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A role of zinc deficiency in the fetal alcohol syndrome

Reginald Howard Rosemond
Atlanta University

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A ROLE OF ZINC DEFICIENCY IN THE FETAL ALCOHOL SYNDROME

A THESIS

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

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BY

REGINALD HOWARD ROSEMOND

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

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There is a recognized pattern of abnormalities in fetuses of chronically alcoholic mothers known as the Fetal Alcohol Syndrome (FAS). Elemental zinc deficiency is also teratogenic and chronic alcoholism with or without overt malnutrition is often accompanied by zinc deficiency. This project was conducted to determine what, if any, role zinc deficiency might play in the FAS and if zinc supplementation could ameliorate the signs of the FAS. Alcohol produces a variety of gastrointestinal tract effects that result in malnutrition. A second part of this investigation was performed to determine if the zinc supplementation regimen could protect the stomach from alcohol-induced mucosal damage.

The animal model used in this investigation was the female Swiss albino mouse. All animals were fed Purina Chow ad lib. Control animals were given water, while alcohol administered animals were either given 12% ethanol in water or 12% ethanol-50 mg/L ZnCl₂. The animals were bred, and
and sacrificed on Day 17 of gestation. Fetuses were removed and examined for signs of the FAS. There was a statistically significant difference between control and ethanol animals in the incidence of internal anomalies. Zinc supplementation had no effect on the incidence of ethanol-induced anomalies.

Animals were chronically exposed to ethanol for 2 weeks and then challenged intragastrically with 50% ethanol. Those animals whose diet had been supplemented with zinc showed a significant reduction in alcohol-induced gastric ulcers.

In summary, Zn supplementation does not significantly ameliorate the signs of the FAS but does seem to have protective properties in the gastrointestinal tract against erosion by ethanol.
ACKNOWLEDGEMENTS

I would like to acknowledge the endless contributions made to this project by my research advisor, Dr. Gordon J. Leitch, without whom this project would not have been possible.
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CHAPTER I

INTRODUCTION

This investigation was conducted to determine whether or not an elemental zinc (Zn) deficiency is a contributing factor to the Fetal Alcohol Syndrome (FAS). The FAS was first described in detail in 1968 by Lemoine et al. (1968), although effects of maternal alcohol consumption on the unborn had been reported for centuries. Alcoholism is known to be associated with Zn deficiency (Weismann, et al., 1976) and alcohol metabolism involves a Zn metalloenzyme, alcohol dehydrogenase (Prasad and Oberleas, 1971).

A distinct dysmorphic condition associated with maternal gestational alcoholism was first described in this country by Jones and Smith in 1973. They found several outstanding congenital defects, such as central nervous system dysfunctions, growth deficiencies, facial abnormalities, and various other major and minor malformations. Other physiological effects included hyperactivity in young children suffering from FAS and growth deficiencies in infant length and weight. A lack of adipose tissue and/or failure to gain weight is often associated with the FAS.

The clinical signs of the FAS in humans has also been described in the mouse (Chernoff, 1977). Some of the observed effects were dose dependent pre-natal death rates and mal-
developments and a low fetal weight. There are significant strain differences in the manifestations of the FAS in the mouse. Pre- and post-natal growth deficiencies have been observed in mice as well as in human infants. Alcohol itself is obviously the direct causative agent of the Fetal Alcohol Syndrome but it may have an indirect effect via alcohol-induced malnutrition. Alcohol in heavy usage is known to erode the lining of the stomach and to cause gastric ulcers - which are further aggravated by continued alcohol consumption (Davenport, 1975). Alcohol is known to produce many nutritional deficiencies (Shanbour, 1979), including a zinc deficiency (Weismann et al., 1976), but it is not known if a Zn deficiency contributes to the prenatal effects of alcohol exposure.

Zinc deficiency (ZD) per se is characterized by several manifestations. In the Middle East, ZD is responsible for delayed sexual maturation and reduced growth rate (Halstead, et al., 1972). It results in an inability to metabolize alcohol due to its role in alcohol dehydrogenase (Prasad and Oberleas, 1971). ZD produces a loss of appetite (anorexia) (Hambridge and Walravens, 1976) which leads to other nutritional deficiencies stemming from a reduced intake of essential nutrients. A deficiency of Zn during pregnancy has some profound effects on the fetus, and when extended throughout gestation causes malformations, extreme growth retardation,
and a high miscarriage rate (McKenzie, et al., 1975).

This project was conducted to determine what, if any, role an incipient ZD might play in the production of the FAS. The albino Swiss mouse was chosen as the animal model. The FAS was induced by presenting female animals with 12% ethanol as their only source of drinking water. Another group of animals was given the ethanol solution but with supplemented Zn in it. The fetuses produced by female mice from these two groups of ethanol-fed animals were then compared with fetuses from mice never exposed to ethanol.

Zinc administration has been shown to protect rat gastric mucosa against stress-induced erosion (Cho and Ogle, 1977). Mice from the three groups described above were challenged intragastrically with 50% ethanol and their stomachs studied to determine if the zinc supplementation used here could protect the gastrointestinal tract from the effects of ethanol.
It was observed that infants of alcoholic mothers often had a
1979. In a report to the British House of Commons in 1834,
children like unto the weasels, morose and languid" (Smith)
child. Aristotle observed that "drunken women bring forth
to drink on their wedding day so as not to produce an abnormal
drink." Early Carthaginian laws forbid newly-wedded couples
an angel warning Samson's mother to "drink no wine or strong
The Old Testament of the Bible in Judges 13:7 tells of

**Fetal Alcohol Syndrome - History of PAS in Man**

role in the Fetal Alcohol Syndrome?
Thus, the question can be asked, does in detoxification play a
(Hev et al., 1998) and anorexia (Sangster et al., 1976)
1972), poor growth, a reduced ability to metabolize alcohol
cause delayed onset of sexual maturity (Hasteed and Ronagh
Y, 1972). Zinc deficiency may be teratogenic and may
et al., 1976). Zinc deficiency is commonly linked with alcoholism (Weisman
excessive alcohol intake depresses body zinc stores and zinc
named the "Fetal Alcohol Syndrome" (Smith and Jones, 1973). And has been
and documented during the past twelve years, and has been

**LITERATURE REVIEW**

**CHAPTER II**
starved, shriveled, and imperfect look (Warner and Rosett, 1975). W.A.F. Brown, a mid-19th century physician, wrote that "...I have two patients who appear to inherit a tendency to unhealthy action of the brain, from mothers addicted to drinking and another, an idiot whose father was a drunkard" (Carpenter, 1849).

In 1899, William Sullivan, a Liverpool prison physician, did the first study by detailed scientific method of the effects of maternal alcohol drinking on offspring. The stillbirth and mortality rate of the children of women alcoholics in the prison was 56 percent, over twice that of the non-alcoholic mothers who bore young. Their deaths occurred most often before the age of two, with convulsions being the most frequent cause of death. This observation by Sullivan was largely disregarded until 1942 when Haggard and Jallinek observed some developmental problems in the children of alcoholic mothers. However, they attributed these problems to poor postnatal nutrition and an unstable childhood environment. Uhlig and co-workers in 1957 made some clinical observations of the malformations, growth deficiency, poor development, and behavioral abnormalities of orphanage children whose mothers were known heavy drinkers or alcoholics.

In 1968, Lemoine and his co-workers published a study of a cohesive pattern of clinical manifestations and abnormalities of children of alcoholic mothers. One hundred and twenty-
seven offspring were described as having uncanny resemblances to each other in displaying certain facial characteristics, growth deficiency, and psychomotor disturbances. These similarities were so unique that Lemoine said that maternal alcohol abuse could be diagnosed by the appearance of this set of features, later termed the "fetal alcohol syndrome."

In 1972, Christy Ulleland published the results of a study she had conducted on the growth patterns of the offspring of alcoholic women. They had a reduced head circumference, were underweight at birth, showed aberrant motor function and behavior, as well as delayed postnatal growth. She also noticed unusual irritability in infants and hyperactivity in older (pre-school) children. In the following year, 1973, Smith and Jones, working independently, expanded upon the range of types of defects peculiar to the FAS. Typically, the birth length of the FAS children was 65% or normal and the birth weight was 38% of normal. They identified this highly recognizable pattern of abnormal development as being of prenatal origin, the result of alcohol toxicity in utero.

Iris Smith (1979) reported on the three major prospective studies of the FAS that are being conducted in the United States at present: the continuing one by Smith and co-workers in Seattle, Washington, one at the Boston City Hospital, and a third one at Loma Linda University in California. Each of
these investigations is attempting to cover as broad a spectrum of racial backgrounds, socio-economic backgrounds, and environmental backgrounds as possible. Preliminary findings from these studies indicate the FAS is the major preventable birth defect in this country. Since the reports of Smith and Jones in 1973, FAS cases numbering into the hundreds have been reported from the United States, Canada, France, Germany, South Africa and several other countries (Streissguth and Smith, 1980). There has been "no complete FAS phenocopy reported in a human being with a negative maternal history of ethanol use" (Clarren and Smith, 1977), thus positively identifying alcohol as the causative agent of the FAS.

Since the FAS has been identified, a more complete list of its signs has been compiled by researchers. A chart of the signs of FAS in man is given on the following pages.

Fetal Alcohol Syndrome - Signs in Man

There has been an elaboration of the signs of FAS since it was first identified and documented (Sullivan, 1899) and as studies continued, more details of the FAS's clinical manifestations have been added. The following is a list of major signs of the FAS in man:

Facial anomalies (Lowry, 1977; Hanson and Jones, 1977)
- short palpebral fissures, microphthalmia (occasional)
- ptosis, strabismus
- epicanthal folds
- short, upturned nose with flattened nasal bridge
- hypoplastic philtrum, thinned upper vermillion
- retrognathia or mild prognathia in adolescence
- hypoplastic maxilla
- cleft palate, occasional cleft lip

**Head anomalies** (Jones and Smith, 1973; Hanson, 1978).
- hydrocephalus, microcephaly
- ear malformations, e.g. posterior rotation, poorly formed concha

**Cardiovascular anomalies** (Jones and Smith, 1973; Noonan, 1976; Loser and Majewski, 1977).
- atrial and/or ventricular septal defect
- pulmonary stenosis
- patent ductus arteriosus
- distorted retinal vessels
- hemangiomata

**Growth deficiency** (Lemoine et. al., 1968; Smith and Jones, 1973; Ulleland, 1972).
- pre- and postnatal growth deficiency
- small head circumference
- reduced weight and height

**Renal anomalies** (Tenbrink and Buchin, 1975; DeBeukelaer, et al., 1977).
- hydrenephrosis, small rotated kidneys
- edematous ureters

**Body and limb anomalies** (Jones and Smith, 1973; Qazi and Masakama, 1976; Lowry, 1977; Abel, 1979).
- joint anomalies
- polydactyly
- abnormal external genitalia, labial hypoplasia
- cryptorchidism
- hirsutism in infancy (occasional)
- abnormal neck vertebrae
- abnormal palmar creases

**Central nervous system and behavioral abnormalities**
(Rowell and Chalmers, 1970; Root *et al.*, 1975; Streissguth, 1976; Hanson and Jones, 1977; Lowry, 1977; Hanson, 1978)
- impaired learning ability (moderate mental retardation)
- poor coordination, impaired motor development
- seizures in early childhood
- hyperactivity in childhood
- muscle hypotonia
- abnormal EEG

**Animal Models of the Fetal Alcohol Syndrome**

Prenatal exposure to alcohol severely (in heavy drinking) affects the morphological development of the mammalian central
nervous system in animals other than man, e.g. rodents, (Sandor and Amels, 1971). Papara-Nicholson and Telford (1957) fed a solution of alcohol orally to guinea pig mothers and found several adverse effects on the offspring. Central nervous system disorders were observed, such as shallow brain fissures, abnormally flat gyri, and cellular lesions in the cortex and basal ganglia. Other signs of impaired fetal development that were found included poor locomotion, sucking and feeding difficulties, and incoordination. Newborn guinea pig offspring had decreased birth weights, more frequent stillbirths and a higher neonatal mortality rate although they did not observe any external structural anomalies.

Nutritional problems have often been associated with the FAS in experimental animals. Such problems may be observed in heavily-drinking mammalian mothers who nurse their young. Support for this was found by Pilstrom and Kiessling (1967) who noted that all rat offspring whose mothers had received a 15% alcohol solution orally died of starvation by postnatal Day 5.

In a paper published by Chernoff (1977), he closely compares the FAS in mice with the syndrome in humans. Cardiac anomalies such as ventricular septal defects were observed at very low levels of ethanol dosages. Mouse fetuses at Day 18 were noted to have prenatal growth deficiency (evidenced
by low fetal weight), cardiac anomalies (as mentioned above) and facial dysmorphogenesis. Tze and Lee (1975) administered alcohol in the drinking water of rats both prior to and during the gestational period and found smaller litter sizes and reduced birth weights in comparison to a pair-fed control group.

Henderson et al., (1979) did a study on chronic and acute alcohol administration and fetal development in the rat. Specific aspects they investigated were fetal viability and fetal organ weight and composition. The effects of chronic alcohol intake on tissue Zn stores were studied since a Zn deficiency is teratogenic and has been reported to occur in chronic alcoholism. The found higher fetal resorption rates in those rats chronically exposed to alcohol on Days 11-13 of gestation than in the controls and there was also an increase in the number of dead fetuses. The dead fetuses were characterized by their lack of response to mechanical stimuli or the earliest stages of resorption. Resorption rates were lower in rats receiving alcohol later in gestation (Day 14-16) and it appears the acute alcohol exposure is most likely to cause fetal death during the first two-thirds of gestation.

There are several clinical manifestations of behavioral anomalies reported in rodents such as hyperactivity (Bond and Degiusto, 1977) and impaired learning ability (Vincent, 1958). An impairment of avoidance conditioning was reported in rats
studied by Bond and DeGuisto (1977) in that offspring exposed to alcohol in utero were impaired in their ability to learn to avoid a noxious stimulus. These offspring are more active than controls, replicating other findings of hyperactivity in children with FAS (Striessguth and Smith, 1978). Thus, in studies conducted in animals to establish similarities of the FAS between animals and humans, it would seem that the FAS in rodents very closely parallels that in humans. However, other mammals are also subject to the FAS, as in the case reported by Ellis and Pick (1976), who, using dogs found that 4.5 g/kg of alcohol was the minimum dose required to produce increased fetal death and abnormalities.

Zinc Deficiency in the Alcoholic

Excessive ingestion of alcohol is known to lead to a severe zinc deficiency (Allan et al., 1975, Weismann et al., 1976). Vallee and co-workers (1956) initially described the abnormal zinc metabolism that occurred in patients with cirrhotic livers. They showed that patients with cirrhosis had low serum zinc, diminished hepatic zinc levels, and oddly, hyperzincuria (an absolute increase in renal clearance of zinc.) From this, they concluded that perhaps zinc deficiency in an alcoholic cirrhotic patient was a conditioned deficiency somehow related to alcohol ingestion. It is known that the Fetal Alcohol Syndrome is a set of clinical
signs peculiar to offspring of alcoholic mothers (Clarren and Smith, 1978; Smith and Jones, 1973). Some of these signs closely parallel those observed in rat fetuses whose mother had their normal zinc intake restricted during gestation (Hurley, 1976). Since excessive alcohol intake depletes body zinc stores to some degree (Weismann, et al., 1976) and given that zinc is required for normal DNA synthesis and cell division (Hurley and Shrader, 1972), a cause and effect relationship between zinc deficiency and the FAS may well be postulated. Hsu et al., (1969) reported that zinc-deficient weanling rats incorporated significantly less $^{14}$C methionine into plasma, kidney, liver and muscle protein than did controls, demonstrating that zinc deficiency affects protein synthesis broadly.

**Teratogenic Effects of Zinc Deficiency**

Zinc deficiency (ZD) *per se* is characterized by several manifestations. In 1961 to 1963, Prasad discovered that a zinc deficiency was often concomitant with an iron deficiency (anemia) (Prasad et al., 1963). This study was conducted in the Middle East in countries such as Iran and Egypt where dietary zinc is low. The investigators found that ZD produced growth retardation, testicular atrophy, and skin changes. In distinguishing iron deficiency from zinc deficiency, zinc supplement was given to adolescent patients with these signs.
Their rate of growth became greater with the zinc supplementation, and their genitalia size and secondary sexual characteristics became more appropriate for their age.

Zinc deficiency during gestation causes malformations, extreme growth retardation, and a high percentage of resorptions (missed abortions in humans) (McKenzie et al., 1975). This is primarily due to the anorexia (loss of appetite) caused by zinc deficiency. The resulting nutritional deficiencies have some profound effects on the fetus.

An article published in *Nutritional Reviews* (March, 1976) gave a summary of two studies of the effects of zinc deficiency in pregnant, fetal and young rats. Zinc deficient animals underwent decreased food intake one day after being deprived of dietary Zn, and because undernutrition resulted from the anorexia, the rats reached only 44% of their expected weight gain. The pregnant animal's food intake decreased progressively near the end of gestation. When pregnant animals were deprived of dietary Zn in the last third of gestation, there was a consequent intrauterine growth retardation which presumably was due to a combined effect of both ZD and undernutrition. Severe anorexia has been shown to appear abruptly within 3 days of dietary Zn withdrawal. Inability to maintain body weight was also noted in the ZD dams, probably due to impaired nutrient utilization. When pregnant rats were fed a Zn diet one to three days before parturition, a significant
reduction in birth weights was noted. In fetuses born of ZD mothers, the brains and livers were very small and the livers were found to incorporate less thymidine. However, the brains of the ZD fetuses were not depleted in zinc, while the livers contained only one-third the total amount of Zn when compared with controls. The fetuses had reduced body weights when again compared to controls (McKenzie, Fosmire, and Sanstead, 1975). This report points out the fact that even in the face of a whole body ZD such a deficiency may be manifested in some tissues, e.g. liver, but not in others, e.g. brain.

In looking at the important role of zinc in nucleic acid metabolism Hurley and Shrader (1972) have proposed that impaired DNA synthesis in Zn-deprived embryos prolongs the mitotic cycle and reduces the number of normal neural cells, leading to malformation of the CNS. It has been observed that the activity of alcohol dehydrogenase (a Zn metalloenzyme) is significantly decreased by a zinc deficiency. The lowered ADH level results in a reduced ability to metabolize alcohol and the FAS could possibly be exacerbated by the increased alcohol levels in the body (Prasad et al., 1967; Prasad and Oberleas, 1971; Huber and Gershoff, 1975).

From a nutritional standpoint adequate Zn is required both to maintain a normal appetite and probably also to maintain a normal gastrointestinal mucosa. Cho and Ogle (1977) showed that Zn administration protected the gastric mucosa
from erosion by stress-induced ulcers.

As ZD often occurs in the chronic alcoholic and as it is teratogenic per se, it was decided to give alcoholic pregnant mice a zinc supplement in an attempt to ameliorate the FAS and thereby demonstrate a role for ZD in this syndrome. A free feeding regimen was employed to allow the manifestation of any ZD related anorexia and the zinc supplement protocol was also used to demonstrate protection of the gastric mucosa against ethanol-induced ulcers.
CHAPTER III
MATERIALS AND METHODS

The animal model used in this investigation was the albino Swiss mouse (ARS-Sprague-Dawley, Madison, WI.). This is one of the larger mouse strains and would be expected to produce large fetuses. The animals were purchased at approximately fifty days of age. They were housed in large plastic gang cages with stainless steel tops. Cedar wood shavings were used as bedding. Ralston-Purina Laboratory Chow (pellets) and tap water were provided ad libitum. One group of female animals in the gang cages was immediately started on a 12 percent ethanol-water solution as their sole source of liquid intake. This group was later subdivided into an ethanol and an ethanol-zinc supplemented group. The remaining animals (controls) were maintained on chow and water. This was continued for 6 to 8 weeks. The animal room was automatically maintained on a 12 hour light-12 hour dark schedule (light cycle 0700h-1900h).

After a minimum of 6 weeks on their respective food and water regimens, the mice were removed from gang cages and housed individually in clear plastic cages, again with stainless steel tops and cedar wood shavings lining the cage floor. Thirty ml graduated drinking tubes were inserted into the cages to measure daily liquid consumption. Daily weight
measurements were also made. Eighteen animals were individually housed at any one time, divided into three groups of six mice each - the control (H₂O) group, the alcohol (12% ETOH) group and the zinc-alcohol group (ZnCl₂ - 12% ETOH). The animals being given the zinc-supplemented alcohol drank a 12 percent ethanol solution containing 50 mg/l of zinc chloride. To insure that no zinc leached from the rubber stoppers into the drinking solution, Saran Wrap was placed between the surface of the liquid and the stoppers.

Two weeks after individual housing, the 3 groups of mice were bred with males of the same age. Males were not exposed to alcohol and were randomly selected for breeding. The breeding was done over a period of 24 hours with the male being taken to the female's home cage. The date of breeding was recorded so that the exact date of impregnation could be determined. The presence of a copulatory mucus plug was tentatively taken to be a sign of impregnation but was found to be an unreliable index of fertilization in our hands because it was difficult to detect in most cases and lasted only a short time. The animals were bred every seven days until pregnancy was confirmed via a significant weight increase. A miscarriage was considered to have occurred if there was a precipitous weight loss in a pregnant animal and no pups were delivered by that animal.
Pregnancies were terminated on Day 17, counting Day 1 as the day following introduction of the male to the home cage. The animals were sacrificed by cervical dislocation and the body cavity was opened immediately to reveal the fetuses in utero. Both horns of the uterus were removed and arranged so that the in vivo configuration and location of the fetuses could be studied. Fetuses and resorption sites were counted and fetuses examined for viability. They were then removed from their membranes, weighed and fixed in Bouin's solution, for later dissection.

The primary areas of the fetal mouse expected to be affected by the experimental procedures were the brain (ventricle shape), the heart (ASD and/or VSD) and the kidneys (Hydronephrosis and edematous ureters).

Gross hypoplasia was used as one of the criteria in assessing the anomalies of the heart. Hydronephrosis is characterized by a large hollow kidney and it is usually associated with swelling of the ureters. Other criteria of the fetuses that were assessed during gross dissection included whether or not the fetus was alive in utero, digit numbers, and abnormal cranio-facial features. These included hydrocephalus, prominent lateral palatine ridges, eye anomalies, and nose shape.

The second part of this investigation involved determining the protective effect of zinc in the gastrointestinal
Experiments were performed to determine if zinc supplementation of the degree used in the FAS study would protect the gastric mucosa from the effects of alcohol. As with the FAS study, chronic alcohol animals and controls of the same ages were housed individually with and without the zinc supplementation for 2-3 weeks, at which time they were challenged with a 50% ethanol solution, and the effects of such a challenge on the gastric and duodenal mucosae were determined.

The animals were deprived of food 24 hours prior to the alcohol administration to reduce the presence of any buffering foodstuff that might lessen the alcohol's effect. The animals were lightly anesthetized with ether to prevent them from biting and damaging the catheter. Then a 1 ml syringe was used to inject 0.3 ml of 50% alcohol directly into the stomach. Three hours after the alcohol injection, the mice were sacrificed (via cervical dislocation) and the stomachs opened for examination of their contents. The stomach and duodenum were rinsed with saline to wash away residual food matter and examined with a magnifying lens. The ulcers were then scored according to the degree of severity of bleeding and preserved in 10% formalin. The ulcers were scored on a scale from 0 to 4; 4 being the most severe bleeding observed. The scale was as follows:

0 Normal
1. Local hyperemia
2. Small ulcers < 2mm
3. One large ulcer > 2mm, with or without smaller ulcers
4. Extensive ulcerations with large ulcers.

The stomachs and duodena were both scored using this index. Only gastric data are included here.

Data are presented in the following tables as means ± standard error of those means, or as ratios of the number of anomalies to the number of fetuses studied or to the number of litters in the experimental group. The rates of many such anomalies were in general so low that no indications of variance are presented, nor were statistical evaluations of individual differences between groups considered justified with such low anomaly incidences. Chi square tests were performed to determine the significance of differences between control and ETOH, and between ETOH and ETOH-Zn groups in their miscarriage rates, number of resorptions, and the rates of appearance of any internal anomalies.

Between-group (treatment) differences were measured in some experiments using a one way analysis of variance.
CHAPTER IV

RESULTS

In conducting this investigation, selection of animals for the various groups was done on a random basis. The same random selection was done in the introduction of a male to females's home cage. To determine whether or not impregnation had occurred, the females were bred every seven days, and weighed daily. If a substantial amount of weight was gained (>6 gm) by the seventh day after breeding day (Day 0), then this was taken to indicate pregnancy. Those animals that were not found to be pregnant were bred weekly thereafter until they were impregnated.

The animals to be chronically exposed to alcohol were immediately started on a 12% ethanol regimen as their sole source of liquid intake. The animals were approximately six weeks old upon arrival and control animals were given tap water. The length of time an animal was exposed to alcohol depended upon how long it took for that animal to become pregnant (range 8 weeks - 5 months).

Daily measurements were taken to determine the amount of alcohol consumed by the ethanol and ethanol-Zn animals. Table 1 shows the mean daily alcohol consumption in gm/kg day of these two groups. The data in this table were from the two days before breeding to one day before sacrifice. The table
Table 1. MEAN DAILY ALCOHOL CONSUMPTION (Gm/Kg Day) OF MICE DURING GESTATION

<table>
<thead>
<tr>
<th>Day</th>
<th>-2*</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETOH</td>
<td>16.3</td>
<td>16.5</td>
<td>15.1</td>
<td>16.7</td>
<td>15.7</td>
<td>18.0</td>
<td>16.2</td>
<td>14.9</td>
<td>15.6</td>
<td>15.0</td>
</tr>
<tr>
<td>± 0.9</td>
<td>± 0.6</td>
<td>± 0.6</td>
<td>±1.1</td>
<td>± 0.8</td>
<td>± 0.5</td>
<td>± 0.5</td>
<td>± 0.8</td>
<td>± 0.7</td>
<td>± 0.5</td>
<td></td>
</tr>
<tr>
<td>ETOH-Zn</td>
<td>14.4</td>
<td>17.8</td>
<td>17.2</td>
<td>17.4</td>
<td>18.3</td>
<td>19.3</td>
<td>18.2</td>
<td>18.1</td>
<td>13.1</td>
<td>14.1</td>
</tr>
<tr>
<td>± 0.8</td>
<td>±1.4</td>
<td>± 0.6</td>
<td>± 0.2</td>
<td>±1.2</td>
<td>± 0.7</td>
<td>± 0.9</td>
<td>± 0.7</td>
<td>±1.00</td>
<td>± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

* 2 days prior to breeding
shows that those animals with zinc supplementation consumed approximately the same amount of alcohol as those animals without the zinc supplement. Statistical evaluation of these data is difficult due to the fact that individual drinking tubes frequently leaked because of manipulation by the animals. As a result of this the data summarized in Table 1 are not made up from two groups of animals, with each animal represented on each day. Instead daily drinking values were only used in those cases where it was certain that there was no spillage.

In order to detect any gross differences in the nutritional status among the three groups, the maternal weights were compared at the time of breeding and on the 17th day of gestation, with and without including the fetus weights. No statistically significant differences were observed.

On Day 17 of gestation, the pregnant dams were sacrificed and various measurements were taken. The number of fetuses in each litter was counted, the number of resorption sites was tallied, and the weight of each fetus was recorded. Table 3 shows the mean litter numbers and weights on Day 17 of gestation and the ratios of miscarriages to pregnancies and resorption sites to total number of pups. There was no statistically significant difference in mean litter weights or mean litter numbers among the three groups, although there was a trend to a decreased mean litter weight in the alcohol
group which was ameliorated by Zn supplementation. Miscarriages were only seen in animals drinking ethanol, while resorption sites were found in all groups.

The crux of this investigation involves the incidence of teratogenic effects among the control, ethanol, and ethanol-zinc animals. These data are summarized in Table 4. Both external and internal characteristics are listed. The zinc supplementation did not reduce the incidence of teratogenic effects seen following in utero alcohol exposure. In the control animals, only one digit anomaly was found (in only one fetus) while none of the other listed anomalies were observed. All the animals that demonstrated open eyes in the ethanol-Zn group were from the same litter.

A group of animals that had been on alcohol for 5 months were individually housed and the ethanol solution of half this group supplemented with Zn as above. Control animals of the same age were also divided into two groups, one of which was given the Zn supplement in their drinking water. After 2 weeks on this feeding regimen the animals were starved for 24 hours, challenged with 0.3 ml 50% ethanol via a gastric tube, and the stomach and duodenum scored for ulcers 3 hours later. Table 5 summarizes these data. There was a trend to a higher gastric ulcer score in animals chronically fed ethanol and a trend to a reduction in ulcer scores when Zn-ethanol
Table 3. MEAN LITTER NUMBERS AND WEIGHT, MISCARRIAGE AND RESORPTION RATES ON DAY 17 OF GESTATION

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ETOH</th>
<th>ETOH-Zn</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean litter number</td>
<td>9.3</td>
<td>9.8</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±.6</td>
<td>±.6</td>
<td>±.8</td>
<td></td>
</tr>
<tr>
<td>Mean litter weight</td>
<td>.885</td>
<td>.715</td>
<td>.797</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±.069</td>
<td>±.058</td>
<td>±.075</td>
<td></td>
</tr>
<tr>
<td>Miscarriage rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\chi^2 = 2.965$</td>
<td>$\chi^2 = 1.115$</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Number of resorptions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>149</td>
<td>117</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\chi^2 = 1.512$</td>
<td>$\chi^2 = 1.700$</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Table 4. ABNORMALITIES OBSERVED IN DAY 17 FETUSES.

<table>
<thead>
<tr>
<th>Anomaly Type</th>
<th>Control</th>
<th>ETOH</th>
<th>ETOH-Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digit Anomalies</td>
<td>1/61</td>
<td>1/56</td>
<td>2/50</td>
</tr>
<tr>
<td>Eyes Open</td>
<td>0/61</td>
<td>0/56</td>
<td>5/50</td>
</tr>
<tr>
<td>Hydrocephalus</td>
<td>0/16</td>
<td>6/16</td>
<td>3/16</td>
</tr>
<tr>
<td>Myocardial Hypoplasia</td>
<td>0/16</td>
<td>2/16</td>
<td>1/16</td>
</tr>
<tr>
<td>Hydronephrosis</td>
<td>0/16</td>
<td>1/16</td>
<td>3/16</td>
</tr>
<tr>
<td>Cryptochidism</td>
<td>0/16</td>
<td>1/16</td>
<td>4/16</td>
</tr>
</tbody>
</table>

Any Internal Anomalies

\[ X^2 = 9.143 \]
\[ X^2 = .625 \]
\[ p < .01 \]
\[ NS \]

* Number of animals examined
** Number of animals dissected
+ All from the same litter
### Table 5. Ulcer Index Scores of Control Animals and Animals Chronically Exposed to Ethanol with and without Zn, Challenged with 50% Ethanol

<table>
<thead>
<tr>
<th></th>
<th>Mean Ulcer Index Score</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.12 ± .37</td>
<td></td>
</tr>
<tr>
<td>Control - Zn</td>
<td>1.43 ± .56</td>
<td>F = 2.44 (V₁ = 3, V₂ = 25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 5.26 (V₁ = 1, V₂ = 27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>ETOH</td>
<td>3.14 ± .55</td>
<td>All Groups</td>
</tr>
<tr>
<td>ETOH - Zn</td>
<td>1.43 ± .40</td>
<td>All Zn against all others</td>
</tr>
</tbody>
</table>
animals were compared to ethanol animals, and when Zn-control animals were compared to control animals. These differences were not statistically significant, however. When the ulcer scores of all Zn animals were compared to the scores of all other animals, there was a statistically significant difference.

Table 6 shows the mean ulcer index scores of control animals and control animals that had their drinking water supplemented with Zn or equivalent Ca or Mg. Again, there was a trend for a lower ulcer index score in the Zn group when compared with the control group, but the variance was such that there were no statistically significant differences between the groups.
Table 6. ULCER INDEX SCORES OF CONTROL ANIMALS AND ANIMALS WITH DIET SUPPLEMENTED FOR 2 WEEKS WITH DIVALENT CATIONS, CHALLENGED WITH 50% ETHANOL

<table>
<thead>
<tr>
<th></th>
<th>Mean Ulcer Index Score</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.18 ± .36</td>
<td></td>
</tr>
<tr>
<td>Zn Supplemented</td>
<td>1.73 ± .52</td>
<td>(4 groups)</td>
</tr>
<tr>
<td>Ca Supplemented</td>
<td>2.80 ± .37</td>
<td>F = 1.93</td>
</tr>
<tr>
<td>Mg Supplemented</td>
<td>2.50 ± .45</td>
<td>(V_1 = 3, V_2 = 38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Zn against control)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F = 4.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(V_1 = 1, V_2 = 20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p &lt; .05</td>
</tr>
</tbody>
</table>
CHAPTER V
DISCUSSION

The primary thrust of this investigation was to determine if a zinc deficiency (ZD) contributed to the signs of the Fetal Alcohol Syndrome (FAS). As a physiological ZD is known to exist even when plasma and tissue zinc levels appear normal it was decided to determine whether or not supplemental zinc ameliorates the FAS. It has been conclusively documented that high levels of alcohol in pregnant mammalian organisms cause a set of clinical signs to appear in the offspring (Lemoine et al., 1968). The severity of the syndrome is dose-related. In those persons who have been diagnosed as being alcoholic, certain dietary deficiencies have been noted, among them ZD (Allan et al., 1975.)

The mouse was chosen as the animal model for these experiments because it has been successfully used as a model for the FAS (Chernoff, 1977; Randall et al., 1977). There are two ways in which any potential ethanol-ZD interaction could be examined in the context of the FAS. In the first way one could attempt to potentiate the teratogenic effects of in utero ethanol with a ZD of a level that was not itself teratogenic. This experiment was initially attempted. The combination of in utero ethanol and ZD in our pilot experiments resulted in such high fetal death rates that there were no
experimental subjects to examine. It was obvious that it would be difficult to titrate the ZD to a point that would allow us to demonstrate a potentiation of the FAS. It was therefore decided to use the second way and supplement the diet of pregnant animals with Zn while they were exposed to ethanol. Thus if an ethanol-induced ZD were to have played a role in the FAS, then the syndrome should have been ameliorated by Zn supplementation.

Alcoholism and zinc deficiency both have the effect of inducing anorexia, i.e., a decrease in appetite (Prasad, 1979). The animals in this study were placed in a free-feeding situation which would more easily permit detection of a zinc-deficiency induced appetite effect. In the more customary pair-feeding experiment an appetite effect would be missed as the animal who consumes the least amount of food is the governing factor in how much the other animal is fed, the other animal's amount being reduced to match the first low-feeding animal. Thus a pair-feeding situation would tend to obscure any appetite effect manifested by ZD and alcoholism.

As stated in the Results, the daily alcohol consumption of the ethanol and Zn-ethanol groups is open to question due to irregular spillage and leakage. It is apparent however, from Table 1 that the two groups of animals consumed approximately the same amount of alcohol per day. This fact, and the data summarized in Table 2, argue against an obvious appetite
Table 2. MATERNAL WEIGHTS (gms) AT TIME OF BREEDING AND ON DAY 17 OF GESTATION

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ETOH</th>
<th>ETOH-Zn</th>
<th>Statistical Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight at time of breeding</td>
<td>30.3 ±.8</td>
<td>30.8 ±1.0</td>
<td>29.3 ±1.0</td>
<td>F = .48 (V1 = 2, V2 = 38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Weight on Day 17 of gestation</td>
<td>46.8 ±1.4</td>
<td>47.3 ±1.2</td>
<td>46.3 ±1.42</td>
<td>F = .07 (V1 = 2, V2 = 38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
<tr>
<td>Maternal weight-fetal weights on Day 17 of gestation</td>
<td>39.0 ±1.1</td>
<td>40.6 ±.9</td>
<td>39.5 ±1.42</td>
<td>F = .46 (V1 = 2, V2 = 38)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>
variation-induced nutritional effect in these experiments. In Table 2 the maternal weights of the three groups of animals were compared on the day of breeding and Day 17 of gestation, both with and excluding the fetus weights. No significant weight differences were found.

As indicated above, the teratogenic effects of maternal ethanol consumption and zinc deficiency are similar. The central nervous system, growth and development, and behavior are all areas that are affected by both the FAS and ZD (Smith et al., 1973; Sanstead, 1977). Zinc is an integral part of the enzyme alcohol dehydrogenase (ADH) which is the initial enzyme in hepatic alcohol metabolism (Prasad and Oberleas, 1971). A ZD then would tend to elevate blood alcohol levels for a given alcohol consumption, thereby increasing the fetotoxic effects, as alcohol has a dose-related effect on the fetus.

Measuring blood alcohol levels in the animals was considered but the idea was dropped owing to the observed variety in drinking patterns. Some of the animals drank a large amount at one time while others only sipped the alcohol. This type of variation is an unavoidable disadvantage of a free-drinking paradigm. It was decided that there was such a high degree of variability in individual drinking patterns that it would not be feasible to continuously measure blood alcohol
levels.

Zinc deficiency is known to impair DNA synthesis and growth (Prasad, 1979). *In utero* ethanol exposure also impairs growth (Ulleland, 1972). Henderson and associates (1979) used a rat model to determine if there was a correlation between FAS signs and maternal and fetal tissue Zn levels. They were unable to find such a correlation, but pointed out that they only measured Zn at the end of gestation.

*In utero* alcohol exposure and ZD in early gestation caused higher rates of fetal death than do later exposures (Henderson, 1979; Jameson, 1976). The period of alcohol exposure and/or ZD determines the nature and degree of the effects on organogenesis. No attempt was made in this study to try to discriminate between periods of gestation, rather the ethanol exposure and the Zn supplementation occurred prior to and throughout gestation in order to maximize any observable effects.

*In utero* alcohol exposure is known to produce a high fetal death rate and a low fetal weight (Sullivan, 1899). In the present study there was no difference between the mean litter numbers of the three groups, and resorption sites were found in all three groups. However, miscarriages were only seen with animals that consumed alcohol. Despite the fact that there was no statistically significant difference between the mean litter weights of the 3 groups, a 20% reduction in
mean litter weight was noted between the alcohol group and the control group. This trend was ameliorated by Zn supplementation, perhaps through its appetite stimulating effects.

In Table 4, a list of observed fetal abnormalities on Day 17 of gestation is shown. Only one minor anomaly was found in the control group, a digit anomaly. In one case, all members of an alcohol-zinc litter were found to have their eyes open, considered to be an anomaly in this species. There were varying rates of anomalies found in both the alcohol group and in the alcohol-zinc group. Some types of anomalies seemed to be found more frequently in one or the other of the two alcohol groups. The severity of these anomalies, particularly the hydronephrosis and hydrocephalus, was less than that reported with other protocols (e.g. Randall et al., 1977). In examining all internal anomalies 44% of the alcohol group fetuses examined showed one or more internal anomalies while 56% of the alcohol-zinc group fetuses showed such anomalies. This indicates that zinc had no ameliorating effect on the teratogenic effects of ethanol.

The second part of this investigation involves the effects of alcohol on gastrointestinal function. Alcohol causes a variety of effects on the gastrointestinal tract, such as gastritis, malabsorption, diarrhea, and vomiting (Davenport, 1975; Shanbour, 1979). Gastric ulcers result from a break in the integrity of the mucosal barrier (Davenport, 1975). The
question was posed whether or not the zinc supplementation could have afforded protection to the gastrointestinal tract as has been reported (Cho, 1977). Animals were challenged with an oral dose of a high concentration of ethanol (50%). Three hours later their gastric mucosae were scored for ulceration and the effect of dietary zinc supplementation was assessed. In order to determine if any effect seen with Zn supplementation was a unique property of zinc or not, the effect of Ca and Mg supplementation on ethanol-induced gastric ulceration was also assessed.

Table 5 summarizes the mean ulcer index scores of female control animals or animals chronically exposed to ethanol, with and without zinc supplementation. These animals used in this gastrointestinal study were of the same group of chronically alcohol-exposed animals that were used in the teratogenicity study. When comparing the mean ulcer index scores among all 4 groups, there were no statistically significant differences in the index scores, although there was a trend for the mean ulcer index score of the chronic ethanol group to be higher than the control group and for zinc supplementation to lower the mean scores when given to either the control or the ethanol groups. When the zinc supplemented animals were compared to all others, the animals with zinc supplementation showed a statistically significant reduction in mean ulcer scores. To determine if the protective property
of zinc is shared by other divalent cations, comparisons were made of the protective effects of two-week supplementation of Zn, Ca, and Mg using control animals. Table 6 summarizes these data. When a one way analysis of variance was used to analyze all groups, no significant difference was found. When the Zn supplemented group scores were compared with the control group scores the difference was significant, indicating that only Zn afforded the gastric mucosa protection against ethanol-induced erosion. This study of the effects of zinc supplementation could be a model for other gastrointestinal effects of zinc and alcohol and their interactions.

In summary, zinc deficiency causes teratogenic effects, causes a loss of appetite, and is induced by chronic alcoholism while zinc supplementation appears to protect certain portions of the gastrointestinal tract against ethanol-induced damage. Nevertheless, in the present study, it has not been possible to demonstrate that zinc supplementation affords protection against development of the Fetal Alcohol Syndrome.
CHAPTER VI

SUMMARY

This investigation was done to determine whether or not an elemental zinc deficiency is a contributing factor in the Fetal Alcohol Syndrome. It has been concluded that:

(1) There may be a role of zinc deficiency in the Fetal Alcohol Syndrome.

(2) There was no significant amelioration of the FAS detected using this protocol and this animal model.

(3) Zinc appears to afford protection of the gastric mucosa against a high concentration of ethanol. This may be a manifestation of a more generalized protection of the gastrointestinal tract.
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