mandible		
Atlas (Cl)		

Dorsolateral View		
VERTEBRAL COLUMN:	Centra	Arches
Cervical (7)		
Thoracic (13)		
Lumbar (6)		
Sacral (4)		
Caudal (28-30)		

GENERAL REMARKS: _____

	Right	Left
FORELIMBS:		
Clavicle		
Scapula		
Humerus		
Radius		
Ulna		
Metacarpus		
(I - V)		
Phalanges		
Ribs (13 pair)		

HINDLIMBS:	Right	Left
Femur		
Tibia		
Fibula		
Metatarsus (I-V)		
Phalanges		

Ventral View

STERNUM: _____

HYOID (BODY): _____

PELVIS:	Right	Left
Ilium		
Ischium		

developmental process in progress. In areas scored as being incomplete, ossification was present and well advanced in part of the bone, but was delayed on the opposite side resulting in a bony assymmetry. Incomplete ossification was limited primarily to the skull bones.

2.5 STATISTICAL ANALYSIS

Data corresponding to fetal weight and crown rump length was collected on 685 fetuses. The skeletal system of 104 additional fetuses was evaluated following alizarin red staining. These fetuses represented the offspring from all six groups. Maternal weight gain, placental weight, litter size, embryolethality and developmental defects were also recorded.

A one-way analysis of variance and Multiple Range Test were performed, using a designed computer program (ST41; Rollwagen, 1973). A file was created for each of the quantitative parameters (fetal weight, placental weight, crown-rump length, maternal weight gain and litter size) and a mean, standard deviation and standard deviation of the mean (standard error) were obtained for each group.

The number of observations, standard deviation and standard error were also obtained as a composite of the six groups. Finally, an analysis of variance and multiple comparisons were performed on the data. Duncan's test of significant studentized ranges for 5% and 1% level (new multiple-range test) was utilized in describing the data.

Data relating to skeletal development, embryolethality and incidence of developmental defects were subjected to Chi-square (X^2) tests (ST22), and multiple comparisons made between the groups. Inherent in the program (ST22) is the limitation that expected values cannot have a numerical value of zero. In view of the relatively low number of fetuses in each group that were examinend for skeletal development and the wide variability observed, Chi-square comparisons were not carried out on all skeletal parameters. Where feasible, the Chi-square test for 5% and 1% confidence limits was utilized. For the remaining parameters, the percentages of the number of fetuses studied in each group are given.

3. RESULTS

3. RESULTS

3.1 EMBRYOLETHALITY AND FETAL GROWTH

Embryolethality (Table 4) and fetal growth (Table 3) were not significantly affected by maternal exposure to nicotine, caffeine or the combination of these two drugs. No significant differences were found among any of the treatment groups with respect to the incidence of resorptions, fetal weight, crown-rump length or placental weight.

3.2 DEVELOPMENTAL DEFECTS

In general, the incidence of developmental defects observed either by external examination (visual inspection) or following Wilson sectioning was low (Table 5). Hydrocephalus occurred to varying degrees in all treatment groups, except for the combined nicotine and caffeine treated group. The incidence of hydrocephalus was significantly increased ($P<0.05$) in the fetuses of animals treated with nicotine, compared to both untreated and nicotine controls, as well as to the nicotine and caffeine treated animals (Figure 1). There were no visceral anomalies observed in any of the treatment groups.

3.3 HISTOLOGICAL EVALUATION

3.3.1 Maternal and Fetal Kidney

In animals treated with nicotine and nicotine and caffeine combined, several glomeruli appeared as dense disorganized basophilic bodies, congested with red blood

cells (RBC's), but devoid of any recognizable microscopic features (Figures 2, 3 and 4). Other glomeruli revealed fragmented capillaries enclosed within a Bowman's capsule (Figure 5). In the control animals, Bowman's capsule enclosed a well defined urinary space and distinct glomerular capillaries were observed, with occasional RBC's, in their lumina. Dense, basophilic nuclei were present within numerous glomerular capillary tufts in tissue sections from animals of all groups, and were therefore not considered to be abnormal.

Proximal and distal convoluted tubules were normal in all treatment groups, displaying cuboidal epithelial cells with prominent round nuclei. The granular cytoplasm of the proximal tubule epithelial cells demonstrated the deeper acidophilia and brush border characteristic of cells in this location, while distal tubular epithelial cells stained less intensely with eosin and demonstrated well defined intact cell borders. At the cortico-medullary junction, the ascending and descending limbs of the loop of Henle were not well preserved or visualized, due to tissue processing and shrinkage. Flat squamous to cuboidal epithelial cells appeared clustered together, while the lumina were generally collapsed.

The collecting tubules and ducts were easily recognizable in the renal medulla. Well defined cuboidal to columnar epithelial cells with distinct borders enclosing a

prominent nucleus were seen surrounding relatively wide, more irregular lumina. There were no intraluminal precipitates present in any part of the renal tubular system, nor any section showed evidence of the vacuolar degeneration of epithelial cell linings often indicative of functional disturbances.

Blood vessels throughout the kidney from animals of all treatment groups demonstrated normal connective tissue and smooth muscle walls lined by simple squamous endothelium.

Fetal kidneys were developmentally immature, but appeared normal in all treatment groups.

3.3.2 Maternal and Fetal Liver

Tissue sections of maternal liver from all animals displayed varying degrees of cellular degeneration. Focal cellular degenerative changes were evidenced by the appearance of scattered cells with pyknotic and sometimes fragmented nuclei. The cytoplasm appeared highly vacuolated in comparison to the majority of normal appearing hepatocytes surrounding them. These cells were pale staining and easily identified. In many instances, degenerative changes involved the majority of hepatocytes constituting an entire hepatic lobule. This hepatocellular degeneration resulted in the section having a "mottled" appearance, with pale and darker staining areas. No appreciable difference could be identified in the degree of these degenerative changes among any of the treatment

groups, approximately 50% of the animals in each group being equally affected.

Seven of the 10 animals treated with nicotine and caffeine (group VI) demonstrated dilatation of hepatic sinusoids and vascular congestion when compared with any other group. The sinusoids were excessively wide, especially in the area approximating the central veins and appeared congested with RBC's. In addition, the central veins in the majority of these animals were completely occluded with blood (Figures 6 and 7). These findings were also seen in the nicotine treated animals, but they occurred to a lesser degree and central venous congestion was not as apparent (Figure 8). The hepatic sinusoids and central veins of caffeine treated animals did not differ in appearance from those of their corresponding controls.

Hepatic cellular organization of fetal liver appeared normal. However, the sinusoidal dilatation and congestion observed to occur in maternal liver was also seen in the fetal liver of the nicotine and caffeine treated group primarily, as well as in animals treated with nicotine (Figure 9).

3.3.3 Placenta

The placentas from all treatment groups revealed variable degrees of degenerative changes, most evident in the decidua basalis and stem (anchoring) villi. Many of the decidual cells were pale staining, with highly vacuolated or

"empty" looking cytoplasm surrounding a pyknotic nucleus. These findings were considered to be the result of normal degenerative changes occurring in the near term placenta. In general, the number of anchoring villi seen in the placentas of nicotine treated animals was diminished relative to other treatment groups.

The decidua basalis of the nicotine, and nicotine and caffeine treated animals, was less developed, when compared with respective controls. Both of these groups were affected to a similar degree. Whereas the decidual cells generally appeared normal in all treatment groups, the decidua was notably thinner following nicotine and both nicotine and caffeine treatment (Figure 10 and 11). Similar findings were observed in the placentas of the caffeine treated animals; however these were minimal in comparison with those already mentioned.

Both nicotine treated and nicotine and caffeine treated animals also demonstrated congestion of intervillous spaces. This was most prominent at the placental margins, and at the interface between the maternal and fetal portions of the placenta, occurring in eight of the ten nicotine treated, and all ten of the combined treated animals. Definitive intervillous dilatation was not evident in these sections (Figure 12).

No evidence of pathological changes or abnormal development were observed in the fetal components of the

placenta in any of the treatment groups. Branch villi appeared normal, with fetal capillaries identified by the presence of RBC's in their lumina.

3.4 EVALUATION OF THE SKELETAL SYSTEM

Observations pertaining to the skeletal system are presented in Tables 6 through 16.

Both the axial skeleton (including the skull and facial bones, hyoid, mandible, sternum, ribs, vertebral column and pelvis), and the appendicular skeleton (including the clavicle, scapula, humerus, radius, ulna, metacarpus, femur, tibia, fibula, metatarsus and phalanges) were studied. Ossification patterns were highly variable among all fetuses examined.

Sternal ossification was affected in the combined nicotine and caffeine treated group of animals. The fetuses displayed a significant decrease ($P<0.05$) in the number of complete sternal ossification centers when compared to animals receiving either of the drugs individually, as well as their corresponding controls (Table 6).

Ossification of the skull bones (Table 7) was significantly delayed in the fetuses of the nicotine and caffeine treated animals ($P<0.05$) when compared with animals treated with only nicotine. In this group, all six fetuses showed partial ossification, evidenced by pale staining skull bones separated by wide cranial sutures. Incomplete ossification was limited specifically to the supraoccipital

bone, which appeared as two small triangular plates, often asymmetrical on right and left sides (Figure 13). In several of the fetuses, a faint irregular bridge of bone could be seen uniting these two plates, giving it a more characteristic dumbbell or bipartite shape. Complete ossification of the skull bones occurred significantly more frequently in the fetuses of nicotine treated animals, compared to their corresponding controls, being 100 and 50% respectively, however no difference was observed between the nicotine treated and the untreated control groups. With regards to the nicotine control animals, fetuses demonstrated an unusually high incidence of delayed ossification of the skull bones relative to any other group. Less variation occurred in the facial bones (Table 7), with 87.5% to 100% of all fetuses showing complete ossification.

Tables 8 and 9 represent a summary of vertebral ossification patterns. Ossification of vertebral arches was well advanced with 100% of all fetuses examined showing complete ossification centers at all levels of the cervical, thoracic and lumbar spines. The vertebral centra displayed complete ossification in the thoracic (exclusive of the first thoracic level; T1) and lumbar spines only, and were completely absent in the cervical spine.

Considerable variation was observed in the sacral area of the vertebral column. Whereas ossification was generally well advanced in the centra of the first three sacral

vertebrae (S1, S2 and S3) in all fetuses examined, it was generally delayed in the vertebral arches, being either partial or absent at all levels below S1. No apparent differences however, were identified among any of the treatment groups. Ossification centers in the vertebral centra and arches were generally absent in the majority of fetuses at the fourth sacral and caudal levels.

Examination of metacarpal and metatarsal ossification centers revealed symmetrical bony development in both forepaws and hindpaws of all treatment groups (Tables 11 and 12). The first metacarpus and metatarsus were completely absent in fetuses of all groups, while ossification of the second to fourth bones was generally well advanced. Ossification of the fifth metacarpus was not complete in any of the treatment groups. However, partial ossification was observed in the fetuses of the nicotine treated and in the corresponding control animals, as well as in the untreated control group. The frequency of partial ossification of the right and left metacarpal bones was generally lower in the nicotine treated group (being 16.7% and 11.1% respectively), compared to the nicotine controls (37.5% and 25% respectively) and the untreated control group (30% and 20% respectively).

A similar ossification pattern was observed with respect to the fifth metatarsal bone. Only the nicotine and nicotine control groups revealed any appreciable degree of

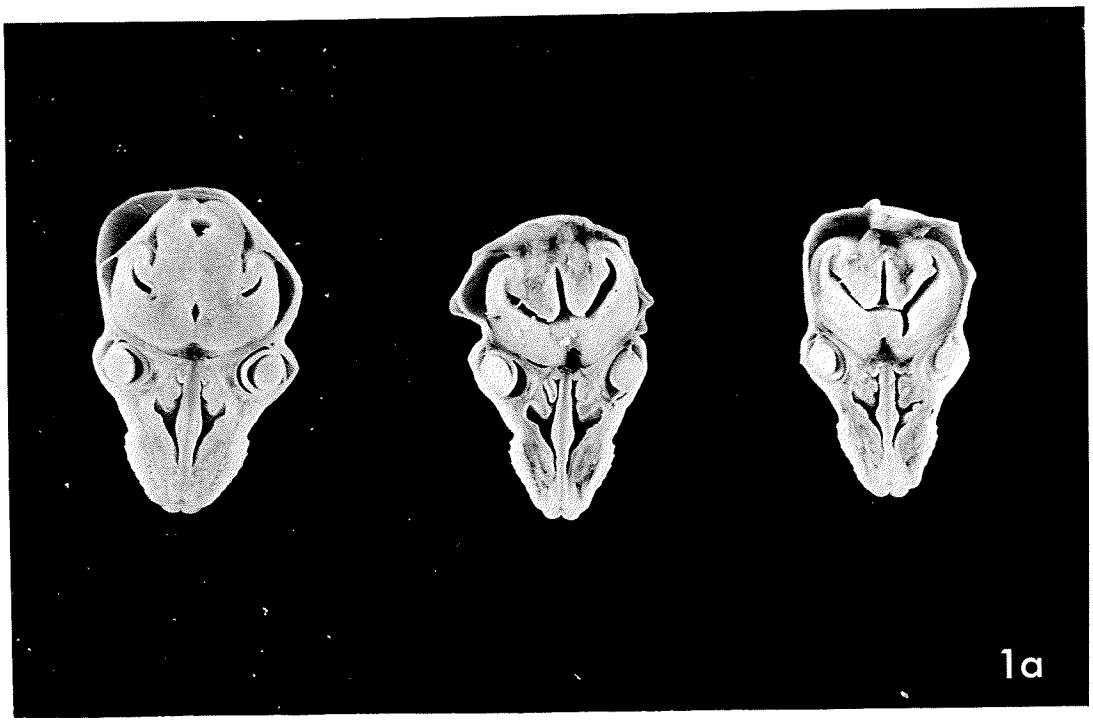
partial ossification of the right and left fifth metatarsal bones. When observed, the incidence of partial ossification was again lower in the nicotine treated group of animals, compared to the controls, being 27.8% and 62.5% respectively. The left hindpaw was missing in one fetus of the caffeine treated group, and showed no ossification centers at all in one fetus of the nicotine and caffeine treated group. Phalanges remained completely unossified in the forepaws and hindpaws of all fetuses examined (Table 13).

Bony development was abnormal in only three of the bones evaluated, these being the vertebrae, ribs and sternum. Bifid vertebral centra were observed in the thoracic and/or lumbar spines of 55% of the nicotine and caffeine treated group of animals. This defect did not occur in any other treatment group and the difference therefore was significant ($P<0.001$) (Figure 14). The occurrence of bifid sternal ossification centers was also restricted to the fetuses of nicotine and caffeine treated animals, however in this instance no significance was demonstrated (Table 10). A low incidence of wavy ribs occurred only in the nicotine and nicotine and caffeine treated groups. The difference, however, was not statistically significant (Table 16, Figure 15).

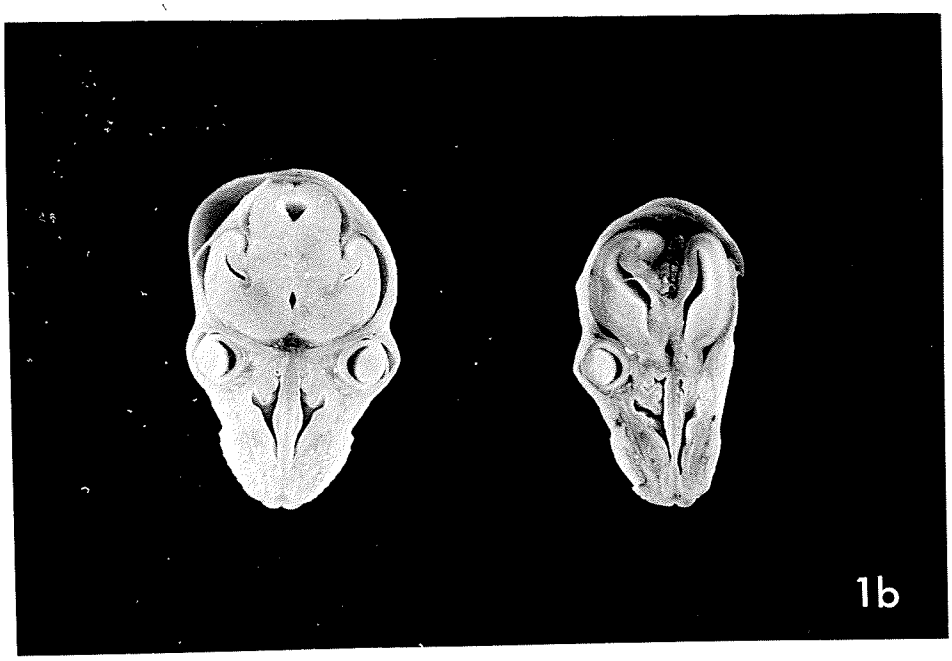
FIGURE: 1 Wilson's section through the head region of a 20 day rat fetus.

1a Left: Control animal.
Right: Nicotine treated animals showing dilatation of the lateral ventricles.

1b Left: Control animal.
Right: Nicotine treated animal. Oblique section clearly reveals hydrocephalus in lateral ventricles.



1a



1b

FIGURE: 2 Cross-section through the cortex of maternal kidney (Nicotine and caffeine treated animal, H and E stain).

Observe scattered dense, congested glomeruli devoid of any internal structure.

(X 180)

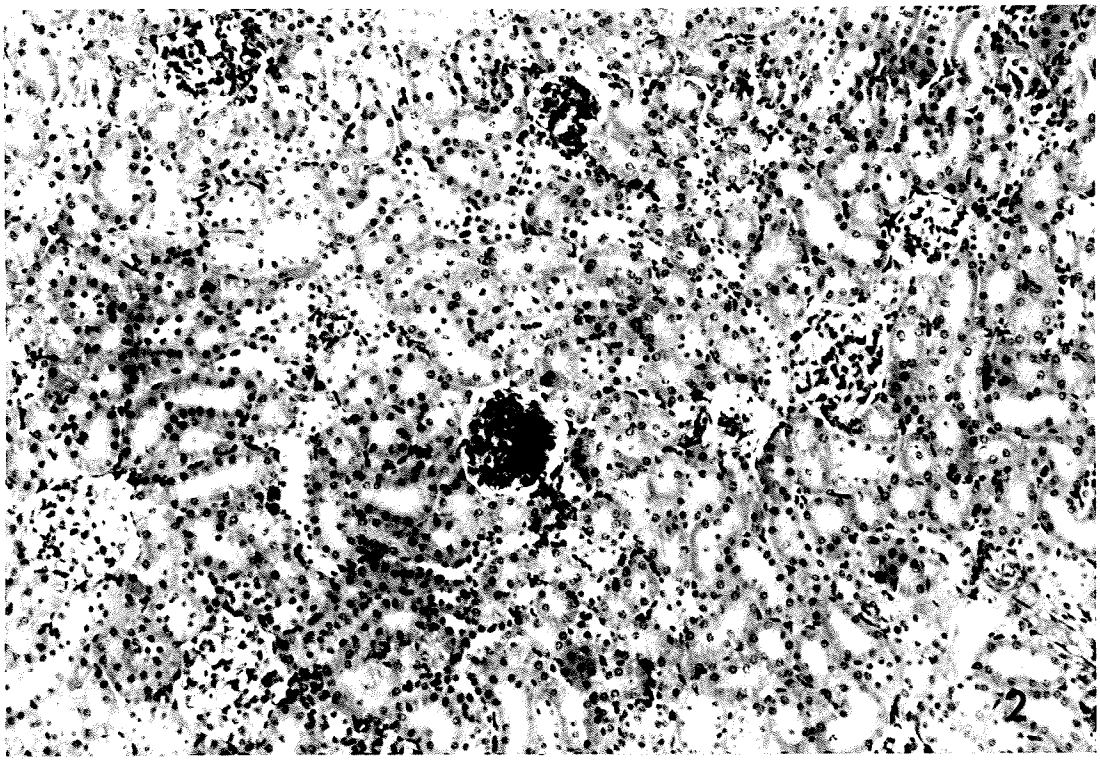
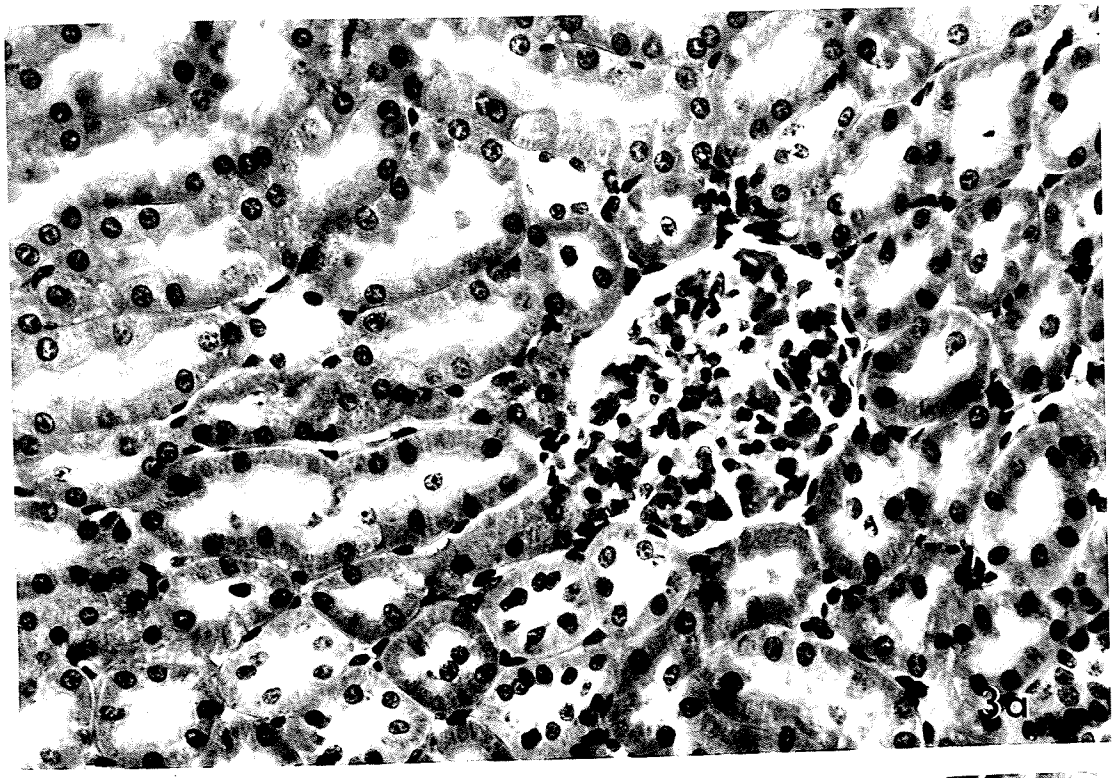

2

FIGURE: 3 Cross-section through the cortex of maternal kidney (H and E stain).

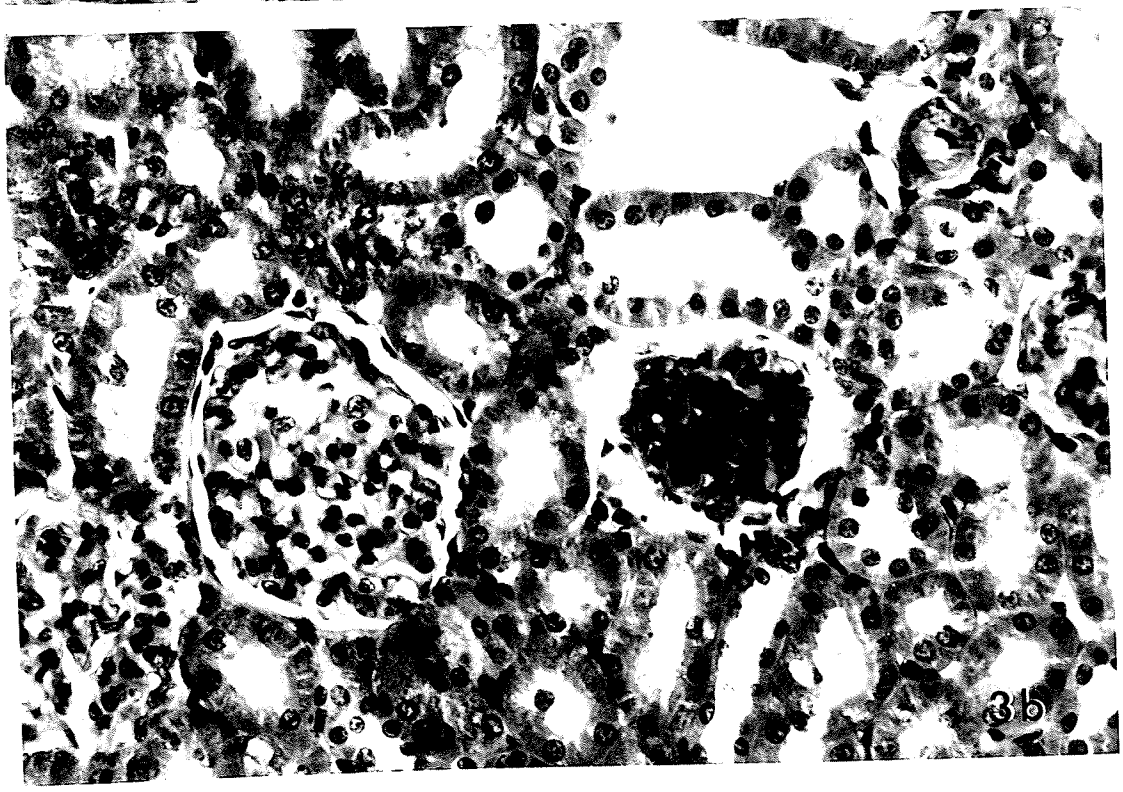
3a Control animal. (X 450)

3b Nicotine and caffeine treated animal. Observe dense, congested glomerulus on right. Compare with the normal appearing glomerulus on left, and with 3a.

(X 450)



3a

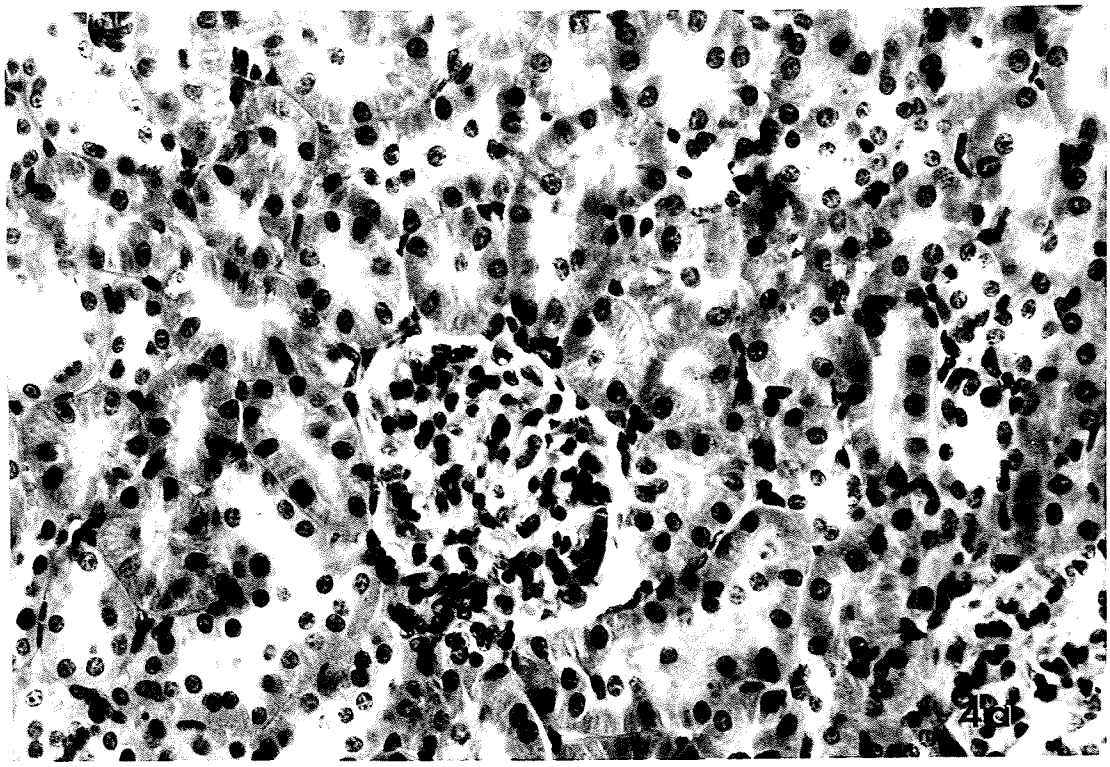


3b

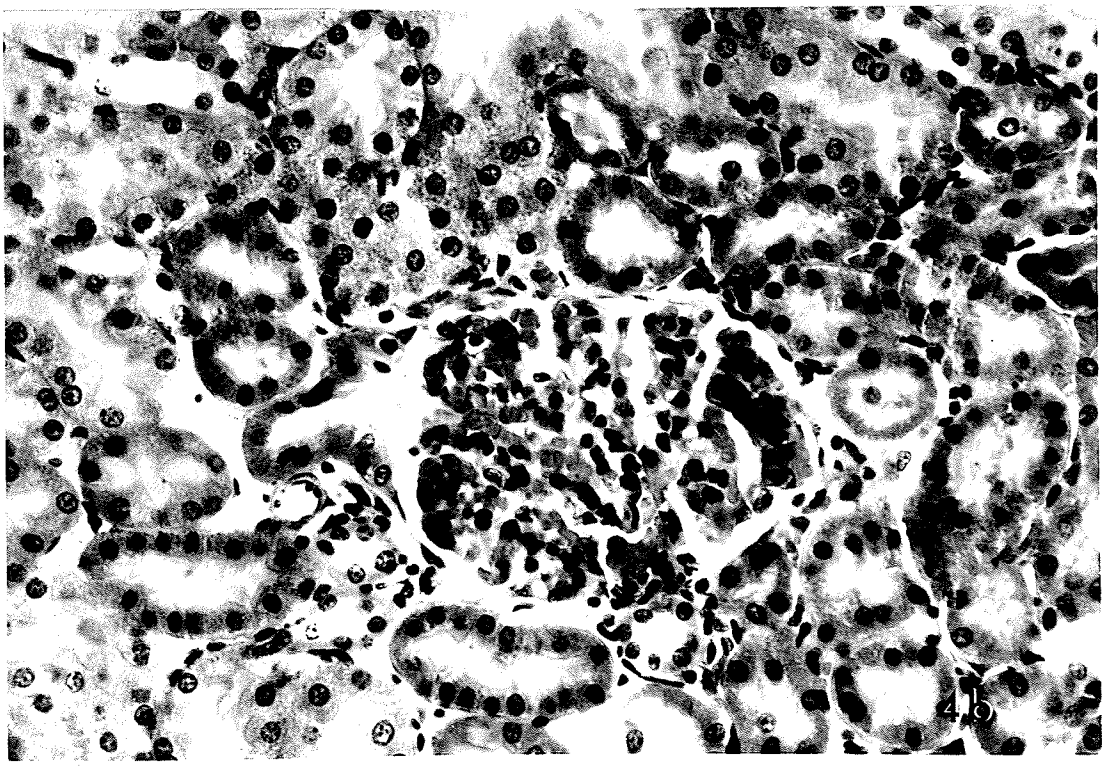
FIGURE: 4 Cross-section through the cortex of maternal kidney (H and E stain).

4a Control to nicotine. (X 450)

4b Nicotine treated animal. Observe the congestion of glomerular capillaries. (X 450)



4a



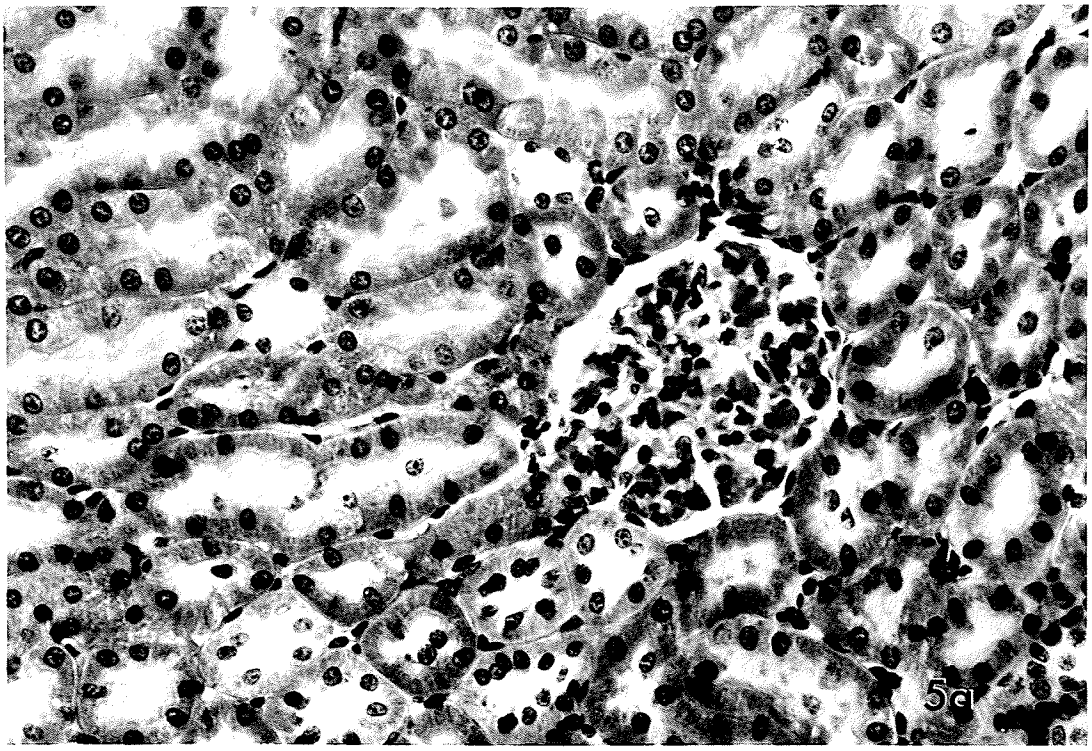
4b

FIGURE: 5 Cross-section through the cortex of maternal kidney (H and E stain).

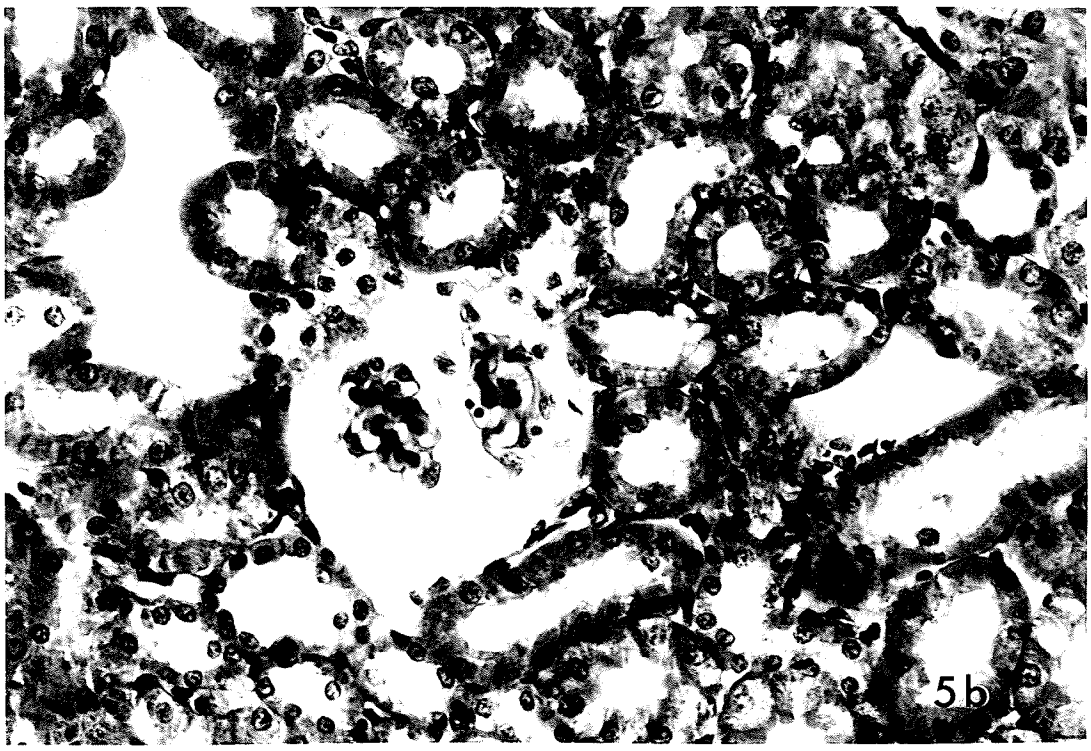
5a Control animal. (X 450)

5b Nicotine and caffeine treated animal showing fragmented capillaries and degeneration of the visceral layer of Bowman's capsule.

(X 450)



5a



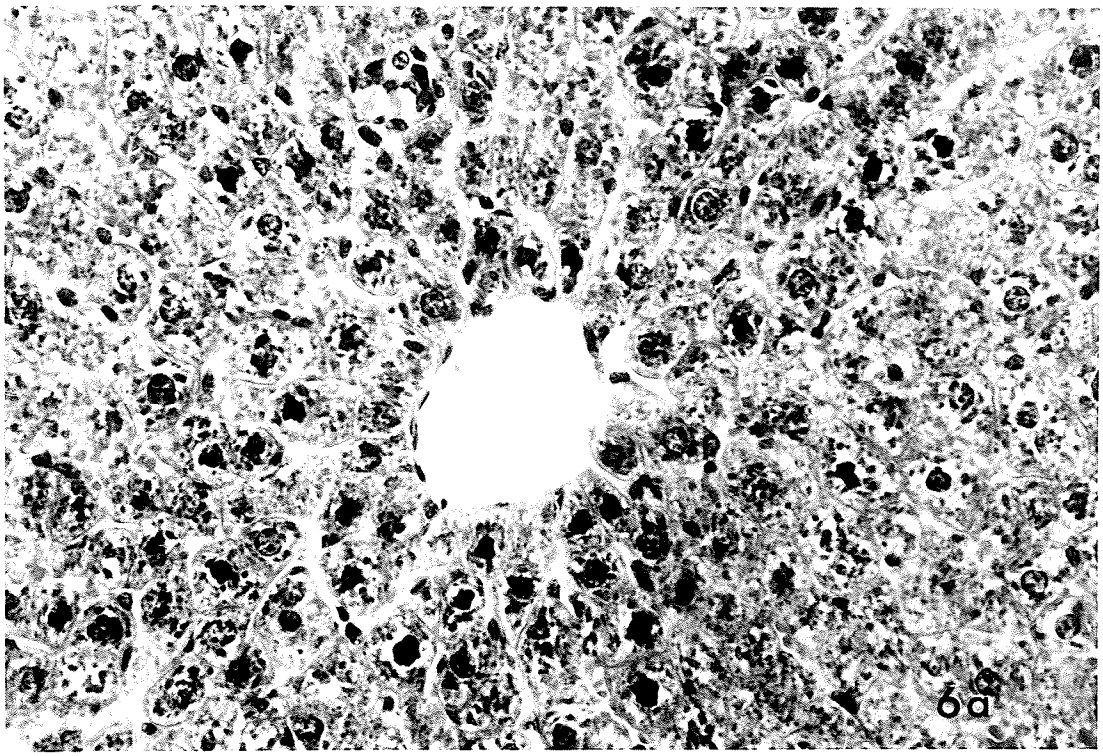
5b

FIGURE: 6 Maternal liver (H and E stain).

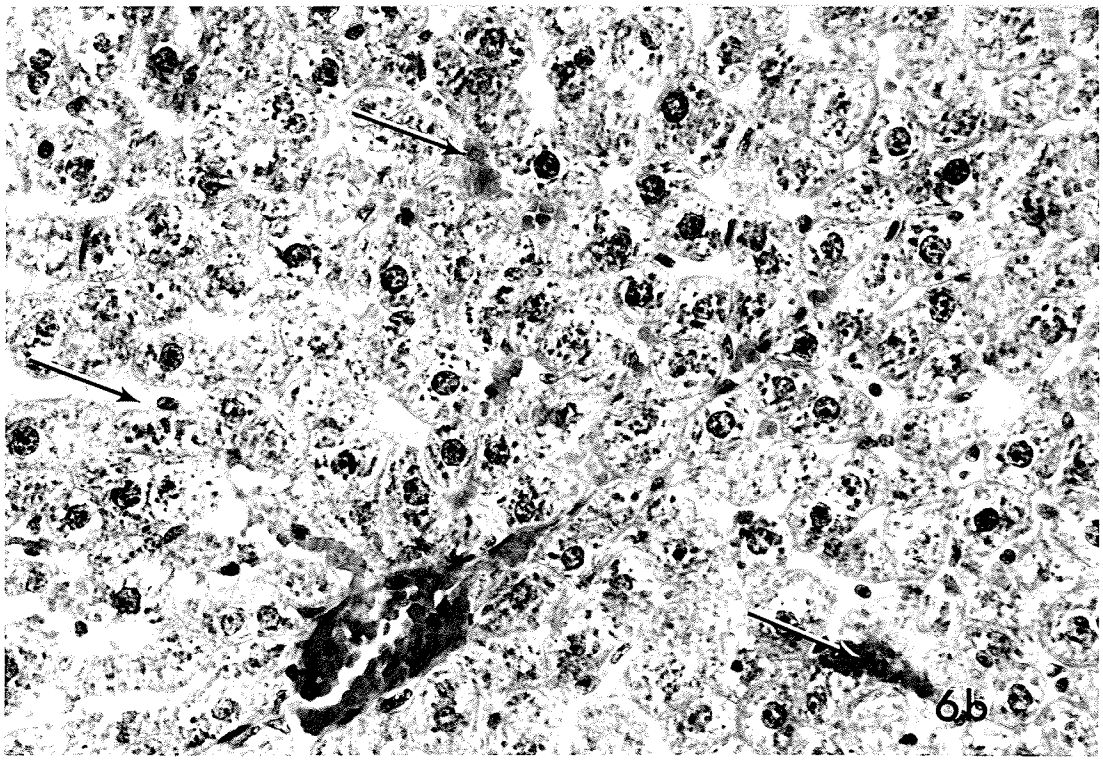
6a Control animal. (X 450)

6b Nicotine and caffeine treated animal. The sinusoids are dilated and congested with red blood cells (arrows).

(X 450)



6a



6b

FIGURE: 7 Maternal liver from a nicotine and caffeine treated animal, showing excessive dilatation of hepatic sinusoids (H and E stain).
CV: central vein

(X 450)

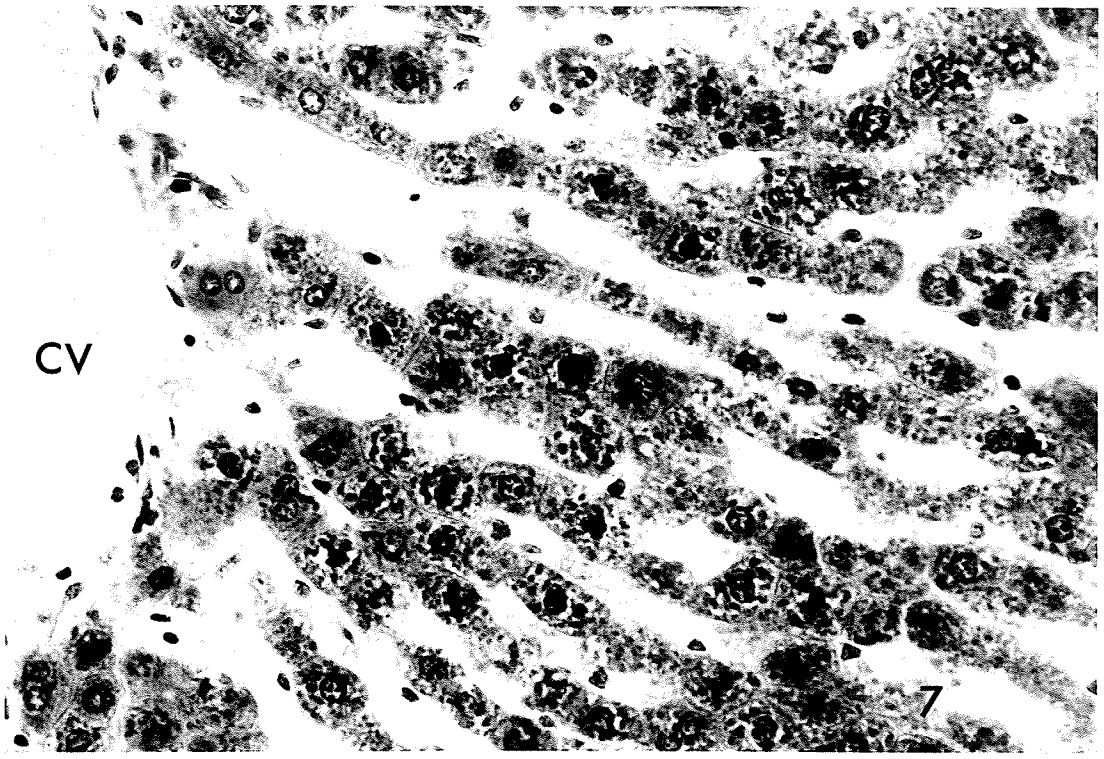
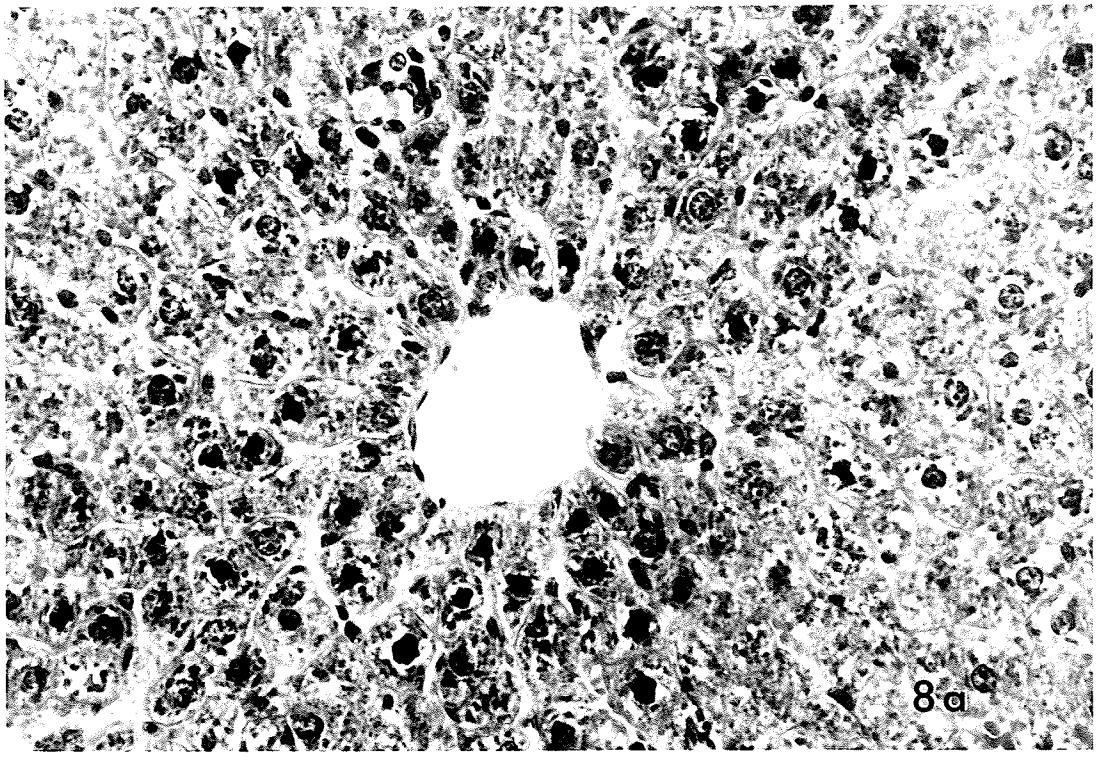

CV
7

FIGURE: 8 Maternal liver (H and E stain).

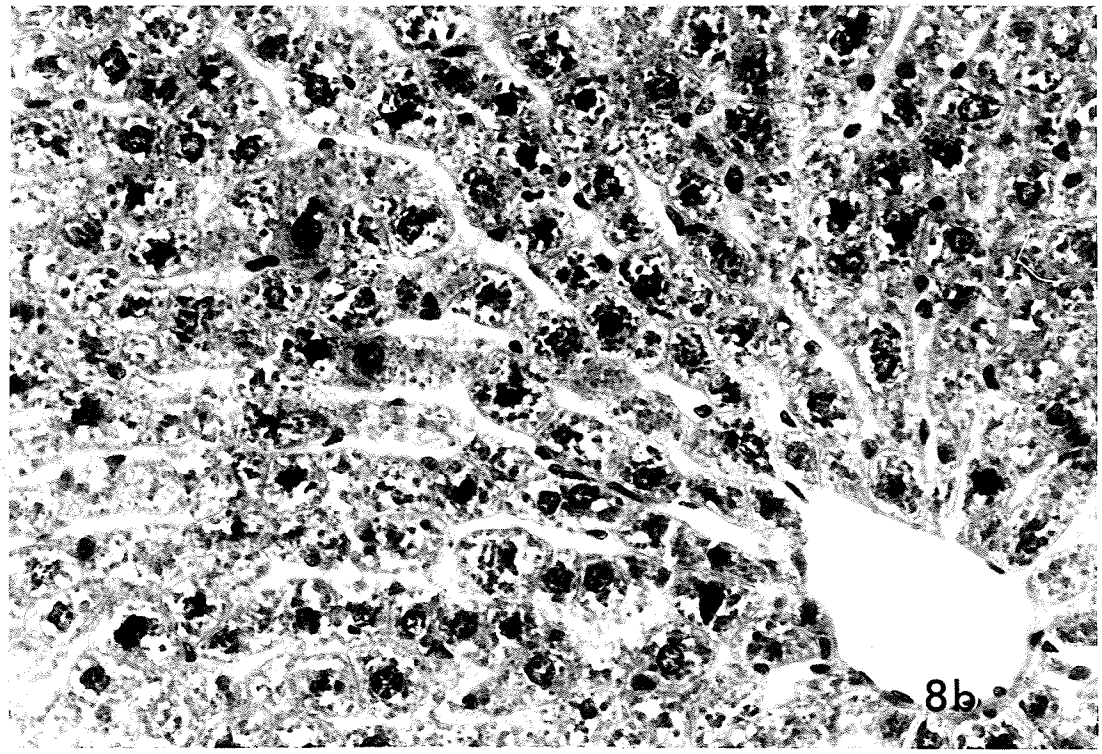
8a Control animal. (X 450)

8b Nicotine treated animal. Though sinusoidal dilatation is evident when compared with the control, it is not as marked as that observed in the nicotine and caffeine treated animals (Figures 6 and 7).

(X 450)



8a



8b

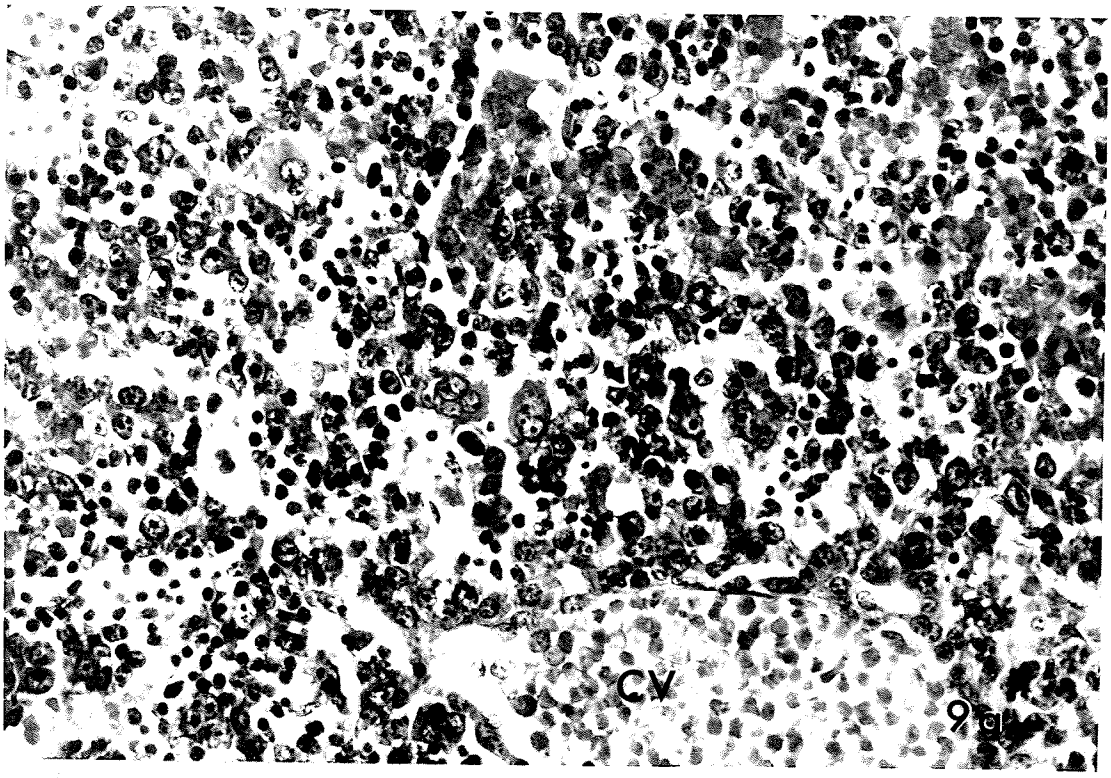
FIGURE: 9 Fetal liver (20 day old rat fetus, H and E stain).

9a Control fetus. Observe the incomplete organization of hepatocytes into hepatic cords, and greater vascularity characteristic of the immature fetal liver, and its role in red blood cell production.
CV: central vein

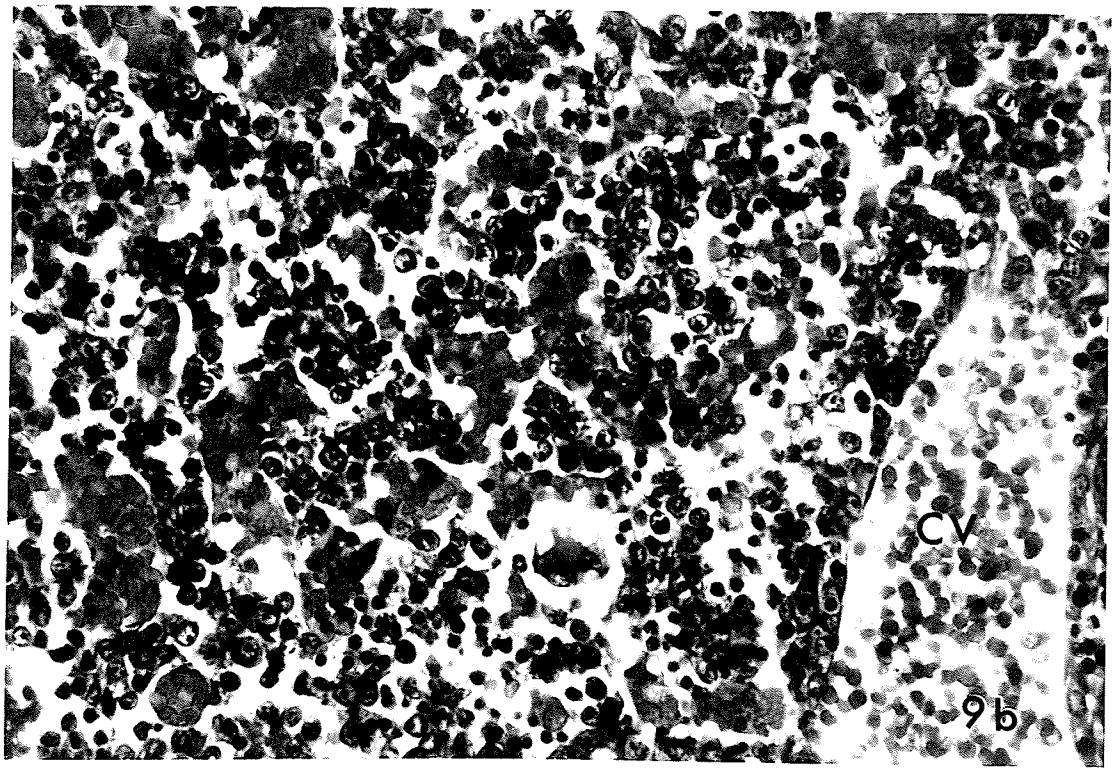
(X 450)

9b Nicotine and caffeine treated fetus. The sinusoidal dilatation and red blood cell congestion observed in maternal liver of nicotine and caffeine treated animals is also apparent in liver sections of their offspring.
CV: central vein

(X 450)



9a



9b

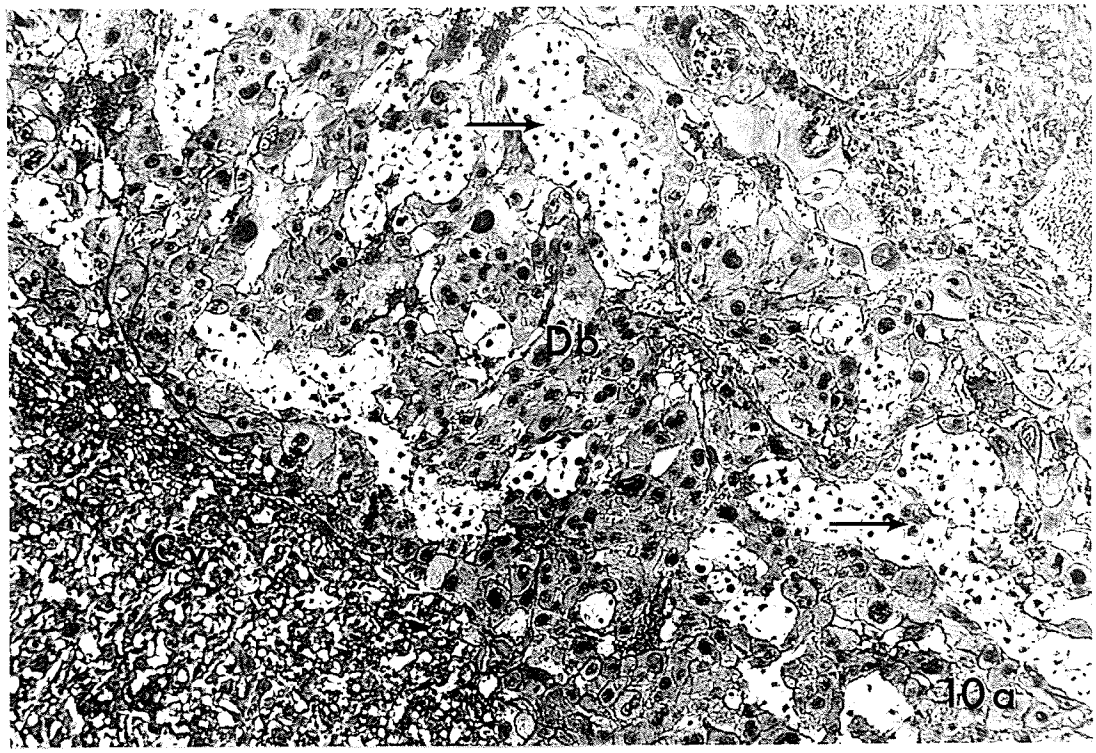
FIGURE: 10 Cross-section of the placenta in the region of the umbilical cord (H and E stain).

10a Control animal showing a well developed decidua basalis (Db) with varying degrees of degeneration (arrows). Chorionic villi (Cv) demarcating the fetal portion of the placenta.

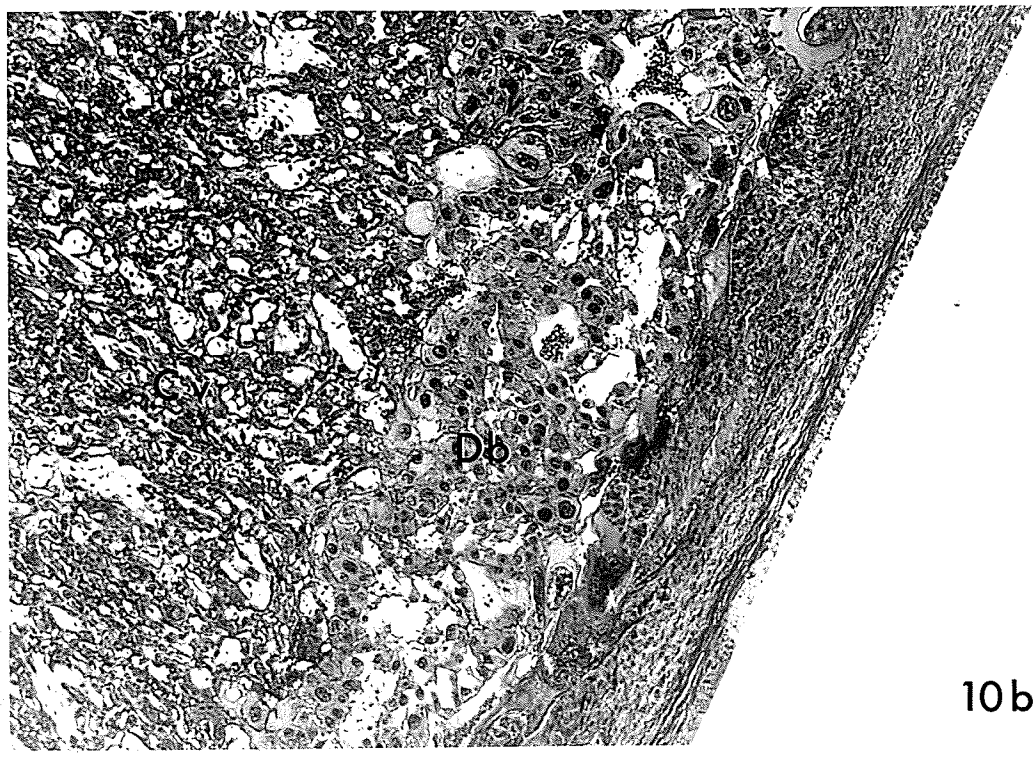
(X 113.4)

10b Nicotine treated animal. Note the thinner decidua basalis (Db) as compared with the control. Chorionic villi (Cv) demarcating the fetal portion of the placenta.

(X 113.4)



10a



10b

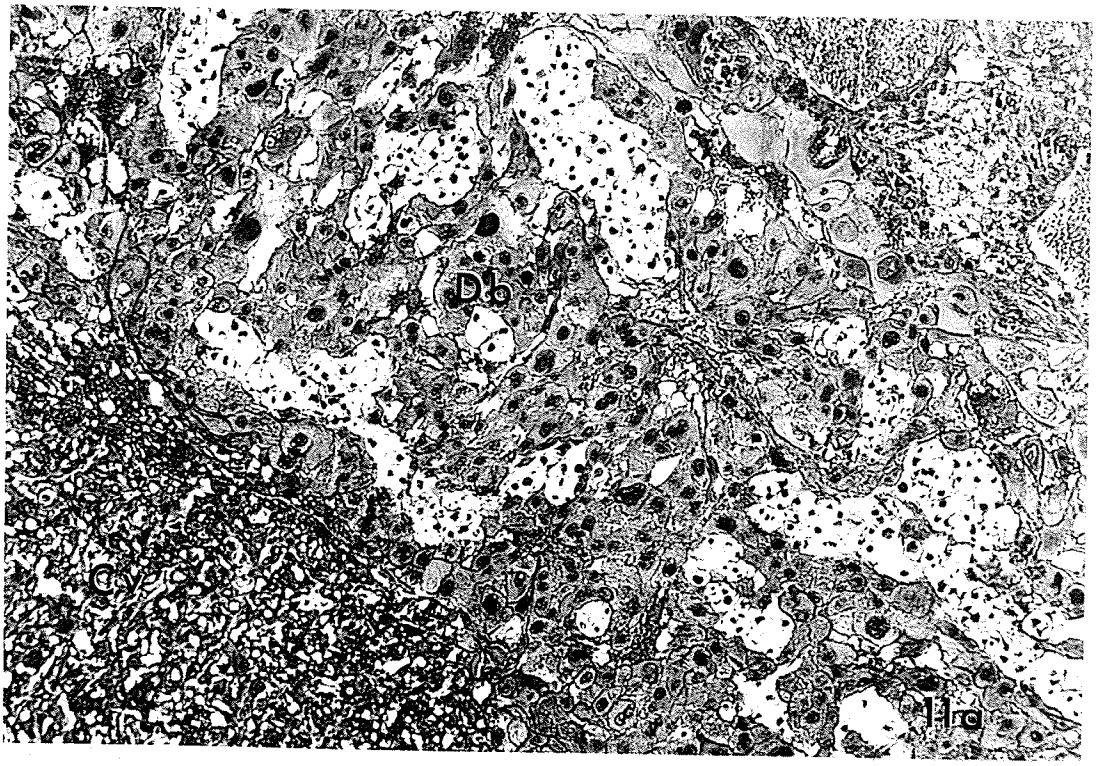
FIGURE: 11 Cross-section of the placenta in the region of the umbilical cord (H and E stain).

11a Control animal. Db: decidua basalis; Cv: chorionic villi.

(X 113.4)

11b Nicotine and caffeine treated animal. Note that the decidua basalis (Db) is notably thinner than that of the control. The chorionic villi (Cv) are evident.

(X 113.4)


Db
C
11a

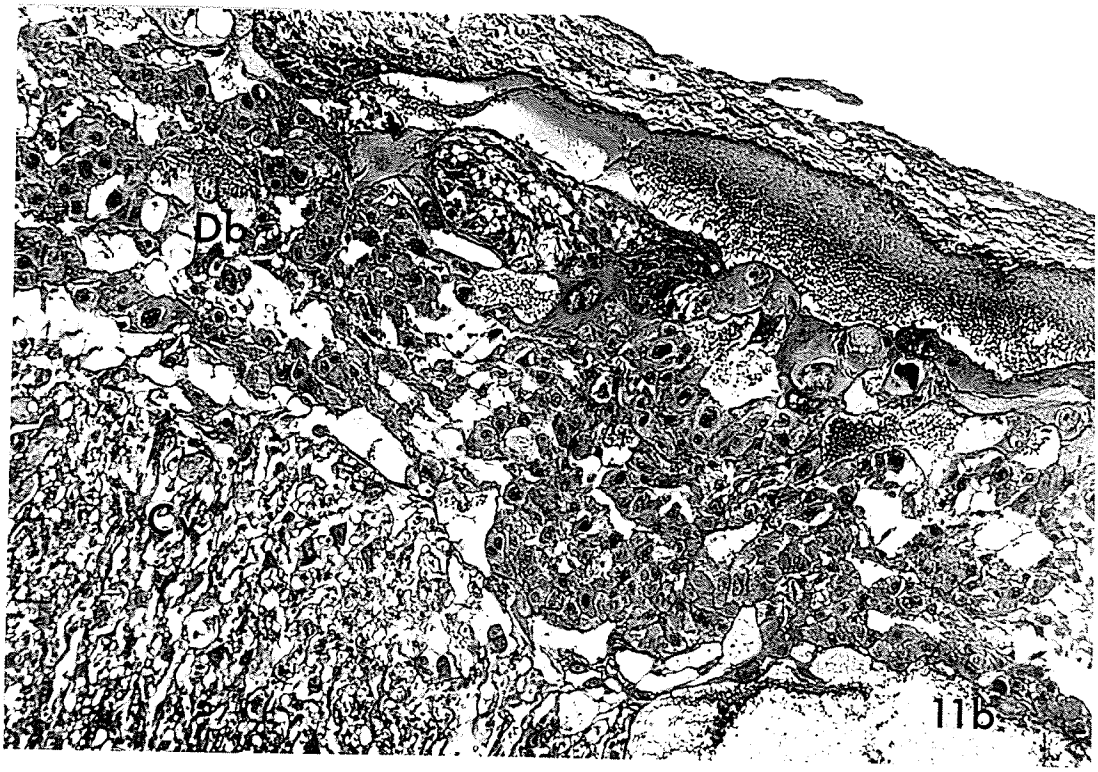
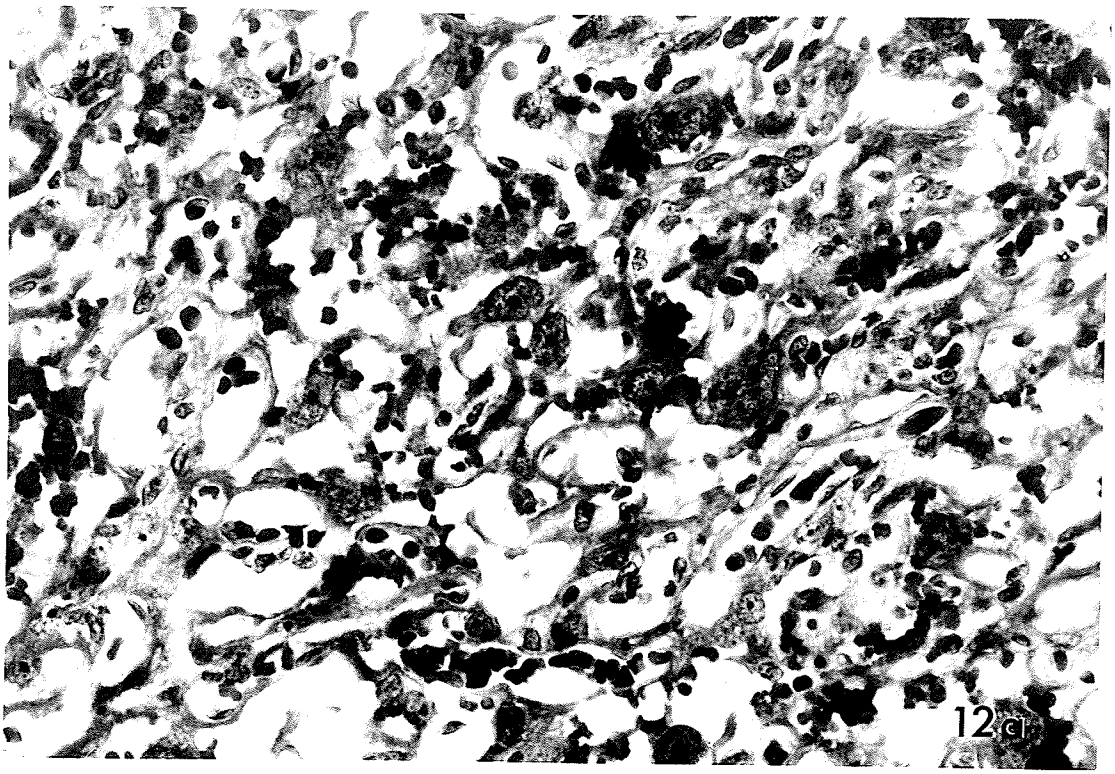

Db
C
11b

FIGURE: 12 Cross-section of placenta, chorionic villi and intervillous spaces (H and E stain).

12a Control animal. (X 450)

12b Nicotine and caffeine treated animal showing congestion of intervillous spaces with red blood cells (arrows). (X 450)


12a

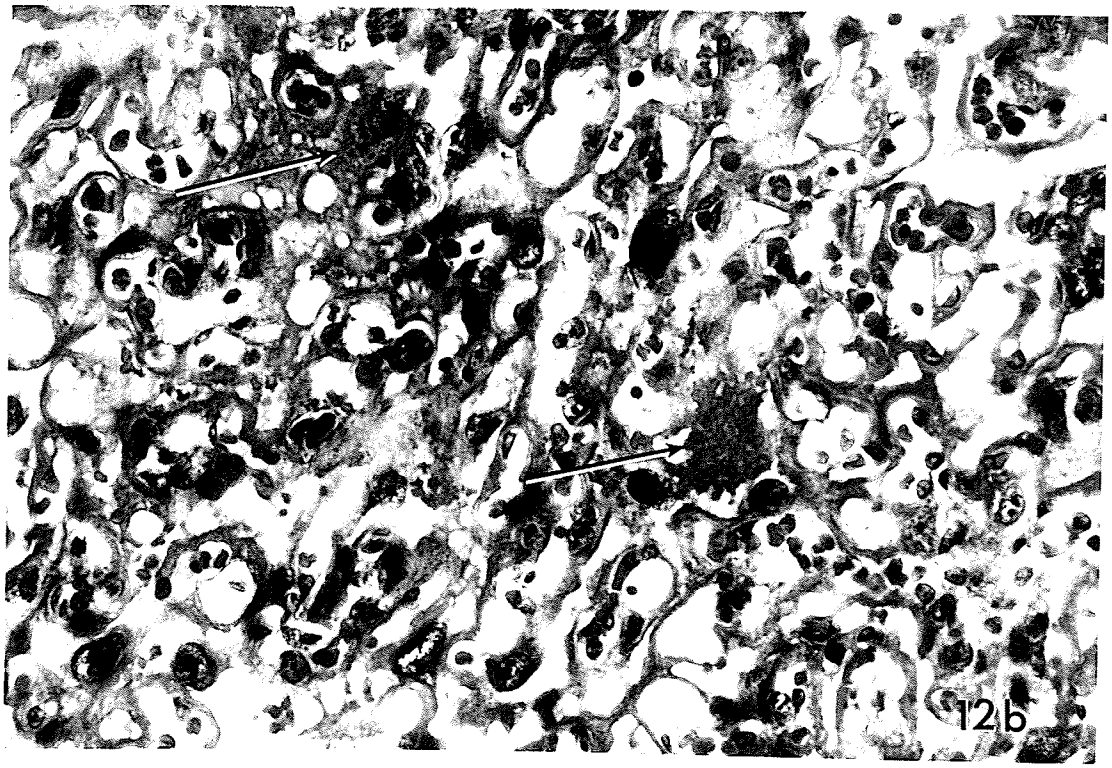
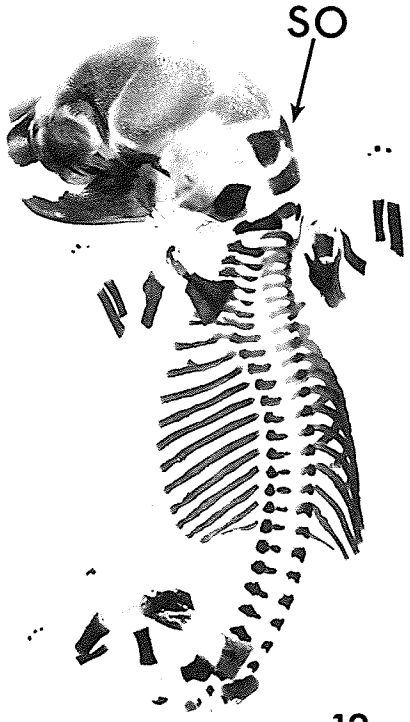

12b

FIGURE: 13 Ossification of the skull (20 day old rat fetus, Alizarin red stain).

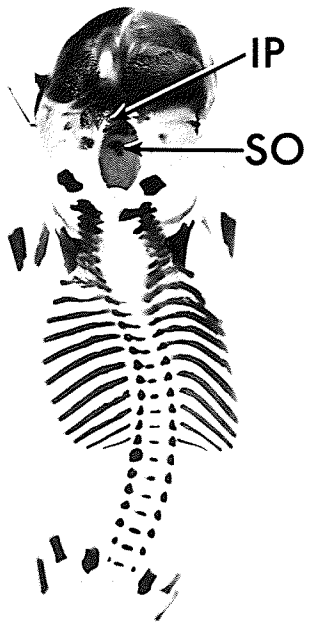
13a Control animal showing complete ossification of the skull bones. SO: supraoccipital bone. The wavy appearance of the ribs is due to refraction from superficial tissues.

13b Nicotine and caffeine treated animal. Observe the delayed ossification of the supraoccipital (SO) and interparietal (IP) bones.

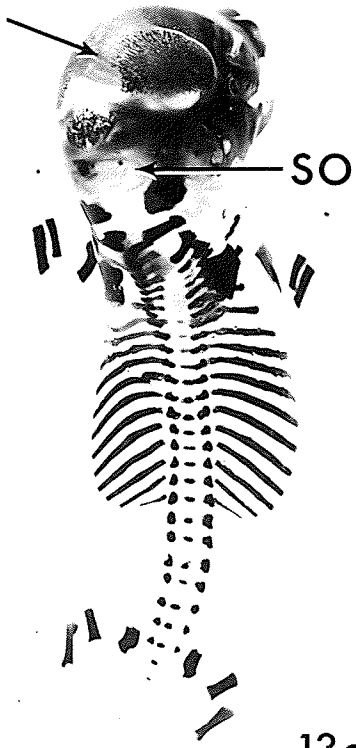
13c Nicotine and caffeine treated animal showing wide cranial sutures (arrow) and incomplete ossification of the supraoccipital bone (SO). Note the assymmetry between right and left sides of this bone and delayed ossification of the interparietal bone.



13a



13b



13c

FIGURE: 14 Ossification of the vertebral column (20 day old rat fetus, Alizarin red stain).

14a Control animal. Note the uniform appearance of vertebral centra in the thoracic and lumbar spines. The wavy appearance of the ribs, is due to refraction from superficial tissues.

14b Fetus of a nicotine and caffeine treated animal showing abnormal ossification of vertebral centra. Observe the bifid shape in the thoracic area, and fragmentation in the lumbar spine (arrows).

14a

14b

FIGURE: 15 20 day old rat fetus of a nicotine treated animal showing wavy ribs (Alizarin red stain).

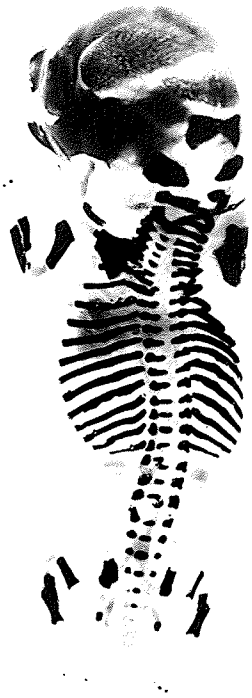


TABLE 3: INFLUENCE OF NICOTINE AND CAFFEINE IN THE PREGNANT RAT.

Treatment groups	No. of animals	Maternal wt. gain* (mean ± SDM)	Litter size* (mean ± SDM)	Fetal weight* (mean ± SDM)	Placental weight* (mean ± SDM)	Crown rump* (mean ± SDM)
I Untreated Control	10	117.3±18.3	13.6±1.0	1.95±0.06	0.34±0.02	2.62±0.03
II Nicotine Treatment (149 µg/hr x 7 days)	10	112.8±5.1	12.8±1.3	2.08±0.04	0.33±0.01	2.61±0.03
III Control to Nicotine	10	90.3±5.7	12.9±1.3	1.93±0.09	0.35±0.02	2.62±0.05
IV Caffeine Treatment (25 mg/kg)	10	120.1±5.0	13.9±0.75	1.96±0.04	0.35±0.007	2.67±0.03
V Control to Caffeine	10	126.1±15.0	12.6±1.3	1.86±0.05	0.34±0.007	2.60±0.03
VI Nicotine & Caffeine Treatment (149 µg/hr x 7 days/25 mg/kg)	10	113.5±7.0	13.1±0.8	1.97±0.05	0.36±0.01	2.67±0.03

Weights recorded in grams; crown-rump recorded in cm.
* Duncan's New Multiple Range Test: No significant differences among the groups ($p<0.05$)

TABLE 4 INFLUENCE OF NICOTINE AND CAFFEINE ON RAT EMBRYONIC DEVELOPMENT.

Treatment groups	No. of mothers*	Total implantations*	Resorptions (%)*	Live fetuses (%)*
I Untreated Controls	10	143	7 (4.9)	136 (95.1)
II Nicotine Treatment	10	132	4 (3.0)	128 (97.0)
III Control to Nicotine	10	133	4 (3.0)	129 (97.0)
IV Caffeine Treatment	10	146	7 (4.8)	139 (95.2)
V Control to Caffeine	10	131	5 (3.8)	126 (96.2)
VI Nicotine and Caffeine Treatment	10	135	4 (3.0)	131 (97.0)

* Data subjected to Chi-Square (x^2) test: no significant differences between groups.

TABLE 5: FREQUENCY OF DEVELOPMENTAL DEFECTS FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE TO PREGNANT RATS.*

Treatment groups	Total no. of live fetuses	Abnormal **	Types of defects (no. of fetuses affected)
I Untreated Controls	136	3(2.2)	hydrocephalus (3)
II Nicotine Treatment	128	7(5.5)*** ($p<0.05$)	hydrocephalus (6) digital anomalies; short forepaw digits (1)
III Control to Nicotine	129	2(1.6)	hydrocephalus (2)
IV Caffeine Treatment	139	3(2.2)	hydrocephalus (1) digital anomalies; missing forepaw digits (1); microcephaly (1)
V Control to Caffeine	126	1(0.8)	hydrocephalus (1)
VI Nicotine & Caffeine Treatment	131	0(0.0)	nil

* Each treatment group consisted of 10 pregnant females.
** Includes external and visceral anomalies.
*** Significant differences between group II and III, II and VI, and II and I (X^2-test).

TABLE 6: INFLUENCE OF NICOTINE AND CAFFEINE ON STERNAL OSSIFICATION.*

Treatment group	No. of fetuses examined	No. of complete sternal ossification centers (%) 0	1	2	3	4	5	6
I Untreated Control	10	10	4(40)	1(10)	3(30)	2(20)	0	0
II Nicotine Treatment**	18	1(5.6)	3(16.7)	7(38.9)	5(27.8)	2(11.1)	0	0
III Control to Nicotine	16	4(25)	6(37.5)	4(25)	2(12.5)	0	0	0
IV Caffeine Treatment**	20	2(10)	3(15)	9(45)	5(25)	1(5)	0	0
V Control to Caffeine	20	6(30)	8(40)	3(15)	2(10)	1(5)	0	0
VI Nicotine & Caffeine Treatment***	20	10(50)	2(10)	6(30)	2(10)	0	0	0

* Bifid ossification centers included as ossification was complete but abnormal. See Table 10.
** Significant differences (X^2-test) between groups II & III, and IV & V ($p<0.001$).
*** Significant differences between groups VI & II, and VI & IV ($p<0.001$).

TABLE 7: INFLUENCE OF NICOTINE AND CAFFEINE ON OSSIFICATION OF THE SKULL[1] AND FACIAL BONES.[2]

Treatment groups	No. of fetuses examined	Ossification of skull		Ossification of facial bones	
		Complete (%)	Delayed[3] (%)	Complete (%)	Delayed[3] (%)
I Untreated Control	10	9 (90)	1 (10)	10 (100)	0
II Nicotine Treatment[4]	18	18 (100)	0	18 (100)	0
III Control to Nicotine	16	8 (50)	8 (50)	14 (87.5)	2 (12.5)
IV Caffeine Treatment	20	17 (85)	3 (15)	18 (90)	2 (10)
V Control to Caffeine	20	15 (75)	5 (25)	19 (95)	1 (5)
VI Nicotine & Caffeine Treatment[5]	20	14 (70)	6 (30)	19 (95)	1 (5)

[1] Skull bones included frontal, parietal, interparietal, supraoccipital, exoccipital and basoccipital bones.

[2] Facial bones included nasal, premaxilla, maxilla, ethmoid, presphenoid, alisphenoid and zygoma.

[3] Delayed refers to both partial and incomplete ossification as described in materials and methods.

[4] Chi-Square test revealed significant differences between group II and III. ($p<0.001$). Performed on skull bones only.

[5] Chi-Square Test revealed significant differences between group VI and II, ($p<0.05$). Performed on skull bones only.

TABLE 8a: INCIDENCE OF OSSIFICATION CENTERS IN VERTEBRAL CENTRA FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.*

Vertebral Centra	Level	I Untreated Control (n=10) Absent (%)	Partial (%)	Complete (%)	II Nicotine Treatment (n=18) Absent (%)	Partial (%)	Complete (%)	III Treated Control to Nicotine (n=16) Absent (%)	Partial (%)	Complete (%)
Cervical	C_1-C_7	10 (100)	0	0	18 (100)	0	0	16 (100)	0	0
Thoracic	T_1	10 (100)	0	0	12 (66.7)	3 (16.7)	3 (16.7)	12 (75)	2 (12.5)	2 (12.5)
	T_2	0	1 (10)	9 (90)	0	3 (16.7)	15 (83.3)	1 (6.3)	2 (12.5)	13 (81.3)
	T_3-T_{13}	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
Lumbar	L_1-L_6	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
Sacral	S_1	3 (30)	0	7 (70)	0	0	18 (100)	0	0	16 (100)
	S_2	3 (30)	0	7 (70)	0	0	18 (100)	0	2 (12.5)	14 (87.5)
	S_3	3 (30)	3 (30)	4 (40)	0	0	18 (100)	11 (68.8)	3 (18.8)	2 (12.5)
	S_4	7 (70)	1 (10)	2 (20)	8 (44.4)	0	10 (55.6)	14 (87.5)	1 (6.3)	1 (6.3)
Caudal	Co_1	8 (80)	2 (20)	0	13 (72.8)	0	5 (27.8)	14 (87.5)	1 (6.3)	1 (6.3)
	Co_2	10 (100)	0	0	16 (88.9)	1 (5.6)	1 (5.6)	16 (100)	0	0
	Co_3+	10 (100)	0	0	18 (100)	0	0	16 (100)	0	0

* Bifid centra included in these incidences, as ossification was complete, but abnormal. See Table 10.

TABLE 8b: INCIDENCE OF OSSIFICATION CENTERS IN VERTEBRAL CENTRA FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.*

Vertebral Centra	Level	IV Caffeine Treatment (n=20) Absent (%)	Partial (%)	Complete (%)	V Treated Control to Caffeine (n=20) Absent (%)	Partial (%)	Complete (%)	VI Nicotine and Caffeine Treatment (n=20) Absent (%)	Partial (%)	Complete (%)
Cervical	C_1-C_7	20 (100)	0	0	20 (100)	0	0	20 (100)	0	0
Thoracic	T_1	16 (80)	3 (15)	1 (5)	18 (90)	2 (10)	0	18 (90)	2 (10)	0
	T_2	0	0	20 (100)	5 (25)	3 (15)	12 (60)	4 (20)	3 (15)	13 (65)
	T_3-T_{13}	0	0	20 (100)	0	1 (5)	19 (95)	0	0	20 (100)
Lumbar	L_1-L_6	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
Sacral	S_1	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
	S_2	0	1 (5)	19 (95)	0	1 (5)	19 (95)	0	2 (10)	18 (90)
	S_3	4 (20)	0	16 (80)	8 (40)	6 (30)	6 (30)	7 (35)	2 (10)	11 (55)
	S_4	11 (55)	4 (20)	5 (25)	18 (90)	1 (5)	1 (5)	17 (85)	1 (5)	2 (10)
Caudal	Co_1	18 (90)	0	2 (10)	20 (100)	0	0	19 (95)	0	1 (5)
	Co_2	19 (95)	0	1 (5)	20 (100)	0	0	20 (100)	0	0
	Co_3+	20 (100)	0	0	20 (100)	0	0	20 (100)	0	0

* Bifid centra included in these incidences, as ossification was complete, but abnormal. See Table 10.

TABLE 9a: INFLUENCE OF NICOTINE AND CAFFEINE ON OSSIFICATION CENTERS OF THE VERTEBRAL ARCHES.

Vertebral Arches	Level	I Untreated Control (n=10) Absent (%)	Partial (%)	Complete (%)	II Nicotine Treatment (n=18) Absent (%)	Partial (%)	Complete (%)	III Treated Control to Nicotine (n=16) Absent (%)	Partial (%)	Complete (%)
Cervical	C_1-C_7	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
Thoracic	T_1	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
	T_2	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
	T_3-T_{13}	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
Lumbar	L_1-L_6	0	0	10 (100)	0	0	18 (100)	0	0	16 (100)
Sacral	S_1	6 (60)	1 (10)	3 (30)	0	0	18 (100)	0	0	16 (100)
	S_2	3 (30)	6 (60)	1 (10)	1 (5.6)	7 (38.9)	19 (55.6)	7 (43.8)	7 (43.8)	2 (12.5)
	S_3	8 (80)	2 (20)	0	15 (83.3)	3 (16.7)	0	15 (93.8)	1 (6.3)	0
	S_4	10 (100)	0	0	18 (100)	0	0	16 (100)	0	0
Caudal	Co_1	10 (100)	0	0	18 (100)	0	0	16 (100)	0	0
	Co_2	10 (100)	0	0	18 (100)	0	0	16 (100)	0	0
	Co_3+	10 (100)	0	0	18 (100)	0	0	16 (100)	0	0

TABLE 9b: INFLUENCE OF NICOTINE AND CAFFEINE ON OSSIFICATION CENTERS OF THE VERTEBRAL ARCHES.

Vertebral Arches	Level	IV Caffeine Treatment (n=20) Absent (%)	Partial (%)	Complete (%)	V Treated Control to Caffeine (n=20) Absent (%)	Partial (%)	Complete (%)	VI Nicotine and Caffeine Treatment (n=20) Absent (%)	Partial (%)	Complete (%)
Cervical	C_1-C_7	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
Thoracic	T_1	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
	T_2	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
	T_3-T_{13}	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
Lumbar	L_1-L_6	0	0	20 (100)	0	0	20 (100)	0	0	20 (100)
Sacral	S_1	0	0	20 (100)	0	2 (10)	18 (90)	1	0 (5)	19 (95)
	S_2	7 (35)	2 (10)	11 (55)	12 (60)	5 (25)	3 (15)	11 (55)	6 (30)	3 (15)
	S_3	18 (90)	2 (10)	0	20 (100)	0	0	19 (95)	1 (5)	0
	S_4	20 (100)	0	0	20 (100)	0	0	20 (100)	0	0
Caudal	Co_1	20 (100)	0	0	20 (100)	0	0	20 (100)	0	0
	Co_2	20 (100)	0	0	20 (100)	0	0	20 (100)	0	0
	Co_3+	20 (100)	0	0	20 (100)	0	0	20 (100)	0	0

TABLE 10: ABNORMAL, VERTEBRAL AND STERNAL OSSIFICATION FOLLOWING TREATMENT WITH NICOTINE AND CAFFEINE.

Treatment groups	No. of fetuses examined	VERTEBRAE: No. of animals with bifid centra (%)	VERTEBRAE: No. of animals with bifid arches (%)	STERNUM: No. of animals with bifid sternal ossification centers (%)
I Untreated Controls	10	0	0	0
II Nicotine Treatment	18	0	0	0
III Control to Nicotine	16	0	0	0
IV Caffeine Treatment	20	0	0	0
V Control to Caffeine	20	0	0	0
VI Nicotine & Caffeine Treatment	20	11* (55)	1 (5)	3** (15)

* Significantly different ($p<0.001$) from other groups (x^2-test). One animal showed fragmented centra and is included in the Table.

** Not significantly different from other groups (x^2-test).

TABLE 11a: METACARPAL OSSIFICATION FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.

Treatment group	No. of fetuses examined	Metacarpus	No. of complete ossification centers Right (%)	Left (%)	No. of partial ossification centers Right (%)	Left (%)
I Untreated Control	10	1st	0	0	0	0
		2nd	5(50)	5(50)	6(60)	4(40)
		3rd	9(90)	10(100)	0	0
		4th	8(80)	9(90)	1(10)	1(10)
		5th	0	0	3(30)	2(20)
II Nicotine Treatment	18	1st	0	0	0	0
		2nd	7(38.9)	8(44.4)	7(38.9)	6(33.3)
		3rd	18(100)	18(100)	0	0
		4th	17(94.4)	18(100)	1(5.6)	0
		5th	0	0	3(16.7)	2(11.1)
III Control to Nicotine	16	1st	0	0	0	0
		2nd	5(31.3)	5(31.3)	11(68.8)	9(56.3)
		3rd	15(93.8)	15(93.8)	1(6.3)	2(12.5)
		4th	11(68.8)	13(81.3)	5(31.3)	3(18.8)
		5th	0	0	6(37.5)	4(25)

TABLE 11b: METATACARPAL OSSIFICATION FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.

Treatment group	No. of fetuses examined	Metacarpus	No. of complete ossification centers Right (%)	Left (%)	No. of partial ossification centers Right (%)	Left (%)
IV Caffeine Treatment	20	1st	0	0	0	0
		2nd	12(60)	12(60)	4(20)	4(20)
		3rd	19(95)	19(95)	0	0
		4th	15(75)	16(80)	1(5)	2(10)
		5th	0	0	0	0
V Control to Caffeine	20	1st	0	0	0	0
		2nd	8(40)	9(45)	9(45)	9(45)
		3rd	20(100)	20(100)	0	0
		4th	18(90)	17(85)	2(10)	1(5)
		5th	0	0	0	0
VI Nicotine & Caffeine Treatment	20	1st	0	0	0	0
		2nd	8(40)	6(30)	3(15)	5(25)
		3rd	20(100)	20(100)	0	0
		4th	17(85)	17(85)	0	0
		5th	0	0	0	0

TABLE 12a: METATARSAL OSSIFICATION FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.

Treatment group	No. of fetuses examined	Metatarsus	No. of complete ossification centers Right (%)	Left (%)	No. of partial ossification centers Right (%)	Left (%)
I Untreated Control	10	1st	0	0	0	0
		2nd	10(100)	10(100)	0	0
		3rd	10(100)	10(100)	0	0
		4th	10(100)	10(100)	0	0
		5th	0	0	3(30)	4(40)
II Nicotine Treatment	18	1st	0	0	0	0
		2nd	18(100)	18(100)	1(5.6)	1(5.6)
		3rd	18(100)	18(100)	0	0
		4th	18(100)	18(100)	0	0
		5th	0	0	5(27.8)	5(27.8)
III Control to Nicotine	16	1st	0	0	0	0
		2nd	12(75)	13(81.3)	4(25)	3(18.8)
		3rd	13(81.3)	14(87.5)	3(18.8)	2(12.5)
		4th	13(81.3)	14(87.5)	2(12.5)	2(12.5)
		5th	0	0	10(62.5)	10(62.5)

TABLE 12b: METATARSAL OSSIFICATION FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.

Treatment group	No. of fetuses examined	Metatarsus	No. of complete ossification centers Right (%)	Left (%)	No. of partial ossification centers Right (%)	Left (%)
IV Caffeine Treatment	20*	1st	0	0	0	0
		2nd	18(90)	17(85)	1(5)	2(10)
		3rd	20(100)	19(95)	0	0
		4th	20(100)	19(95)	0	0
		5th	0	0	1(5)	1(5)
V Control to Caffeine	20	1st	0	0	0	0
		2nd	17(85)	17(85)	2(10)	2(10)
		3rd	17(85)	18(90)	3(15)	2(10)
		4th	20(100)	19(95)	1(5)	1(5)
		5th	0	0	0	1(5)
VI Nicotine & Caffeine Treatment	20**	1st	0	0	0	0
		2nd	14(70)	13(65)	3(15)	5(25)
		3rd	17(85)	17(85)	3(15)	2(10)
		4th	17(85)	17(85)	3(15)	2(10)
		5th	0	0	0	0

* In one fetus the left hind paw was entirely absent.
** In one fetus the left hind paw showed no ossification centers.

TABLE 13: OSSIFICATION PATTERNS OF THE MANDIBLE, FORELIMBS, HINDLIMBS AND PHALANGES.

Treatment Group	No. of Fetuses Examined	Mandible (%)		Forelimbs* (%)		Hindlimbs** (%)		Phalanges*** (%)	
		Complete	Partial	Complete	Partial	Complete	Partial	Complete	Partial
I Untreated Control	10	10 (100)	0	10 (100)	0	10 (100)	0	0	10 (100)
II Nicotine Treatment	18	18 (100)	0	18 (100)	0	18 (100)	0	0	18 (100)
III Control to Nicotine	16	16 (100)	0	16 (100)	0	16 (100)	0	0	16 (100)
IV Caffeine Treatment	20	20 (100)	0	20 (100)	0	20 (100)	0	0	20 (100)
V Control to Caffeine	20	20 (100)	0	20 (100)	0	20 (100)	0	0	20 (100)
VI Nicotine & Caffeine Treatment	20	20 (100)	0	20 (100)	0	20 (100)	0	0	20 (100)

* Forelimbs: i) Includes the clavicle, scapula, humerus, radius and ulna
ii) Bilateral representation
** Hindlimb: i) Includes the femur, tibia and fibula, bilaterally
*** Includes digits 1 to 5, bilaterally

TABLE 14: OSSIFICATION OF THE HYOID BONE.*

Treatment group	No. of fetuses examined	No. of animals with complete ossification (%)	No. of animals with delayed** ossification (%)	No. of animals with partial ossification (%)	No. of animals with absent ossification (%)
I Untreated Controls	10	8 (80)	2(20)	0	2(20)
II Nicotine Treatment	18	11 (61.1)	7 (38.9)	1 (5.6)	6 (33.3)
III Control to Nicotine	16	9 (56.3)	7 (43.7)	6 (37.5)	1 (6.3)
IV Caffeine Treatment	20	15 (75)	5 (25)	0	5 (25)
V Control to Caffeine	20	16 (80)	4 (20)	0	4 (20)
VI Nicotine & Caffeine Treatment	20	14 (70)	6 (30)	1 (5)	5 (25)

* No significant differences between the groups (x^2-test).
** "Delayed" includes partial and absent ossification centers.

TABLE 15: PELVIC OSSIFICATION FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.

Treatment groups	No. of fetuses examined	ILIUM			ISCHIUM		
		Complete(%)	Partial(%)	Absent(%)	Complete(%)	Partial(%)	Absent(%)
I Untreated Control	10	10(100)	0	0	10(100)	0	0
II Nicotine Treatment	18	18(100)	0	0	18(100)	0	0
III Control to Nicotine	16	16(100)	0	0	13(81.2)	3(18.8)	0
IV Caffeine Treatment	20	20(100)	0	0	18(90)	1(5)	1(5)
V Control to Caffeine	20	20(100)	0	0	19(95)	0	1(5)
VI Nicotine & Caffeine Treatment	20	20(100)	0	0	16(80)	1(5)	1(5)

TABLE 16: OSSIFICATION PATTERN OF THE RIBS FOLLOWING ADMINISTRATION OF NICOTINE AND CAFFEINE.*

Treatment groups	No. of fetuses examined	RIBS		
		Complete (%)	Absent (%)	Wavy (%)
I Untreated Controls	10	10 (100)	0	0
II Nicotine Treatment	18	16 (88.9)	0	2 (11.1)
III Control to Nicotine	16	14 (87.5)	2 (12.5)	0
IV Caffeine Treatment	20	20 (100)	0	0
V Control to Caffeine	20	20 (100)	0	0
VI Nicotine & Caffeine Treatment	20	17 (85)	2 (10)	1 (5)

* No significant difference among the groups (x^2 Test).

4. DISCUSSION

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Birth defects are a major cause of perinatal and neonatal mortality. In recent years, mortality rates attributed to congenital malformations have declined far less than those related to other causes (for example infectious and nutritional diseases). Two to three per cent of all newborns present with major malformations and 20% of all stillbirths and infant deaths are associated with severe developmental defects. Though recent advances have contributed greatly towards a better understanding of human developmental processes, the etiology of most major malformations remains unknown (Persaud, 1985).

Researchers have focused much attention on the role of environmental factors since Gregg (1941) first identified the rubella virus as a cause of human malformations. The thalidomide tragedy twenty years later provided the first causal relationship between maternally ingested drugs and specific birth defects (McBride, 1961).

Wilson (1974) referred to developmental toxicity as "encompassing all deviations in developmental processes originating between fertilization and postnatal maturity, including fetal death, malformation, growth retardation and functional deficiency". Identification of environmental teratogens represents a complex toxicological problem, in that the response of the developing conceptus is influenced by multiple other factors acting either individually or

collectively (Persaud, 1985). These include the type of drug, level and duration of exposure, maternal modulation of dosage, access to the conceptus, developmental stage at time of treatment, disposition within the conceptus and susceptibility of species and the individual (Wilson, 1974). In today's society, pregnant women are frequently exposed to a wide variety of different chemical agents, including industrial pollutants, over-the-counter pharmaceutical preparations and socially abused drugs. Thus it is difficult to attribute deleterious effects on development to any single agent.

Nicotine and caffeine represent two of the most common pharmacologically active chemicals to which pregnant women are exposed throughout the course of their pregnancy. Exposure of the conceptus to these drugs occurs primarily as a result of maternal cigarette smoking and consumption of caffeine containing beverages, especially coffee.

The adverse effects of cigarette smoking during pregnancy are well documented in the current literature. However, no clear association between nicotine use and developmental defects has to date been established (Christianson, 1980; Hemminki et al., 1983; Kelsey et al., 1978; Lindenschmidt and Persaud, 1980; Persaud, 1982; Shiono et al., 1986). Though numerous studies have demonstrated the teratogenicity of caffeine in several species of animals (Fujii et al., 1969; Fujii and Nishimura, 1969 and 1972;

Gilani et al., 1983; Lee et al., 1982; Palm et al., 1978; Scott, 1983; Tanaka et al., 1984), experimental results can only be applied to isolated cases due to the variability of caffeine dose, exposure time and species variation.

At this time, only a single study involving the combination of nicotine and caffeine has been reported in the literature (Gilani and Persaud, 1986). In view of increasing concern regarding the potentially deleterious effects of these agents on the embryo, the teratogenicity of nicotine and caffeine at dose levels approximating human consumption was investigated on Sprague-Dawley rats.

Because ethical and legal considerations prohibit the use of humans for teratological evaluations, such investigations are carried out in laboratory animals. Teratologic screening represents an important parameter utilized by pharmaceutical industries and regulatory agencies for assessing the toxicity of various drugs and chemicals during pregnancy.

Due to species variations in genotype and metabolic processes, extrapolation of the results from animal models to humans is not possible. However, the results of carefully planned animal studies are important in predicting potential harmful effects on the human fetus, as well as identifying the mechanisms operating at various stages of intrauterine development (Persaud, 1979 and 1985).

In determining the effects of drugs or chemicals, the offspring of animals exposed to a specific agent(s) are compared to those documented as having a normal pattern of growth and development. Thus, information regarding the normal development of several animal species is essential in order to conduct teratological studies (Edwards, 1968).

The World Health Organization as well as the regulatory agencies in most countries (including Canada and the United States), recommend that toxicological evaluation be performed on at least 2 mammalian species of animals, one rodent (mice or rats) and one non-rodent species (eg. rabbits). The use of virgin Sprague-Dawley rats throughout this investigation was based on the following: a) this strain of rat has a low incidence of spontaneously occurring congenital malformations, b) a relatively short gestational period and c) large litter size, from 8 to 12 pups per litter. Additional factors included economical considerations and the fact that a considerable volume of reproductive data on the rat is already available. Though non-human primates probably represent the closest approximation to human metabolic and developmental events, their use in teratological screening studies is limited due to excessive maintenance costs, lengthy gestational period and a relatively small number of offspring (Persaud, 1985).

According to Wilson (1964) all chemicals are capable of producing some embryonic effect if given in adequate dosages

during critical stages of development. One of the major difficulties encountered in the interpretation and subsequent practical application of the research literature relates to the dosages of nicotine and caffeine utilized to induce teratogenic effects in laboratory animals. This is particularly evident with regards to caffeine. Whereas numerous investigators have demonstrated various teratogenic effects of caffeine in rodents (Fujii and Nishimura, 1969; Fujii et al., 1969; Scott, 1983; Palm et al., 1978; Kimmel et al., 1982), the dosages utilized were far in excess of those generally consumed by humans.

According to a recent report by Lelo et al. (1986) the average daily human caffeine intake of moderate to heavy consumers ranges from approximately 300 to 600 mg/day, or from 3 to 6 cups of coffee (assuming 100 mg/cup). The dosage level therefore in a person weighing 70 kg ranges from approximately 4.3 to 8.6 mg/kg/day. In comparison, caffeine doses administered to laboratory animals ranged from 30 mg/kg (Palm et al., 1978) to 250 mg/kg (Fujii and Nishimura, 1969). Caffeine is known to have a rapid metabolic rate in both man and animals (Rall, 1980; Palm et al., 1978). However, even when species variation is taken into account, the practical application of the results obtained from many of these animal experiments to the human condition is unrealistic due to the excessive dose levels administered.

In studies investigating the effects of nicotine, dosage regimens are also high relative to human consumption. However, when the rapid metabolic rate of this drug in rodents and other species of animals is taken into account, they are not as excessive as those reported in the literature related to caffeine.

Other important considerations in studying the teratogenic potential of test agents include the mode of administration (Fujii et al., 1969) and the developmental stage during which the conceptus is exposed (Wilson, 1964). In the present study, a total dose of 25 mg nicotine was administered to pregnant rats over a seven day period. The mode of administration was via continuous subcutaneous infusion from an osmotic mini-pump. Nicotine treatment commenced on gestational day 6 to coincide with implantation of the blastocysts, and continued through the 12th day of pregnancy. In the rat, the 9th to 12th gestational days are developmentally important, as they represent the major period of organogenesis. During this time, differentiation of nearly all organ primordia occurs (Hebel and Stromberg, 1986). Also, humans consume nicotine in small continuous doses, over an extended period of time through inhalation of cigarette smoke. Thus, the rat embryos were exposed to nicotine during their critical stages of development in a manner similar to human consumption. At an infusion rate of 149 micrograms/hr, each animal received approximately 3.6 mg

nicotine/day. Based on an average maternal weight of 300 grams, this can be translated to a dose of 0.5 mg/kg/hr, or the nicotine equivalent of 12 cigarettes, assuming each cigarette contains 1.0 mg nicotine (Taylor, 1980).

A moderate dosage level of 25 mg/kg caffeine was administered as a single intravenous injection on gestational day 6. Fujii et al. (1969) demonstrated that in mice, whereas embryolethality is related to the duration of caffeine exposure, teratologic effects are more dependent on a sufficiently high concentration of the drug. Though intravenous injection does not simulate human caffeine consumption the method of caffeine administration in the present study was the most expedient and in accordance with that utilized by others.

In the rat, fertilization usually occurs in the uppermost part of the oviduct at the time of coitus. Tubal passage of the fertilized ova into the uterine horns requires three days. During this time, the blastocyst begins its segmentation and differentiation, and enters the uterus at the late morula stage (Hebel and Stromberg, 1986). As stated previously, implantation occurs six days following coitus.

Caffeine can act as a mitotic poison in mammalian embryos (Spindle and Wu, 1985). Furthermore, Fabro and Sieber (1969) demonstrated that caffeine penetrates the preimplantation blastocysts in rabbits. In the present

study, caffeine was administered to the pregnant rats on the 6th gestational day in order to study the teratogenic effect on early embryonic development.

With regards to teratogenic evaluation, the methods described by Wilson (1964) have the advantage of providing information on a whole litter in a manner that is both cost and time efficient. Gross developmental anomalies are readily identified by external examination of the fetuses. Malformations such as neural tube defects, micropthalmia, various musculoskeletal malformations (for example cleft lip and/or palate, micrognathia, limb and digital defects including micromelia, ectrodactyly, polydactyly and others) and umbilical hernia can all be identified by careful visual inspection.

The Wilson technique of free hand razor blade sectioning (Wilson, 1964) provides considerable information regarding the structure and development of internal organs. Whole fetuses are sectioned in a craniocaudal direction. Sequential examination of these sections reveals the presence of numerous visceral anomalies. Sections of the head region reveal abnormalities of the palate, nasal cavities, eyes and brain. Malformations that can be identified in sections through the thorax include various cardiac defects, malformations of the great vessels, tracheoesophageal fistula and herniated abdominal viscera due to diaphragmatic defects. Abdominal sections have

revealed anomalies such as inverted abdominal viscera, ectopic and/or fused kidneys, renal hypoplasia and agenesis and hydronephrosis. External genitalia are evaluated by internally inspecting the pelvic cavity for evidence of abnormalities such as cryptorchidism or various degrees of hermaphroditism.

Nicotine has long been considered to be the primary substance in tobacco smoke responsible for the pharmacological response to smoking, although carbon monoxide, carbon dioxide and cyanide have also been implicated (Kelly et al., 1984; Phillip et al., 1984; Luck et al., 1985). The most consistently observed effect of maternal smoking during pregnancy is intrauterine growth retardation, with a clear dose-response relationship existing between the number of cigarettes smoked and the birth weight deficit (Meyer et al., 1976; Nieburg et al., 1985; Lubs, 1973; Cnattingius et al., 1985; Phillip et al., 1984; Mochizuki et al., 1984; Harrison et al., 1983; Spira et al., 1975).

In all of the above mentioned studies, observations are based on retrospective data from large human populations. Babies were evaluated at birth, and the mothers smoked cigarettes throughout the entire gestational period. While the growth retardant effect of nicotine on fetal growth cannot be disputed under these circumstances, questions

remain as to when does this effect occur and what is (are) the mechanism(s) involved.

In the present experiments, maternal exposure to nicotine during the early stages of pregnancy had no significant effect on fetal growth evaluated near term, either alone or in combination with caffeine. These results are in accordance with those reported by Meyer et al. (1976) who revealed that mothers who stopped smoking during pregnancy gave birth to babies of normal weight and length. Thus, I.G.U.R. secondary to nicotine consumption (smoking) during pregnancy is dependent at least in part, upon fetal exposure throughout the entire gestational period.

One of the proposed mechanisms responsible for the retarded fetal growth in smokers, is impairment of uteroplacental circulation secondary to the vasoconstricting effect of nicotine (Mochizuki et al., 1984; Phillip et al., 1984; Suzuki et al., 1980). Despite discontinuation of nicotine treatment after the 12th gestational day, histologic changes noted to occur in tissue samples of maternal liver, kidney and placenta obtained on the 20th day of gestation were suggestive of some degree of functional circulatory disturbances secondary to nicotine. This was evidenced by dilation and congestion of hepatic sinusoids, glomerular congestion associated with degenerative changes and congestion of placental intervillous spaces. The poor development of the decidua basalis observed in the nicotine

and the nicotine and caffeine treated animals may be the result of impaired uteroplacental circulation. The dilation and congestion of hepatic sinusoids observed to occur in fetal liver are in support of the hypothesis that nicotine has a direct effect on the fetus, causing circulatory responses similar to those observed in the mother (Eriksen and Marsal, 1984; Suzuki et al., 1980).

Of practical importance is the observation that although histological changes were observed in the maternal component of the placenta, the chorionic (branch) villi of the fetal compartment appeared normal. Whereas fetal plasma concentrations of nicotine have been shown to surpass those of the mother, the amount of nicotine in the entire fetus accounts for only 1 to 4% of the total dose administered to the mother (Suzuki et al., 1980). Maternal metabolic processes and placental metabolism play an important role in determining the dosage of a drug to which the fetus is exposed (Wilson, 1974; Persaud, 1985; Yaffe and Juchau, 1974). Though based on limited numbers, the histological changes noted in this investigation suggest that even a moderate dose of nicotine administered over a limited time period has a persistent adverse effect on the circulatory function of maternal tissues. While some evidence suggesting a direct effect of nicotine on the fetus was observed, the normal appearance of the kidney, and in particular the fetal component of the placenta, indicates

that the functional development of fetal tissues was not adversely affected by nicotine.

The acute effect of nicotine on fetal tissues cannot be assessed from this study, because development was allowed to continue to near term. Factors such as maternal modulation of dosage and the highly proliferative nature of embryonic tissues may account for the lack of observed effects of nicotine treatment. The compensatory placental hypertrophy reported by Spira et al. (1975) was not evident in this investigation.

To date evidence associating maternal nicotine consumption and embryolethality (early fetal loss) remains inconclusive. Whereas several studies on human populations reported a higher incidence of spontaneous abortion in mothers who smoked (Hemminki et al., 1983; Kline et al., 1977 and 1983), all of the confounding factors known to influence human embryonic development could not be statistically controlled, nor were the reported increases statistically significant. Nasarat et al. (1986) reported a higher incidence of perinatal mortality in mice, associated with maternal exposure to nicotine. In this study, however, perinatal mortality was defined according to the observed number of neonatal deaths and stillborn infants, and embryolethality, as evidenced by the number of resorption sites in the uterine horns, was not evaluated. In the present study, nicotine had no effect on embryolethality.

Similar results were reported by Persaud (1982) and Lindenschmidt and Persaud (1980).

Whether or not cigarette smoking during pregnancy represents significant teratologic risk to the fetus remains a controversial topic. Factors such as conflicting definitions and classification of various birth defects, lack of control for potential risk factors in the human population and species variation make the interpretation of research literature very difficult. With regards to human populations, recent studies indicate that moderate doses of nicotine (<1 package/day) do not have a teratogenic effect on the developing conceptus (Christianson, 1980; Hemminki et al., 1983; Shiono et al., 1986).

The overall low incidence of birth defects observed throughout this investigation are likely due to the normal low incidence of birth defects in the Sprague-Dawley strain of rats. Thus, extremely large study populations would be required to detect any significant increase in these defects. In animals treated with nicotine, however, a significantly higher incidence of hydrocephalus was observed relative to any other group which may represent a manifestation of observations reported by Lajtha and Sershen (1986) and Seidler et al., (1986). These authors have revealed that early fetal exposure to nicotine causes interruption of both DNA synthesis and subsequent cell replication (Seidler et al., 1986) and protein metabolism

and synthesis in central nervous system neurons (Lajtha and Sershen, 1986). They propose that the biochemical interference with early developmental events may contribute to the formation of some central nervous system defects.

Skeletal ossification is generally considered to be an indicator of developmental maturity. In the rat, it normally begins during the seventeenth day after conception with ossification of the mandible and ribs and then proceeds very rapidly, adhering to a precise time schedule (Walker and Wirtschafter, 1957; Hebel and Stromberg, 1986). Delayed or retarded skeletal ossification at a given time near term is indicative of a non-specific retardation of fetal growth and development. Thus, evaluation of skeletal maturity is an important criterion in teratological studies. Although several investigators have described the normal skeletal development of the rat (Aliverti et al., 1979; Walker and Wirtschafter, 1957), subtle variations between the different strains of rats studied do not allow direct comparisons with the results obtained in the present study.

The perinatal ossification patterns of the Sprague-Dawley strain of rats was documented by Fritz and Hess (1970). According to these investigators two days before term, the skull, scapular region, ribs, hip bones and extremities (with the exception of phalangeal nuclei) were ossified. In this instance, however, "two days and one day before term" were defined corresponding to 21 and 22 day old

fetuses respectively. In this study, skeletal evaluation was performed on 20 day old fetuses, and as a result ossification was not as advanced in certain locations when compared to normal ossification patterns reported by Fritz and Hess (1970).

Specifically, in a 20 day old fetus, ossification was generally complete in the skull, scapular region and ribs in all of the fetuses examined. In the pelvic region, whereas ossification of the ilium was complete, the ischium showed variable degrees of partial ossification in all animals. In the extremities, ossification was well advanced in the long bones (humerus, radius, ulna, femur, tibia and fibula), but less advanced in the metacarpal and metatarsal bones. Because Fritz and Hess (1970) do not make specific mention of the metacarpal and metatarsal bones, it has been assumed that they have included these bones in their reference to the "extremities".

These authors also reported complete ossification centers in both the centra and vertebral arches in the thoracic, lumbar and sacral spines. Cervical centra were generally absent, with ossification visible only in the cervical vertebral arches. Vertebral ossification observed in the present investigation is in accordance with these findings, with the exception of the sacral spine, which demonstrated considerable variations in the degree of

ossification present, particularly with respect to the vertebral arches.

Finally, whereas Fritz and Hess (1970) reported ossification in all 6 sternebral centers in over 90% of the 21 day fetuses examined, the maximum number of sternal ossification centers observed in the 20 day fetuses in the present study was four, and the frequencies among them were highly variable. These variabilities are normal and represent intermediate stages of ossification between the 20 and 21 day old fetus.

Among the fetuses examined in this investigation, it is likely that the variations observed in metacarpal and metatarsal ossification, as well as in the sacral spine are representative of intermediate stages of ossification, rather than a teratogenic effect of nicotine, in view of the overall bony symmetry observed and the rapid ossification process occurring at this stage of development.

Maternal exposure to drugs may cause an increase in the frequency of spontaneously occurring phenomena in the rat (Fritz and Hess, 1970). This may account for the very low incidence of wavy ribs observed to occur in the fetuses of both the nicotine and the nicotine and caffeine treated animals.

Intravenous injection of a moderate dose of caffeine (25 mg/kg) on gestational day 6 was found to have no embryolethal or teratogenic effects on the developing fetus.

These results are in agreement with those reported by Nolen (1981). Although other investigators have reported significant increases in embryolethality and developmental defects in several animal species (Fujii and Nishimura, 1969 and 1972; Gilani et al., 1983; Lee et al., 1982; Scott et al., 1983), the dosage levels utilized in those experiments were excessively high, and when involving rodents, approached near lethal maternal levels.

Caffeine has been shown to penetrate the preimplantation blastocysts in rabbits (Fabro and Sieber, 1969). Spindle and Wu (1985) have proposed that caffeine, acting as a mitotic poison may cause developmental failure of early embryonic tissues. In those experiments, however, mouse blastocysts were cultured in-vitro and exposed to concentrations of caffeine of up to 4 mM. Results from the present investigation suggest that a dosage level of greater than 25 m/kg is required to induce significant embryolethal effects on rat fetal development.

With regards to the teratogenic potential of caffeine, in virtually all studies reported in the literature, fetal exposure to the drug occurred during the major period of organogenesis of the species being studied. Numerous developmental anomalies in laboratory rodents have been related to caffeine exposure either as a single dose (Fujii and Nishimura 1969; Fujii et al., 1969; Scott, 1983; Kimmel et al., 1982) or throughout the entire gestational period

(Fujii and Nishimura, 1972; Palm et al., 1978; Tanaka et al., 1984) despite the route by which it was administered. In the present study, all parameters of fetal growth and development, histological studies and skeletal ossification patterns were normal in the caffeine treated animals. This evidence indicates only that caffeine, at moderate dose levels, has no apparent teratogenic effects on early embryonic tissues because it was administered prior to organogenesis. In view of this, no conclusions can be made regarding its effect on fetal development. Also, it is possible that due to the highly proliferative and reparative nature of embryonic tissues (Anders and Persaud, 1980), any effects that may have occurred at the time of treatment were no longer evident near term.

Even though there is no conclusive evidence to associate caffeine consumption with adverse outcome of human pregnancy, warnings administered by the Food and Drug Administration continue to warrant serious consideration. Caffeine is a methylxanthine which resembles the purines found in genetic material, and therefore has the potential to interfere with cellular differentiation and proliferation. In view of the tragic nature of many congenital birth defects, women are advised to refrain from consuming caffeine containing beverages and foods during pregnancy.

Individuals who consume large amounts of caffeine tend to smoke cigarettes as well (Cagguila et al., 1986; Martin, 1982). Because maternal exposure to a variety of chemical agents is rarely an isolated event, there is currently increasing concern regarding the effects of chemical interactions on the developing conceptus.

Subcutaneous infusion of nicotine on gestational days 6 through 12 coupled with intravenous injection of caffeine on gestational day 6 resulted in delayed and abnormal skeletal ossification in the offspring, revealing a coteratogenic effect between these two drugs with regards to development of the skeletal system. Fetuses exposed to nicotine and caffeine revealed a significant decrease in the number of complete sternal ossification centers, as well as in the incidence of complete ossification of the skull bones (delayed ossification) when compared to those exposed to either of the drugs individually. In addition, abnormal ossification patterns evidenced by the appearance of bifid sternal and vertebral ossification centers occurred only in the combined treatment animals.

In general, although musculoskeletal defects are associated more with fetal exposure to caffeine, nicotine has also been implicated to induce orofacial clefts and some nonspecific musculoskeletal defects (Hemminki et al., 1983). Both nicotine and caffeine have a vasoconstrictive effect on peripheral blood vessels, though their mechanisms of action

are slightly different (Rall, 1980; Taylor, 1980). Also, caffeine has been shown to potentiate the teratogenic effects of various chemical substances in experimental animals (Ritter et al., 1982; Kwasigroch and Skalko, 1985; Nakatsuka et al., 1983; Fujii and Nakatsuka, 1983; Skalko et al., 1984).

Histological changes in both maternal and fetal tissues similar to those observed to occur in nicotine treated animals alone were also evident in the combined treatment animals, and in the case of the latter, were more pronounced. Whereas nicotine treatment alone caused some degree of functional circulatory disturbance in the mothers, these were not severe enough to induce any teratological effects in their offspring. As in the nicotine treated animals, the combination of nicotine and caffeine did not affect embryolethality or fetal growth. However, disturbances in the development of the fetal skeletal system did occur in the combined treatment animals. These results may be due to potentiation of the vasoconstrictive effects of nicotine by caffeine. Thus, caffeine acts as a coteratogen when administered together with nicotine.

Finally, in any investigation involving the subjective analysis of qualitative data, the possibility of introduction of investigator bias must be considered. Although applicable to the current study, extreme caution

was exercised in evaluation of qualitative data to avoid unintentional introduction of bias.

APPENDIX 1

Classification of Smokers:

- Light Smoker: 1-10 cigarettes per day.
- Moderate Smoker: 10-20 cigarettes per day.
- Heavy Smoker: 20+ cigarettes per day (>1 package/day).

In studies reported by Meyer (1976) and Meyer et al. (1978), light and moderate smokers were grouped together, ie. 1-20 cigarettes per day, or <1 package; heavy smokers consumed 20+ cigarettes per day, ie. >1 package/day.

Intrauterine Growth Retardation: One mechanism responsible for small for gestational age babies.

Low Birth Weight: Birth weights less than or equal to 2500 grams.

Prematurity: Refers to infants born prior to 37 weeks gestation. Premature infants are often of low birth weight, but are normal in length for their gestational age. Low birth weight babies are not necessarily premature.

Small for Gestational Age: For each gestational age, corresponding birthweight and fetal length have been correlated. Refers to babies that are of lower birthweight and shorter length than normal for their gestational age.

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