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Guy, Julia Freeman

TERATOGENIC EFFECTS ON THE CD-1 MOUSE EMBRYO EXPOSED TO
CONCURRENT DOSES OF ETHANOL AND ASPIRIN

The Ohio State University

PH.D. 1986

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TERATOGENIC EFFECTS ON THE CD-1 MOUSE EMBRYO
EXPOSED TO
CONCURRENT DOSES OF ETHANOL AND ASPIRIN
DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Julia. F. Guy, B.S., M.S.

The Ohio State University
1986

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A handwritten signature in cursive script, reading "Martha E. Sucheston". The signature is written in dark ink and is positioned above the printed name "Martha E. Sucheston".

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To My Parents
James R. and Ruth S. Freeman

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INTRODUCTION

Since the fetal alcohol syndrome, FAS, (Jones et al '73) first gained wide publicity, many human and animal studies have been described which verify the association between chronic maternal alcohol consumption and the range of malformations it produces. The syndrome includes craniofacial defects, developmental delay, pre-and post-natal growth deficiency, and central nervous system involvement.

It is now an understood fact that alcohol, like almost all other drugs with molecular weights under 600 and many between 600 and 1000 (Calabrese '78), crosses the placenta. And, when ingested, some drugs can augment, diminish, or totally alter the effect of other drugs on the human body (Hansten '79; Hayes '81; Seeler '79). The two drugs which will be administered simultaneously in this study are ethanol and aspirin. Alcohol is considered by many to be the greatest available known teratogen (Smith '81; Sulik '83), and it is being used by as many as 75% of pregnant women (Rosett '76; Streissguth '78). Clarren ('78a) estimates that perhaps three to five out of 1000 live

births show some degree of alcohol embryopathy. Aspirin, not even considered a drug by many, has been proven to be teratogenic in animals given four to five times the human therapeutic dose (Kimmel '71; Klein '81; Trasler '65; Warkany '59) and is highly suspect in the human (Collins '75; McNeil '73; Richards '69). Estimates place the number of pregnant women taking aspirin at over 50% (Collins '81; Hill '77). Both drugs are listed as teratogens by Shepard ('80). While several studies document aspirin/alcohol augmentation of gastric mucosal irritation (DeSchepper '78) and pancreatic duct permeability (Reber '79), as well as on hemostasis (Deykin '81), no previous studies have been reported which explore the possibility of augmentation of fetal effects when aspirin is ingested by a pregnant animal in which alcohol exists.

There are several factors which would indicate that a potentiation effect may be seen when these drugs exist at the same time in the developing fetus. A description of the minimal expected effect, an additive effect, can best be understood after describing the teratogenic observations from retrospective, prospective and experimental work with the individual drugs.

Alcohol: History and Human Studies

An historical survey of American and British literature on the effect of drinking on offspring has been prepared by Warner ('75). He included the following references: from Aristotle, "foolish, drunken or hare-brain women, most part bring forth children like unto themselves, morsos et languidos."; in The Bible, an angel appears to Samson's mother saying "Behold now, thou art barren and bearest not: but thou shalt conceive, and bear a son. Now therefore beware, I pray thee, and drink not wine nor strong drink." (Judges 13:3-4): and, Gellius, quoted in the 1600s, "if a drunken man get a child, it will never likely have a good brain." These statements indicate a very early concern which would seem to have been based on some type of negative observations.

Cause and effect began to be documented with England's gin epidemic (1720-1750) when observations made by medical writers were recorded. In spite of good crops and wages during that period, birth rates dropped, infant death rates increased, and in 1726, the College of Physicians petitioned parliament for control of the distilling trade, calling gin "a cause of weak, feeble and distempered children." (Warner '75)

Over the next two centuries many harmful effects of alcohol were postulated and debated. In 1899, a physician

named Sullivan (1899) investigated the outcome of the pregnancies of 120 female alcoholics in a Liverpool prison, and concluded that the mortality and stillbirth rate was two and a half times higher in the alcoholics than in their non-drinking relatives. But in the early 1900's, instead of conducting experiments, moral and scientific statements were simply presented, argued, and reviewed. While many still supported the view that alcohol intake during pregnancy was harmful, others came out very strongly against such a view (Warner '75). Little was written during the 1940's, 1950's, and early 1960's, until a study by Lemoine ('68) was done in France and reported in a little known publication. He described facial abnormalities, motor disturbances, low birthweight, and malformations in children of women who were alcoholics. About the same time, but independent of Lemoine's report, similar abnormalities were reported by Jones and Smith ('73). In this 1973 study, the collective abnormalities were considered the Fetal Alcohol Syndrome (FAS).

The Jones studies ('73, '75) describe the pattern of morphological defects in sixteen children born to alcoholic mothers. Following are the typical FAS characteristics found in the Jones study and references to other investigations documenting similar findings in human infants. Observed craniofacial defects include: short

palpebral fissures (Clarren '78a; Havers '80), maxillary hypoplasia (Clarren '78a; Havers '80; Lemoine '68), epicanthal folds (Clarren '78a; Havers '80), and cleft palate (Herrmann '80; Olegard '79). Growth features and performance deficits he saw were: developmental delay (Clarren '78a; Darby '81; Landesman-Dwyer '81; Staisey '83), microcephaly (Clarren '78a; Havers '80; Ouellette '77), prenatal growth deficiency (Kaminsky '78; Landesman-Dwyer '81; Little '77; Ouellette '77; Rosett '80; Streissguth '80a), and postnatal growth deficiency (Havers '80; Landesman-Dwyer '80; Olegard '79). Central nervous system involvement was indicated by fine motor dysfunction (Landesman-Dwyer '81; Ouellette '77; Staisey '83) and low IQ (Lemoine '68; Olegard '79; Streissguth '80). All the preceding anomalies except cleft palate were seen in at least half the infants. There were also limb anomalies such as altered palmar crease patterns and joint problems. Some infants had increased incidence of hemangiomas, and there were some cardiac defects. Prenatal growth deficiency, more severe in regard to length than weight (unlike what is seen in malnutrition) and mental deficiency, with IQs ranging from 50 to 83, were shared by all children. His figures indicate that 43% of all alcoholic women have adverse outcomes of pregnancy. Of the offspring of 23 alcoholics he cites a 17% fetal mortality

rate versus 2% in offspring of 46 non-drinkers, and 32% full fetal alcohol syndrome versus 0% for non-alcoholics.

An autopsy was performed on one infant who had been diagnosed FAS (Jones '75). Extensive developmental anomalies of the brain were seen, and were reported to be a result of an aberration of neuronal migration. There were sheets of neuronal and glial cells which had migrated over the surface of the cerebral hemispheres. These are similar to findings of other investigators in both clinical and experimental situations (Clarren '78; Reyes '83). Many of the developmental delay problems could be a result of this type of brain disorganization.

Since these typical features of children born to alcoholics were described by Lemoine, and Jones et al, many retrospective and prospective human studies have been published (see review article by Streissguth '80a). Problems exist in these studies because of numerous variables. These are compounded by the need to depend on the memory and the honesty of the women being interviewed.

Sokol ('80) has addressed some of these problems in study designs for prospective clinical experiments. In his own work ('80a) he determined that 204/12,127 (1.7%) of the births at a single institution were complicated by alcohol. Five of these had enough stigmata to be labeled fetal alcohol syndrome infants. During this study he proved that

alcohol and smoking do have an additive effect on the fetus. Therefore, smoking, as well as other concomitant risk factors such as nutrition, maternal age, demographics, and socioeconomic background, must be considered in the epidemiologic experimental design. Considering these factors, he still estimates that 50% of women who abuse alcohol endanger their offspring.

In a prospective plan proposed by Ouellette, Rosett, et al ('77) at Boston City Hospital, 633 women agreed, on their first prenatal visit, to be involved in the study. Such factors as smoking and other drug use were considered. Alcohol intake of five or more drinks on occasion and consistent daily average of more than 45 ml absolute alcohol classified the subject as a heavy drinker; moderate drinkers were those who drank more than once a month without meeting heavy drinking requirements; abstinent or non-drinkers drank less than once a month. Microcephaly and congenital anomalies occurred twice as frequently in the heavy drinking group. Major decreases in growth measurements were also seen. No significant problems were seen in the moderate and light drinking groups, but, in a later study (Rosett '80a) less growth retardation is observed when alcohol consumption was reduced before the third trimester. A growth spurt occurs at this time of fetal development.

When Little ('77) considered one ounce of absolute alcohol daily to be "moderate" drinking in a similar prospective study of 263 women members of a health maintenance organization, she did find lower birth weights. This finding was supported by Kaminski's ('78) prospective study of 9236 pregnancies in France. In the latter study, Kaminski also noted increased fetal death in what he considered to be relatively light daily drinking of approximately 1.6 oz. of absolute alcohol.

One conclusion which becomes apparent when reading epidemiologic human studies is the lack of consistency in ascertaining the drinking level categories. Most studies use an average alcohol/day consumption, but when 2913 pregnant women were screened in Michigan it occurred to the investigators that it would seem to be more accurate to determine the amount used on "drinking" days (Sokol, '80a). This would increase the estimated blood ethanol level in certain patients and might explain why some infants are affected to a greater degree than others.

Because it is apparent that there are differences in degree of damage to the fetuses of mothers who drink, some investigators suggest using the term fetal alcohol effects (Olegard 79; Smith '81) instead of syndrome. The diagnosis of FAS, as suggested in a report to the Research Society on Alcoholism (Rosett '80), should be confined to patients who

meet minimal criteria. This would include only patients who have signs in each of three categories: A.) growth B.) CNS involvement, and C.) facial dysmorphology with at least two of the following signs: microcephaly, microphthalmia and/or short palpebral fissures, or poorly developed philtrum, thin upper lip, and flattening of the maxillary area. The CNS involvement can be signs of neurologic abnormality, developmental delay or intellectual impairment.

In addition to these accepted and well-documented guidelines for FAS, however, alcohol is reported to cause other defects in FAS children which should not be overlooked. Herrmann ('80) has reported a significant number of short metacarpals and metatarsals and tetraectrodactyly in his case studies of eleven FAS patients. He also saw abnormal scalp hair patterns associated with significant brain involvement. Reports of 110 FAS patients in West Germany (Havers '80) included nine cases of malformations of the urinary tract. Frequency of cardiac malformations is controversial, but in 76 cases of patients with documented features of FAS, 31 (41%) had cardiac lesions (Sandor '81). Decreased muscle tone among nine-day-old infants of alcoholic mothers has also been reported (Staisey '83).

While the full syndrome may be manifest only in the children of alcoholic mothers, it is suggested that lesser

degrees of involvement may be attributed to "social" or moderate drinking. Speech problems (Iosub '80) may be one manifestation of such neurological problems, while behavior (Landesman-Dwyer '81) in which offspring of moderate drinkers were seen to be "less attentive, less compliant with parental commands, and more fidgety at mealtimes," may be another. This study took into account the differences in home environment. In her review of epidemiologic and experimental studies, Little ('81) mentions many of the "subtle effects" caused by maternal alcohol intake, and her own interpretation is that the large body of reports indicating adverse effects of moderate drinking are too consistent to discount.

Having established high doses of ethanol as a human teratogen, many questions are raised as to the timing, dosages, and mechanisms which result in fetal damage. A dose-response relationship is indicated in some of the previously documented human studies (Landesman-Dwyer '81; Little '81; Rosett '80a). Because of ethical problems, genetic differences, and uncontrollable multivariants this cannot be explored with accuracy in retrospective or prospective human studies. In order to answer some of the questions, animal models must be used (Sokol '80).

Alcohol: Animal Studies

In recent years, investigators have worked with chicks (Pennington '83), dogs (Ellis '80), rats (Messiha '83; Rawat '77; Reyes '83; Samson '84; Sorette '80; Strahlendorf '83), sheep (Kirkpatrick '76), rabbits (Schwetz '78), tadpoles (Nakatsuji '83), monkeys (Clarren '82; Scott '84), and mice (Boggan '79; Chernoff '77, '80; Crabbe '83; Giknis '80, '80a; Kronik '76; Randall '77; Sandor '80, '80a; Schwetz '78; Sulik '81, '83). The results of this body of research show many marked similarities between the effects of alcohol on the animal and human fetuses. The mechanisms and results which will be documented in the following discussion of animal research, appear to parallel the types of damage which have actually been seen in the developing human exposed to alcohol in utero.

One concern of many investigators involved in designing alcohol research studies is eliminating the possible adverse effects caused by poor nutrition. Not only does poor diet affect alcohol metabolism (Wiener '80), but, alcohol has been shown to slow the absorption of nutrients from the GI tract (Abel '80) and, in high doses, to have an anorexigenic effect on animals (Sorette '80). Carefully controlled animal experiments (Chernoff '80; Ellis '80; Goad '82; Randall '81; Sorette '80) demonstrate that it is the alcohol and not poor nutrition which is

responsible for the fetal damage, and a diet which supports healthy development of mouse fetuses in alcohol experiments has been formulated (Goad '82).

Since the mouse will be used in this study, it will often serve as the animal of reference when metabolic and developmental comparisons are needed. Genetic differences do alter alcohol metabolism and therefore its teratogenic effects (Chernoff '80; Crabbe '83; Giknis '80; Webster '80), but these can be controlled by using the same animal strain. Rodents, as well as man, have a microsomal oxidizing system which has the capacity to increase in activity adaptively after ethanol feeding (Lieber '70). Since the fetus has not developed this system, levels of alcohol handled by the maternal system could harm the fetus. There are still differences in animal and human metabolism of alcohol (Abel '80). Mice, for example, metabolize alcohol at a rate of 300 mg/kg/hr compared to 100mg/kg/hr for man (Wallgren '70). This means that the duration of the same peak drug levels will be longer in man. Perhaps this is favorable in terms of extrapolating from the mouse to the human in teratological experimentation since each stage of development is of shorter duration in the mouse.

Like genetic differences, the day or days of drug administration and dosage levels are variables which can be

controlled in animal studies, and they are important in understanding the mechanism of the effect of alcohol on the developing fetus. Indications of teratogenicity range from embryoletality to gross malformations and include a great variety of more subtle abnormalities, both morphological and behavioral. Some of these problems, such as heart anomalies (Tuchmann-Duplessis '83) and genitourinary problems (Havers '80), may not be recognized until several years after birth. With a better understanding of the timing of the damage, more corrective measures can be planned.

Animal researchers have examined the dose-response relationship and/or the timing of the dosage, and have concentrated largely on embryoletality, fetal weight, and gross malformations which resemble those of the human fetal alcohol syndrome. Many of the individual characteristics of the human FAS infant have been seen, and, in some cases, several are combined in the same animal fetus. The general conclusion is that maternal alcohol consumption has an adverse effect on the developing animal fetus, and that it can be used to answer some of the questions related to the human syndrome.

Trying to simulate the condition of the chronic alcoholic human mother, Chernoff ('80) used two strains of mice, CBA and C3H, which were fed a liquid diet of 15-35%

alcohol-derived calories. He controlled for nutritional effects by using a liquid diet proven to be more than adequate for normal development. The treated mice received alcohol diet thirty days before gestation and throughout pregnancy. And, although blood alcohol levels may not be equivalent in pregnant and non-pregnant animals (Abel '80; Kesaniemi '75), he used non-pregnant animals given the alcohol diet so the pregnant animals would not have that added stress. Blood alcohol levels (BAL) ranged from 73-398 mg/100 ml blood. A strain difference in BAL was noted which showed a direct relationship to the number of anomalies. This might partially explain the differences seen in offspring of women who drank approximately the same amounts of alcohol (Kaminsky '78; Ouellette '77).

The most common skeletal anomaly seen by Chernoff was a missing supra-occipital bone, and, at higher doses there were rib and vertebra anomalies. Skeletal defects were reported in the human study by Herrmann('80), and in several experiments using mice (Kronick '76; Webster '80, '83; Randall '77). A high percentage of brain anomalies was seen in both strains at high and low dosages. This, of course, is consistent with the human studies and has been seen in rats (Burns '84; Rawat '77; Reyes '83; Samson '84; Strahlendorf '83), monkeys (Clarren '82), tadpoles (Nakatsuji '83), and mice (Nakatsuji '84; O'Shea '81;

Randall '77; Sulik '83; Webster '83). Cardiac anomalies were seen in this Chernoff study in a dose-dependent number, and Randall ('77) reported similar defects in her mouse study. Cardiac problems in the human were documented earlier (Jones '73; Sandor '81). Cleft palates, reported in the human (Jones '73; Herrmann '80), occurred in dogs (Ellis '80), and mice (Sulik '83; Webster '83). Renal anomalies were seen in human studies by Lemoine ('68) and Havers ('80) and have been reproduced in dogs (Ellis '80) and mice (Boggan '79; Randall '77). Other craniofacial abnormalities characteristic of FAS have been seen in numerous animal studies (Clarren '82; Nakatsuji '84; Samson '84; Sulik '83; Webster '83). Chernoff also saw decreased litter size and decreased prenatal growth. Both these characteristics of maternal ethanol consumption were seen in all but a few of the many animal studies which have been documented (Clarren '82; Randall '77; Schwetz '78).

Many of the animal experiments mentioned were not designed as chronic studies, and much evidence is available from animal (Nakatsuji '84; Sulik '83; Thadani '79; Webster '83) as well as epidemiologic reports (Clarren '78; Herrmann '80; Warner '75) to indicate that damage can occur to the fetus of the occasional or "binge" drinker. This theory is strengthened by the dose-response relationships documented in many animal studies (Abel '80; Brown '79;

Clarren '82; Dexter '83; Ellis '80; Giknis '80; Pennington '83; Samson '84). FAS-like fetal insult has resulted when dosing has been done during short periods of embryo development.

In 1980, Webster studied both inbred (C57BL/6J) and outbred (QS) mice to test the binge drinking theory. He gave the mice one single IP injection of 25% ethyl alcohol in normal saline at 0.030%(5.8g/kg), 0.022%(4.3gm/kg) or 0.015%(2.9g/kg) body weight on one of gestation days 7-11. The blood alcohol level in the 0.030% group reached 800mg/100ml thirty minutes after injection, and the mice were comatose for four to six hours. Lower doses produced BALs of 600mg/100ml and 350mg/100ml, but he does not report on the condition of the mice at lower doses. With this design, a definite dose-response relationship in the teratogenicity of alcohol was established, as well as a relationship between the type of malformation and the timing of the dose. Maxillary hypoplasia, seen most often in doses given on gestation day eight, associated with reduced eye size and dysplasia of brain and hypophysis, correspond with typical characteristics of human FAS. Limb defects were more common in animals dosed on gestation days nine, ten, or 11. While there were strain differences, results from both strains supported the conclusion that alcohol was teratogenic in binge drinking.

Webster ('83) recently reported that with two doses of ethanol four hours apart the BAL increases after the second dose, but if the doses are six hours apart they do not. In correlating this information with other aspects of his experiment, he added to the evidence that it is the BAL which is most responsible for the level of fetal insult. Again, Webster found more head anomalies in animals dosed on gestation days seven and eight and more limb anomalies if treatment was on days nine or ten.

In a similar study, Sulik ('83) gave two acute doses of ethanol (25% v/v 0.015gm/kg in saline) intraperitoneally to C57BL/6J mice. She injected on gestation day seven plus or minus four hours to correspond with the time of gastrulation. In an earlier, similar study, Sulik ('81) compared this with the third week of human pregnancy. She used a gas chromatographic technique to determine BALs at 20 to 30 minute intervals for eight hours following the initial dose of ethanol. Peak BALs ranged from 150-218 mg/100ml within 30 minutes. These are much lower than the levels achieved with the same dose by Webster, and could reflect the method of assay. When the first dose was given at six days 20 hours the embryo absorption rate was low (9.5%) but, by gestation day 14, 59.6% exhibited craniofacial malformations. In addition to abnormal nasal and upper lip regions there were many eye malformations.

The C57BL/6J mouse strain is genetically predisposed to eye malformations, but there was still a significant difference. In animals in which treatment was initiated at seven days zero hours, the resorption rate was 18.2% and the craniofacial malformation rate was 44.4%. The neural plate in this group appeared particularly small, and the "blebbing" of neural epithelium reported in other studies (Bannigan '82; Nakutsuji '83) was seen. The experimental group which was dosed on gestation day seven plus four hours had a 45.9% resorption rate with 27.5% abnormal fetuses.

Sulik concludes that the typical FAS craniofacial features result from acute ethanol insult during the time the anterior neural plate is forming. This would account for the eye abnormalities and the medial nasal prominence abnormalities seen in the medial aspect of the nose, the philtrum and the primary palate. Her hypothesis is supported by Nakatsuji's ('83, '84) tadpole and mouse studies in which his observations lead him to suggest that neural tube related craniofacial defects may result from inhibition of mesodermal migration.

Alcohol: Mechanism of Action

It has been stated by Tuchmann-Duplessis ('83) that "the high susceptibility of the embryo to exogenous agents

is due to cellular multiplication and differentiation and to the lack of development of the enzyme systems necessary for the detoxification of chemicals." Ethanol is toxic to cells (Guth '83), and, when ingested by the mother is soon found in at least equal amounts in fetal tissue (Kesanieni '75; Mann '75). Therefore, it is understandable that alcohol levels could be quite high in the developing neural tube and that it could exert its toxic effect there. Since the central nervous system is one of the most strongly predetermined tissues (Jacobsen '66), the toxic effects of alcohol could begin affecting it even before placental flow is established. This would be possible because of the rapid diffusion of alcohol to all cells, and because the sites of action of ethanol are considered to be membranes, both cellular and subcellular (Tuchmann-Duplessis '83).

Evidence strongly suggests that alcohol is also damaging to other developing tissue. In order to determine its exact distribution, Akesson ('74) injected ¹⁴C-labeled ethanol into eight pregnant albino mice. He discovered that it distributed quickly to the fetus, being equal in fetal and maternal tissues in only ten minutes. In some tissues the levels of alcohol continued to rise beyond maternal concentrations. One such tissue is the brain. The concentration here could be due to the high lipid content of the brain (Stave '78). The highest levels of

alcohol were reached in the liver and in bone. The concentration in bone could be responsible for the skeletal malformations often seen in FAS. An alcohol-induced decrease of glycosaminoglycans in rabbit articular cartilage (Chacha '80) might support this theory.

While some investigators (O'Shea '79; Veghelyi '77) have presented evidence that acetaldehyde, the primary metabolite of ethanol, is the embryotoxic substance, others (Bannigan '82; Webster '83) show no increase of fetal effects in their initial studies with acetaldehyde. Brown ('79) has cultured rat embryos in ethanol and seen growth deficiencies similar to those in FAS, implicating ethanol and not its metabolites. Kesaniemi ('75) shows that only 25% of the maternal acetaldehyde level in rats is found in the placenta, while none could be detected in fetal tissue. Humans, rats and mice have the same basic alcohol metabolizing system with about 80% metabolism occurring in the liver (Leake '66; Mann '75) via the hepatic enzyme alcohol dehydrogenase. When Chernoff ('80) measured the alcohol dehydrogenase level in his experiment using three mouse strains, CBA, C3H, and C57, he found an indirect relationship in the level of the maternal enzyme and in fetal abnormalities. Since the enzyme system is not developed in the early embryo, and, since acetaldehyde apparently has little ability to cross the placenta, the

ethanol itself must be the toxic substance. This evidence supports the theory of Bing ('82) and Messiha ('83) that the difference in the maternal ability to metabolize alcohol, possibly a genetic difference, plays a role in FAS.

Hormonal and neurotransmitter levels have been affected in offspring of alcohol treated subjects. Druse ('81) reviewed the experimental work on this subject, but, the connection with FAS, if there is one, has not been clearly established. Rawat ('81) feels that the decreased levels of acetylcholine, GABA, and glutamate may cause some neurological anomalies. In his rat experiments, Thadani ('79) discovered lower levels of growth hormone which might decrease the amount of ornithine decarboxylase. Since ornithine decarboxylase is the rate limiting step in cell proliferation, this could account for the FAS anomalies. Reduced cellular proliferation has been indicated in many studies (Brown '79; Pennington '83; Rawat '75; Sorette '80) which show reduced cell number and/or reduced DNA content in the developing fetus.

Another explanation for this reduced protein synthesis, however, is poor fetal nutrition. This deficiency is not due to maternal diet, but to the effect of alcohol on placental function, or, to the vasospasms reported to be an ethanol induced response, reducing blood

flow in umbilical vessels (Altura '82). Chromosomal damage could be a factor also. Obe ('79) has examined the lymphocytes of 100 alcoholics and has seen exchange-type aberrations in alcoholics with about five times the frequency as in non-alcoholics. His literature review of ethanol induced damage is controversial and not convincing. Other studies (Veghelyi '77; Henderson '81) show no genetic defects produced in experimental work.

As can be seen, there are many questions concerning the mechanism of ethanol teratogenicity. The pattern which seems to be emerging when considering both human and animal studies is that ethanol itself is teratogenic in a dose-response relationship. The amount of fetal insult is determined by the timing of high blood alcohol levels. Since the chronic drinker reaches these levels more frequently during all stages of embryonic and fetal development, she is more likely to bear infants with manifestations of the full fetal alcohol syndrome. Binge, or moderate drinkers bear infants who may show lesser degrees of damage, the particular insult dependent on genetic susceptibility and the timing of high blood alcohol levels, as well as other variables, which include simultaneous use of other drugs.

Aspirin: History and Human Studies

One of the most frequently ingested drugs is aspirin, acetylsalicylic acid (ASA) (Reid '83). It was discovered as a byproduct of coal tar in 1853, but it was not used medicinally or made synthetically until the end of the century (Nault '74). Although studied pharmacologically in the late 1800s, no reports of damage to the human fetus were mentioned until Jackson ('48) published a case study in 1948. A stillborn infant was born to an eight month pregnant female suffering from salicylate poisoning. In the journal article reporting this case, Jackson refers to findings of an investigator named Binz, whose concern about possible abortion in his rat experiments led to warnings that aspirin should not be used in large amounts in pregnancy. The case study encouraged Jackson's own animal research which convinced him that, while aspirin does cross the placenta in high concentrations, it was not harmful to fetal tissue.

However, in 1959, Warkany and Takacs ('59) proved salicylates to be teratogenic in rats if given at approximately five times the human dose. Then, in the late 1960s epidemiologic studies began to be reported (see review article by Corby '78). In order to discover the etiology of 833 defective infants born in South Wales between 1964 and 1966, a retrospective investigation was performed

(Richards '69). Salicylates used in the first trimester proved to be a significant factor in the "all systems defects" category. They were implicated specifically in anomalies of the central nervous system and the alimentary canal, which includes cleft lip and palate, and in the occurrence of talipes. When Saxen ('75) studied 599 children with oral clefts, the incidence was three times greater in infants whose mothers took ASA than in their matched controls.

Nelson and Forfar ('71) reported, in their study of 458 infants with congenital abnormalities, that a significantly high number of the mothers took aspirin during pregnancy. Those taking aspirin in the first 28 days had babies with achondroplasia, hydrocephalus, congenital heart disease, mongolism, congenital dislocation of the hip, hydrocele, and talipes. In 390 babies with congenital heart disease, the mothers took aspirin significantly more often than the control group (Rothman '79).

In case studies reported by McNeil ('73) and Wright ('81), skeletal, cardiac and CNS defects appear to be related to high aspirin ingestion; and, Turner ('75) and Collins ('75) indicated a possible association between aspirin and perinatal mortality. Perhaps the perinatal mortality is related to the effects of aspirin ingested

later in pregnancy. Premature closure of the ductus arteriosus is known to be induced with aspirin (Heymann '76; Ishikawa '79; Prasad '82; Rudolph '81, '81a), and, hemostatic problems have also been reported (Rumack '81; Stuart '72).

The human evidence implicating aspirin as a teratogen is not overwhelming. Retrospective study (Shapiro '76; Slone '76) suggests that no relationship exists between ingestion of aspirin and fetal malformations. In Slone's design, however, his description of a heavy user is questionable, and Shapiro excluded pregnancies of less than seven months duration. Some of the information discovered in the other epidemiologic and clinical studies might also account for questionable conclusions in human studies. For instance, Palmisano ('69) found high levels of salicylates in the blood of women who said they had taken no "aspirin". And, Nelson ('71) discovered that five times more pregnant women self-medicated with aspirin than had them prescribed. Certainly the need for more investigation is indicated when such questionable human statistics are combined with convincing experimental animal data.

ASPIRIN: Animal Studies

In the Warkany ('59) study rats dosed with a single subcutaneous injection of methyl salicylate on the ninth or

tenth day of gestation had 298 young, 40.3% of which were abnormal. There was a high incidence (12/298) of craniorachischisis, an unclosed skull and spinal column similar to spina bifida and exencephaly in the human (Corby '78). Other animals had brain abnormalities, a few with hydrocephalus, and there were some facial clefts, eye defects, gastroschisis, and skeletal defects (75/201). In a later, similar study (Takacs '68) investigators examined internal tissue from 42 specimens which showed external abnormalities and found 25 fetuses with non-uniform cardiac anomalies.

Trasler ('65) soon reported strain differences in aspirin-induced cleft lip in A/J and C57BL/6 mice after two or three high doses of 500mg/kg on gestation days eight and nine or nine and ten. The C57BL/6 mice showed a 28% incidence of overall abnormalities compared with 3% for the controls and 24% in the A/J strain. Exencephaly, microcephaly and various skeletal anomalies were seen in both strains. There was no significant difference between strains.

Strain differences were also noted by Larsson ('66) and Eriksson ('71) in fetal resorption rate, but, most note-worthy was the increase in resorption rate seen when salicylates were given on progressively later days. Exencephaly, gastroschisis, and skeletal anomalies were

seen in fetuses which were not resorbed.

Other studies have shown salicylates to be teratogenic in rats (Becci '82; Butcher '72; DePass '82; Kimmel '71; Klein '81; Wilson '77; Ungvary '83) and mice (Larsson '66, Saito '82), as well as in hamsters (Laponte '64), dogs (Robertson '79), chicks (Ishikawa '79) and monkeys (Wilson '77). Daily doses producing teratogenesis in the above animals range from 250-1000 mg/kg. With a single dose of either 500 or 625 mg/kg, rats dosed on day ten (DePass '82) exhibited skeletal and cardiac abnormalities, hydrocephaly, and cleft palate that were both strain and dose-response related. In his dog study, Robertson ('79) demonstrated a 50% dose-dependent rate of malformations similar to those in humans and other animals.

Aspirin: Mechanism of Action

The mechanism of aspirin teratogenicity in the animal, and possibly the human, is not understood. Cell toxicity has been shown in adult human (Leonards '73) and animal cells (Hingson '71), and if the concentration and duration of exposure to fetal tissue is high enough, perhaps cell damage could account for the observed anomalies. In Klein's ('81) rat study showing skeletal malformations, he hypothesizes that the decreased umbilical blood flow caused by prostaglandin inhibition prevents proper nutrition

resulting in the observed mesodermal cell death. Prostaglandin inhibition could also result in the premature closure of the ductus arteriosus, and cause an aspirin-induced stillbirth rate increase (Prasad '82; Rudolph '81), as well as the hemostatic problems indicated in studies of fetal damage later in pregnancy (Stuart '72).

It is not known whether it is the ASA or its metabolites causing the fetal damage. Aspirin has long been known to cross the placenta (Jackson '48) in both animals and in man. According to Boulos ('72), this is due to the high solubility coefficient of salicylates. However, it is the primary metabolite, salicylic acid which has been measured in most cases. That salicylic acid is found in fetal plasma in levels even higher than in adult tissue (Levy '75) is probably due to lower protein binding capacity. Many investigators (Kimmel '71; Levy '74; Saito '82) believe it is salicylic acid, not its metabolites, which is the toxic form of the drug.

Kimmel ('71) theorizes that, since acetylsalicylic acid defects are indistinguishable from those of methyl salicylate and sodium salicylate (Warkany '59; Takacs '68), the ASA molecule must not be needed to produce the teratogenic effect. Kimmel also bases her hypothesis on the fact that ASA is hydrolyzed so rapidly it cannot be measured in blood. Morgan ('82), however, measured peak

amounts of ASA twenty to thirty minutes after aspirin ingestion in the adult. The other supporting evidence cited for the toxicity of salicylic acid is the increased number of defects produced when salicylate levels are raised by addition of benzoic acid, as originally reported by Levy ('74). In Saito's ('82) experiment which produced teratogenic defects in the rat, injections of ASA and salicylic acid into the pregnant rat resulted in deformed fetuses. All the other salicylate metabolites failed to cause significant anomalies.

We have seen that aspirin, like ethanol, is teratogenic in a dose-dependent manner, but the primary metabolite, salicylic acid, may be teratogenic instead of, or in conjunction with, acetylsalicylic acid. Genetic predisposition, as well as the timing of the dose, is obviously a factor in the fetal insult.

Alcohol/Aspirin Interaction

Ethanol consumption and aspirin ingestion occur often in the same person. And, as Hansten ('79) states, it is obvious to most health professionals that drug combinations have the potential to cause adverse effects. Possible interactions described are: pharmacokinetic, in which drugs affect the absorption, distribution, metabolism, or excretion of other drugs, and, pharmacologic, in which

effects may be additive, synergistic or antagonistic.

The only well-documented adverse effect of ethanol plus aspirin is a synergistic increase in gastric mucosal irritation (Hansten '79; Hayes '81; Seixas '79; Stern '84). However, in Brodie's experiment ('71) aspirin was injected through a tail vein, so the irritation was not just topical as had been assumed earlier; and, since sodium salicylate did not produce the fecal blood loss seen with ASA (DeSchepper '78; Leonards '73) apparently the ASA molecule, not salicylate only, is toxic. And, according to Morgan ('82), it is the ASA molecule itself which is therapeutic.

There are several recent studies which use ethanol and ASA together and show a drug drug interaction. Reber ('79) used the pancreatic duct of the cat and treated it with doses of ASA, ethanol, and a combination of the two. Each substance caused an increased permeability in the duct, but used together the effect was greater. He then determined, by adjusting the amount of the drugs and the pH, that the effect of ASA on the pancreatic duct is potentiated by small amounts of ethanol at all pH levels.

Using human volunteers, Deykin ('81, '82) gave 125cc of 100 proof vodka and tested bleeding time for five days. After a five day rest, the volunteers took one 325 mg tablet of ASA and had bleeding time tested for five days. After another five day rest he gave the ethanol and ASA

together. The ethanol alone did not increase the average 3.3 minute bleeding time. The ASA alone increased the time to 7.2 minutes, the peak being reached at 12 hours and remaining significantly high for 24 hours. When the ethanol and ASA were taken together the rise in mean bleeding time was brisk, more than doubling in two hours. It peaked at 11 minutes and remained high for 72 hours. These results showed a marked potentiation of ASA with ethanol.

Diabetic flushing with ethanol is an effect often seen. Strakosch ('80), using human observations, reported a decrease in the flushing when the patients were treated with ASA. He theorizes that prostaglandin inhibition caused by the ASA is responsible for this reaction. Ethanol also affects prostaglandin levels (Horrobin '80), so there is possibly an antagonistic interaction. Prostaglandins were measured in fetal rat and lamb organs by Pace-Asciak ('76). Because of the pattern of prostaglandin levels compared with those in corresponding adult rat and lamb tissue, he concluded that prostaglandin levels may play a role in tissue maturation and differentiation.

Only one fetal study has been reported in which both aspirin and alcohol were given concurrently. Randall ('84) found that an aspirin pretreatment antagonized the

deleterious effects of acute alcohol on the developing C57BL/6J mouse fetus.

Obviously some pharmacologic or pharmacokinetic action is involved in these situations. And, there are several individual aspirin or alcohol studies which would provide possibilities for additive, synergistic, or potentiating effects on the development of the fetus.

Using mice, Chen ('68) tested the extensor seizure-threshold and found it to be increased with ASA, indicating some central depression; ethanol is also known to be a CNS depressant (Rodman '68). Ethanol decreases reflexes in the lamb fetus (Kirkpatrick '76), and, in the adult human, aspirin decreases the mono-synaptic reflex (Eke-Okoro '82).

Ethanol was shown to produce umbilical vasospasms (Altura '82), and, Klein ('80) and Rudolph ('81) have shown vascular constriction with the use of ASA. Low birth weight can occur as a result of reduced placental flow (Robinson '79), so the drugs may have an additive effect.

Since there is a dose-response relationship between the amount of either ethanol or aspirin and their teratogenic effects, the increase in fetal levels of either drug should increase the possibility of fetal insult. Gupta ('79) has performed an in vitro investigation to determine the effect of other drugs and chemicals on the

degradation of ASA. When ethanol was used, it was found to inhibit the action of aspirin esterase. Since this enzyme is needed in the metabolism of aspirin, the toxic effects of the intact molecule would impinge on the fetal cells for a longer time, and would reach higher concentrations.

Another way in which the level of ASA in the fetus might be increased is due to the renal excretion of ASA (Levy '75). Barnes ('73) has reported that urine acidification favors resorption of salicylates. Mann ('75), experimenting with fetal sheep, has shown alcohol to cause fetal metabolic acidosis.

The ethanol-induced decrease in the effectiveness of the blood brain barrier (Lee '62), is noteworthy since the most frequently seen expression of teratogenic malformations of both ethanol and salicylates are on the CNS. In studies of both drugs, one of the "moderate" effects also relates to the CNS in the form of behavioral teratology. Lee ('62) injected radioactive iodinated albumin into rabbits pretreated with varying amounts of ethanol. While the control showed no radioactivity in the brain, the brains of the treated animals showed increased amounts of radioactivity as the concentration of ethanol increased. In an attempt to extrapolate the actual level of alcohol which would cause this amount of permeability in the human brain, Lee suggested it would probably be about

0.2%. That is the level at which a human normally feels "dizzy" (Hansten '79), or which is low for an alcoholic (Veghelyi '77), and so is certainly within human range. Since Akesson ('74) found higher levels of alcohol in the fetal brain than in the maternal brain, perhaps the increased brain permeability of the fetus could increase the toxic effects of ASA.

The clinical, epidemiologic and animal experimental research reported here attribute many similar defects to ethanol and salicylates. These include: central nervous system disorders ranging from anencephaly, exencephaly, and microcephaly to low intelligence and behavioral problems; oral clefts; anomalies of the eye, heart, and urinary tract; many skeletal defects; and, low birth weight, increased incidence of abortion, and perinatal mortality.

While the clinical manifestation of alcohol teratogenicity is now widely accepted, it is only when the group of defects occur together that alcohol is blamed. There are obviously common defects which can be occurring at different times during development, therefore if one defect appears alone in a non-alcoholic mother ethanol is not suspect. Since many of those same defects have been attributed to aspirin-induced damage in some human and several animal studies, perhaps a harmful combination has been overlooked due to the type of malformations: ones that

get lost in the statistics as "spontaneously occurring".

Yaffe ('78) speculates that, had thalidomide caused a less dramatic type defect, it might have been much longer before its damage would have been noticed.

Levels of alcohol and aspirin which would not be toxic alone, and which might not seem noteworthy to either the pregnant woman or the epidemiologist, could interact or cause an additive effect to account for some of the 60% of fetal defects of unknown etiology (Fabro '83). The purpose of this study was to determine if a binge episode of alcohol in the presence of a non-teratogenic dose of aspirin (both drugs listed as teratogens by Shepard '80), would result in augmentation or interaction to produce a teratogenic effect in the CD-1 mouse fetus.

MATERIALS AND METHODS

Animals

Nulliparous CD-1 mice (Charles River Breeding Laboratories) were housed in polypropylene cages in a temperature-, humidity-, and light-controlled (12 hour light/dark cycle) environment. They were fed Purina Lab Chow and tap water ad libitum while they acclimated to the lab and matured. Adult mice were mated, for the first hour of the light cycle, in a ratio of two females to one male. The presence of a vaginal plug was designated day 0 of gestation. Pregnant females were weighed daily. On 8 days + 4 hr of gestation pregnant animals were divided into the following treatment groups.

Experimental Design

Group I. (Untreated Control) An untreated control group of ten pregnant animals was weighed daily.

Group II. (Treated Control) A treated control group of ten pregnant females was given three doses of saline, two 0.02 ml/gm doses (IP) two and one half hours apart, and, one hour later, a 0.5 ml dose by intubation.

Group III. (250mg/kg Aspirin) Ten pregnant females were given two IP injections of 0.02 ml/gm saline two and one half hours apart. One hour after the last dose of saline the mice received, by intubation, 250mg/kg acetylsalicylic acid (aspirin, Mallinckrodt USP) in approximately 0.5 ml solution. The aspirin solution was freshly prepared for each treatment with two drops of Tween 80 (to reduce surface tension) in 10 ml of physiological saline.

Group IV. (500mg/kg Aspirin) Ten pregnant females were treated as those in Group II, but received only 500mg/kg aspirin.

Group V. (Ethanol) Ten pregnant mice were given two intraperitoneal (IP) injections each of 0.02 ml/gm of 25% v/v of 95% ethanol/physiological saline solution two and one half hours apart. One hour after the second ethanol dose the mice received approximately 0.5 ml physiological saline by intubation.

Group VI. (Ethanol+Aspirin) Ten pregnant females were given two 0.02 ml/gm IP injections of the ethanol solution. One hour later they were intubated with 250mg/kg aspirin.

Group VII. (Aspirin+Ethanol) Ten pregnant females were pretreated with 250mg/kg aspirin (by intubation). One hour later they received the first of two doses

of 0.02 ml/gm ethanol; the second dose of ethanol was given two and one half hours later.

All pregnant animals (except Group I) were denied food and water for six hours to approximate the time the alcohol sedated animals were unable to eat and drink.

Blood alcohol levels were determined in ten separate pregnant females treated as those in Group V (two doses of ethanol) and Group III (two doses of ethanol followed by 250 mg/kg ASA). Blood samples (n=2 in each group) were taken from the retro-orbital sinus 15 minutes after the first ethanol treatment; ten minutes before the second treatment; 15 minutes after the second treatment; and, one hour and 15 minutes after the second alcohol treatment (15 minutes after the ASA in the aspirin treated group). The blood was collected in heparinized capillary tubes, centrifuged (IEC model PR-2) for 30 minutes at 2000 RPM, and the plasma was analyzed for alcohol content using the Sigma Chemical Company diagnostic kit. (Sigma Diagnostics Alcohol Procedure no. 332-UV, 1983). Absorption was measured using a Gilford spectrophotometer.

Examination of Fetuses

On gestation day 18 each pregnant dam was weighed and sacrificed by cervical dislocation. The uterine horns were examined for live, dead and resorbed fetuses. The live

fetuses were removed, weighed, sexed, and examined for external malformations.

One-third of the live fetuses were fixed in 95% ethanol for skeletal examination using Inouye's technique ('76). The fetuses were examined for skeletal abnormalities and developmental delay. The remaining two-thirds were placed in Bouin's fixative for visceral examination. Palate and facial features were inspected for abnormalities, and the brains were then removed, weighed, and examined for ventricular enlargement. The remainder of each fetus was examined for visceral defects using Barrow's technique ('69). To reduce the observational bias, all control and treated dams were assigned a code number unknown to the examiner.

Statistical Analysis

The data obtained were analyzed by one way analysis of variance and covariance using the litter as the unit of analysis and litter size as the covariate. Tukey's and Newman-Keuls served as methods for post hoc multiple comparisons with significance set at the 0.05 level. Additional data was analyzed by chi-square contingency tables using the fetus as the unit of analysis and adjusted standardized residuals as follow-up measures. Summary data is presented in the form of means, standard deviations, and frequency counts.

RESULTS

Litter Effects

Treatment effects on the number of dead and resorbed fetuses and on mean fetal weights are shown in Table 1. In an analysis of variance and covariance, no significant difference was found in the number of dead and resorbed fetuses among treatment groups.

There was a difference ($p < .01$) among groups in average fetal weight. Newman-Keuls test identified the experimental group pretreated with 250mg/kg aspirin prior to receiving two doses of ethanol as having significantly lower mean fetal weights than those in all other groups. None of the other groups showed statistically significant differences in fetal weight.

The total fetal brain weight in the group pretreated with aspirin was also significantly lower ($p < .0001$) than all other groups, while the other groups did not differ significantly. Percentage brain weight, however, showed no significant difference among groups. (See Appendix A for mean values in fetal weights and fetal brain weights).

Fetal Malformations

Table 2 shows the effect of maternal treatment on frequency and type of malformation. Malformations included cleft palate (Fig. 1), hematoma, kinky tail (Fig. 2), club foot (Fig. 3), and exencephaly (Fig. 4), as well as hydronephrosis (Fig. 5b), enlarged bladder (Fig. 5c), and an ectopic ovary. Chi-square analysis using the total number of fetuses affected showed a significant difference ($p < .05$) among treatment groups. The aspirin pretreatment group was significantly different from all other groups in an analysis of adjusted standardized residuals. Analysis of variance and covariance using the litter as the unit of measurement showed no significant difference among treatment groups when external malformations and visceral defects were considered separately.

Skeletal Defects

As shown in Table 3 and Figures 6-8, skeletal anomalies and delayed ossification occurred in all treatment groups. The anomalies consisted largely of supernumerary ribs, rib fusions, and extra sternbrae. In a chi-square analysis of the total number of fetuses examined for skeletal defects, significance ($p < .0001$) was found among treatment groups. Interpretation of adjusted standardized residuals analysis indicated that both the

Aspirin+Ethanol and the Ethanol+Aspirin treatments caused statistically significant ($p < .05$) differences in the number of affected fetuses when compared to all other groups.

In an analysis of variance among litters, statistically significant differences were seen in percent developmental delay and in total percentage of fetuses showing treatment effects on the skeleton. (See Appendix B for mean values in developmental delay and total percentage of skeletal defects.) The percentage of developmental delay in the Aspirin+Ethanol group was significantly different ($p < .05$) from all other groups using Tukey's Critical Difference. The percentage of skeletal defects in the Alcohol+Aspirin group was significantly different ($p < .05$, Newman-Keuls) from all other groups except the Ethanol+Aspirin. The Ethanol+Aspirin group had the second highest incidence, but it was not significantly lower than Aspirin+Ethanol or significantly higher than the other groups.

Summary Data

To complete the analysis of variance and covariance, percentage figures were also analyzed for the following measures: dead and resorbed, external malformations, and visceral defects. Statistical significance was seen only in skeletal defects. When all measures were ranked, however, the sum of ranks indicated the combination of

alcohol and aspirin caused considerably more insult to the developing fetus than any other treatment (Table 4).

Blood Alcohol Levels

Mean blood alcohol levels were highest 15 minutes after the second dose, $659 \pm 30\text{mg}/100\text{ml}$. Levels 15 minutes after the first treatment were $417 \pm 55\text{mg}/100\text{ml}$, and they had decreased to $215 \pm 30\text{mg}/100\text{ml}$ ten minutes before the second treatment. One hour and 15 minutes after the second treatment the levels were $611 \pm 21\text{mg}/100\text{ml}$. No difference between groups was evident after the last measurement suggesting that the aspirin did not affect the alcohol metabolism.

DISCUSSION

The results of this study demonstrate that the CD-1 mouse embryo is adversely affected by a single maternal ethanol exposure when the mother is also treated with aspirin. The main effect of the treatment was developmental delay, one of the characteristics consistent with the fetal alcohol syndrome in the human (Jones '73; Little '77, 81; Streissguth '80; Abel '85) and in animal studies in the monkey (Clarren '82), mouse (Boggan '79; Webster '80), chick (Pennington '83), dog (Ellis '80) and rat (Reyes '83; Sorette '80).

Embryolethality in the ethanol treated mouse has been reported in binge studies using the C57Bl/6J strain (Webster '80; Sulik '83; Randall '84), the BALB/c (Stuckey '84), the B6D2F1/J (Kronick '75), the MF1 (Padmanabhan '84), and the CD-1 (Blakely '84). An increase in mortality rate was not seen in this study. These results are in agreement with those of Giknis ('80) and Hood ('79) who also used CD-1 mice. Strain differences, documented by several investigators (Giknis '80; Stuckey '84; Webster '80; Chernoff '77, '80) could partially account for this

difference in the rate of fetal mortality. Although the CD-1 mouse was used by Blakely, significant differences in resorptions were seen only when maternal ethanol doses were higher than those used in the present study. Several investigators have reported dose related increases in other effects of alcohol on the developing mouse fetus (Webster '80, '83; Giknis '80; Blakley '84; Randall '79; Chernoff '80).

Aspirin has been shown to produce a significant increase in fetal mortality in A/J and CBA mice (Larsson '66; Eriksson '71), but only when the treatment was administered during the last three or four days of pregnancy. The lowest single dose of aspirin reported to cause teratogenic effects in the mouse is 500mg/kg (Larsson '66). A pilot study was performed in our lab to determine the effect of a single dose of aspirin in the CD-1 mouse on day eight of gestation. One dose of 100mg/kg, 250mg/kg, 400mg/kg or 500mg/kg caused no apparent fetal damage. In the present study, 500mg/kg given on gestation day eight, caused no embryoletality or other gross fetal damage. The highest human therapeutic dose of aspirin is approximately 250mg/kg (Schardien '77), half the single dose suspected of being teratogenic in mice. Since the 500mg/kg dose was not embryotoxic, and 250 mg/kg is within the human therapeutic range, it was chosen as the non-teratogenic dose for this study.

Contrary to many reports on acute ethanol studies (Padmanabhan '84; Giknis '80; Randall '84; Blakley '84), the average fetal weight of the Group V (ethanol only) animals, while decreased, was not significantly lower than the controls. Strain, dose level, timing of dose, or treatment protocol may account for the differences. Hood ('79), using CD-1 mice, did not find a significant decrease in fetal weight. The Group VII animals (pre-treated with aspirin) did, however, show significantly lower mean fetal weights, while those post-treated with aspirin did not. It is possible that greater damage occurred because pre-treatment allowed a longer period of cellular exposure to higher levels of ethanol during peak aspirin levels. The aspirin half life in the pregnant mouse is eight hours (Eriksson '71), whereas more rapid metabolism of ethanol has been demonstrated in the mouse; plasma ethanol concentrations decreased by half in only one hour (Gentry '83). Maternal-fetal equilibrium of ethanol is reached approximately ten minutes after an intraperitoneal injection (Akeson '74); this is also the time of peak concentration (Gentry '83). Information is not available on aspirin maternal-fetal equilibrium in the mouse, but in the rat equilibrium is reached in approximately an hour (Wilson '77). Aspirin crosses the placenta rapidly and freely so the slow establishment of equilibrium is probably

due to plasma protein binding (Boulas '72). The elimination of aspirin is even slower in the fetus than in the mother (Levy '74, '75). Therefore, to have maximum concentrations of the ethanol/aspirin combination over longer periods of time, the aspirin would need to be present when the ethanol was injected.

Mental retardation is another human FAS characteristic. Animal studies have shown reduced anterior neural tube and cerebral hemispheres in the C57Bl/6J mouse (Sulik '81, '83; Webster '83), and decreased brain growth in the chick (Boyd '84) and rat (Schapiro '84; Weinberg '85). Weinberg reported a chronic study which showed the decrease in brain weight was not as pronounced as the decrease in body weight, and so considered a possible "brain sparing" effect. In the present study, the decrease in brain weight corresponded to the decrease in body weight (therefore the brain was not "spared" the ethanol damage), and was significantly lower only in the aspirin pretreatment group. Therefore, if brain damage occurred, it was not exhibited by lower brain weight.

The malformations seen in the treatment groups in this study were similar to those seen in the other binge episode studies in the mouse (Kronick '75; Webster '80; Blakley '84; Randall '84; Stuckey '84), and, as in those studies, the incidence of no single malformation is statistically

significant. When considering the total number of fetuses showing either external or visceral malformations, however, the aspirin pretreatment group reported here shows a statistically significant increase in defects (Table 2).

Cleft palate (Fig. 1), seen frequently in FAS children (Jones '73; Olegard '79; Herrmann '80) and possibly caused by aspirin in the human (McNeil '73; Richards '69; Saxen '75), has been reported in both alcohol and aspirin experimentation. Using ethanol, cleft palates were reported in dogs (Ellis '80), and mice (Giknis '80; Sulik '83; Webster '83; Padmanabhan '84); aspirin treatment resulted in cleft palates in the dog (Robertson '79), rat (DePass '82; Kimmel '71; Klein '81) and mouse (Trasler '65; Eriksson '71).

Hematomas are not widely reported in human literature, but experimental support for the observations in this study are reported in ethanol studies using mice (Martinez '85; Padmanabhan '84) and in aspirin studies using hamsters (Laponte '64) and mice (Eriksson '71).

Kinky tail (Fig. 2) has been reported primarily in aspirin studies (Kimmel '71; Saito '82; Robertson '79); while club foot (Fig. 3), seen in aspirin studies in mice (Saito '82) and in rats (Kimmel '71), has been reported in human FAS children (Herrmann '80). Havers ('79) describes limb defects in human FAS which could be compared to the

hindlimb malformations seen in this study.

The occurrence of exencephaly (Fig. 4) has been reported in ethanol experiments on other mouse strains (Randall '79; Padmanabhan '84; Webster '80; Kronick '75) with greater frequency than was seen in the CD-1 mouse in this study. This malformation has also been reported in aspirin studies with rats (Warkany '59), and in the human FAS (Corby '78).

Genito-urinary tract defects, particularly hydronephrosis, occurred in all ethanol-treated litters more frequently than any other malformation. High incidence of hydroureter and hydronephrosis is reported in other ethanol studies in the mouse (Randall '84; Boggan '79; Giknis '80; Blakley '84; Kronick '75). This developing system is also sensitive to alcohol in the dog (Ellis '80) and in the human (Lemoine '68; Havers '80; Sokol '80).

Skeletal anomalies and delayed ossification occurred with the greatest statistical significance in the combined aspirin/ethanol treatment (Table 3). Neither aspirin nor ethanol alone produced significant levels of skeletal defects. Extra sternebrae, supernumerary ribs and rib fusions were seen in all groups treated with alcohol, the highest frequency occurring in the fetuses of mothers which received both aspirin and alcohol. Similar anomalies have

been reported in other ethanol studies on mice (Chernoff '80; Blakley '84; Stuckey '84) and in aspirin studies on mice (Larsson '66; Eriksson '71), rats (Butcher '72; Kimmel '71; DePass '82), and dog (Robertson '79). In a recent case study follow-up, 13 pairs of ribs were reported in a FAS child (Streissguth '85).

Delayed ossification, determined in careful studies in the rat (Aliverti '79) is considered a better indication of developmental delay than low fetal weight. The normal development of ossification centers (Rugh '68; Theiler '72; Wirtschafter '60) in the calcaneus, metacarpals and metatarsals was used as a guide in studying the skeletons in this study. The animals treated with both aspirin and alcohol had greater frequencies of delayed ossification. Support for these observations has been reported in other mouse strains using ethanol alone (Schwetz '78; Chernoff '77; Stuckey '84). When the total number of fetuses with either skeletal anomalies or delayed ossification were considered, a statistically significant ($p < .05$) number of the animals which had been exposed to both ethanol and aspirin were adversely affected. The developmental delay seen in these studies is noteworthy in light of the fact that in other animal studies (Abel '79; Messiha '83; Ihemelandu '84) and in the human (Streissguth '85; Kyllerman '85) catch-up growth does not occur.

When all parameters in this study were ranked, a definite trend emerged (Table 4). The groups which received both ethanol and aspirin accumulated almost twice the total score achieved by the sum of ranks of all other groups.

Blood alcohol levels, which ranged from approximately 215 mg/100ml to as high as 659 mg/100ml over a period of several hours, were comparable to levels which produced teratogenic results in other binge alcohol studies (Stuckey '84; Giknis '80; Sulik '83; Webster '80). The observations from this work demonstrate that, even though these levels did not cause significant damage alone, the binge episode theory of fetal damage with alcohol is valid.

The results of this study indicate an additive effect of aspirin and ethanol on fetal development, but the mechanism of action is not known. Previous studies have shown both aspirin (Leonards '73; Hingson '71; Klein '81) and ethanol (Guth '83) to be cytotoxic. It has also been demonstrated that ethanol decreases the amount of aspirin esterase causing aspirin to metabolize more slowly in the presence of ethanol (Gupta '79). The total concentration of these drugs might, therefore, have reached the level where cell damage could not be repaired.

Another possibility with this particular drug combination is an imbalance in prostaglandin (PG) levels.

Since both ethanol (Rotrosen '80; Pennington '81; Horrobin '80) and aspirin (Smith '71; Vane '71) change the rate of PG synthesis, it is possible that an imbalance in PG levels could be at least partially responsible for the fetal damage. Horrobin ('80) has suggested that a PGE1 deficiency could be responsible for fetal alcohol syndrome. Several investigators are trying to determine the role of prostaglandins in fetal development (Shemesh '83; Caldwell '81; Pennington '81).

Another physiological parameter altered by both aspirin and alcohol is the acid-base balance. Alcohol has been determined to cause a metabolic acidosis (Mann '75; Lieber '84). Aspirin is itself an acid, so the combined direct effect could be detrimental or could help lead to hypoxia as a result of the Bohr effect (Mann '75). Hypoxia is an FAS mechanism suggested by several investigators due to either a change in placental function resulting from ethanol damage (Amankwah '84; Gordon '85), or to contraction of umbilical vessels caused directly by ethanol (Altura '82). It is definitely a consideration in this study where further umbilical vessel constriction could be caused by the aspirin (Klein '80, '81).

The volume of clinical, epidemiologic, and animal experimental research reported demonstrates that maternal alcohol consumption at high levels, and probably even at

"social drinking" levels, is not safe for the developing human fetus. If mechanisms of action and confounding detrimental factors, such as use of aspirin, can be determined, it might offer some hope of corrective treatment during pregnancy for those whose infants are already endangered. Effective use of such information might also decrease the percentage of women of childbearing age who use or abuse alcohol and aspirin.

APPENDIX A
ANALYSIS OF VARIANCE
MEANS FOR FETAL AND BRAIN WEIGHTS

APPENDIX A

Analysis of Variance for Average Fetal Weights

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Prob.
Treat.	0.12772	6	0.02129	3.20	0.0084
Litter size	0.01781	1	0.01781	2.68	0.1068
Error	0.41250	62	0.00665		

Analysis of Variance for Total Fetal Brain Weight

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Prob.
Treat.	0.12987	6	0.02165	2.87	0.0157
Litter size	0.00586	1	0.00586	0.78	0.3814
Error	0.46785	62	0.00755		

Analysis of Variance for Percent Fetal Brain Weight

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Prob.
Treat.	0.57682	6	0.09614	1.41	0.2245
Error	4.29255	63	0.06814		

APPENDIX B

ANALYSIS OF VARIANCE
MEANS FOR SKELETAL MEASURES

APPENDIX B

Analysis of Variance for Percent Developmental Delay

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Prob.
Treat.	34733.57320	6	5788.92887	7.32	0.00000
Error	49856.10749	63	791.36679		

Analysis of Variance for Percent with Skeletal Defects

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Prob.
Treat.	5722.29647	6	953.71608	4.18	0.0014
Error	14389.35832	63	228.40251		

APPENDIX C
TABLES

TABLE 1. Effect of Treatment on Live Births and Fetal Weight

Treatment Group (Ten litters per group)	Total Dead or Resorbed	Total Live	Mean Adjusted Fetal Weight ± Std. Dev.	Mean Adjusted Brain Weight ± Std. Dev.
Untreated Control	9	114	1.353±0.043	0.081±0.0045
Treated Control	6	123	1.313±0.091	0.079±0.0041
250mg/kg Aspirin	5	123	1.293±0.066	0.080±0.0039
500mg/kg Aspirin	3	114	1.314±0.093	0.076±0.0052
Ethanol	3	121	1.297±0.050	0.079±0.0015
Ethanol+ Aspirin	12	110	1.296±0.115	0.078±0.0055
Aspirin+ Ethanol	6	118	1.201±0.095*	0.071±0.0049**

*p<.01 ANOVA

**p<.0001 ANOVA

Aspirin+Ethanol group significantly different from others (Newman-Keuls).

TABLE 2. Treatment Effect on External and Visceral Malformations

Treatment Group (Ten Litters per Group)	Number Litters Affected	*Number Fetuses Affected(%)	Cleft Palate	Hema- toma	Kinky Tail	Club Foot	UG Tract
Untreated Control	1	1(0.88)	-	-	-	-	1
Treated Control	2	2(1.63)	-	1	-	-	1
250mg/kg Aspirin	5	5(4.07)	1	1	1	2	2
500mg/kg Aspirin	3	4(3.51)	-	1	1	1	1
Ethanol	5	6(4.96)	-	-	-	2	3
Ethanol+ Aspirin	4	8(7.27)	1	2	-	1	5
Aspirin+ Ethanol	5	10(8.47)**	1	3	-	-	7

*Chi-square = 12.83, df=6, $p < .05$

#One fetus in the ethanol group was exencephalic.

**Significantly different using adjusted standardized residuals.

TABLE 3. Treatment Effects on Skeleton

Treatment Group	No. Fetuses examined	No. with anomalies (%)	No. with dev. delay(%)	*Total no. affected(%)
Untreated Control	34	5 (14.7)	3 (8.8)	5 (14.7)
Treated Control	36	5 (13.9)	7 (19.4)	11 (30.6)
250mg/kg Aspirin	38	8 (21.1)	3 (7.8)	10 (26.3)
500mg/kg Aspirin	34	4 (11.8)	8 (23.5)	10 (29.4)
Ethanol	38	9 (23.7)	3 (7.9)	11 (28.9)
Ethanol+ Aspirin	33	13 (39.4)	10 (30.3)	19 (57.6)**
Aspirin+ Ethanol	36	9 (25.0)	28 (77.8)	30 (83.3)**

*Chi-square 149.97, df=6, $p < .0001$.

**Statistically different ($p < .05$) using adjusted standardized residuals.

TABLE 4. Percentage of Affected Fetuses

Treatment Group	Dead/ resorbed (rank)	External Defects (rank)	Visceral Defects (rank)	Skeletal Abnor. (rank)	Skeletal Delay (rank)	Sum of Ranks
Untreated Control	6.88(6)	0.00(1)	1.25(2)	15.83(2)	12.50(3)	14
Treated Control	4.75(5)	0.71(2)	1.67(3)	22.50(4)	19.50(4.5)	18.5
250mg/kg Aspirin	3.70(3)	3.41(6)	2.68(4)	17.00(3)	7.83(1)	17
500mg/kg Aspirin	2.96(2)	2.92(5)	1.11(1)	7.00(1)	19.50(4.5)	13.5
Ethanol	2.59(1)	2.67(3)	4.92(5)	25.50(6)	11.50(2)	17
Ethanol+ Aspirin	10.15(7)	2.85(4)	7.38(6)	40.00(7)	30.00(6)	30
Aspirin+ Ethanol	4.58(4)	4.50(7)	8.05(7)	24.17(5)	77.50(7)	30

APPENDIX D

FIGURES

Fig. 1. Ethanol+Aspirin treated 18-day-old CD-1 mouse
fetus with cleft palate.

Fig. 2. Aspirin treated 18-day-old CD-1 mouse fetus with kinky tail (left) compared with smallest fetus from a treated control litter.

Fig. 3. Ethanol+Aspirin treated 18-day-old CD-1 mouse
fetus with club foot.

Fig. 4. Eighteen-day-old CD-1 mouse fetus with exencephaly from ethanol treated litter. Note open eye, protruding tongue, and shortened maxilla in affected fetus.

Fig. 5. a. Normal kidney (k) and urinary bladder (u) in an 18-day-old untreated control CD-1 mouse fetus. b. Bilateral hydronephrosis (k) in Aspirin+Ethanol treated 18-day-old CD-1 mouse fetus. c. Enlarged urinary bladder (u) seen in Aspirin+Ethanol treated 18-day-old CD-1 mouse fetus.

Fig. 6. a. Alizarin Red S and Alcian Blue stained skeleton from an 18-day-old untreated control CD-1 mouse fetus. Note ossification of phalanges of both fore- and hind-limbs, and calcaneus in hind-limbs. b. Forelimb ossification centers present only in metacarpals of 18-day-old CD-1 mouse fetus treated with both aspirin and ethanol. c. Hindlimb of Ethanol+Aspirin treated 18-day-old CD-1 mouse fetus shows the absence of ossification center in the calcaneus.

Fig. 7. Alizarin Red S and Alcian Blue stained skeleton of Ethanol+Aspirin treated 18-day-old CD-1 mouse fetus with 14 pairs of ribs.

Fig. 8. Alizarin Red S and Alcian Blue stained skeleton from an 18-day-old Aspirin+Ethanol treated CD-1 mouse fetus. Note complex rib fusion. On the left (arrow), ribs seven and eight fuse and bifurcate and ribs nine and ten fuse and bifurcate; laterally, ribs eight and nine fuse and bifurcate. On the right (arrow), ribs nine and ten fuse and bifurcate.

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