THE COMBINED PRENATAL EFFECTS OF ETHANOL AND NICOTINE ON DEVELOPMENT IN THE RAT

A THESIS

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

The present investigation was undertaken to establish the effects of prenatal exposure to ethanol, nicotine, and the joint usage of ethanol and nicotine on neonatal parameters and postnatal development, physiology, morphology, and behavior. In order to fulfill these objectives, three separate studies were performed. Study I accessed any differences between the offspring of anesthetic control dams, saline implanted control dams, and ethanol treated dams by measuring the following: neonatal parameters, post gestational parameters, offspring developmental measurements, offspring physiological measurements, offspring behavioral measurements, and offspring morphology.

Study II attempted to access any differences between the offspring of saline implanted dams, nicotine treated dams, and ethanol plus nicotine treated dams by measuring the following: neonatal parameters, post gestational parameters, offspring developmental measurements, offspring physiological measurements, offspring behavioral measurements, and offspring morphology.

Study III sought to determine the effects of earlier in utero exposure to nicotine by administering nicotine to pregnant dams one day earlier than in the previous study.

Study III attempted to access any differences between the offspring of saline implanted dams and nicotine treated dams by measuring the following: neonatal parameters, post gestational parameters, offspring developmental measurements, offspring physiological measurements, and offspring behavioral measurements.

Alcohol and nicotine are the two most readily accessible drugs used in today's society. Both have cost taxpayers millions of dollars in terms of lost productivity, lost wages, illness and disease (Plavt, 1967; Worick, et al, 1977). While these are the most obviously debilitating results of the abuse of either drug, little is known about their combined effects postnatally and even less prenatally.

The interaction of ethanol on membranes (cellular and subcellular) is thought to be the basis for alcohol action in the central nervous system (CNS). Ethanol is a small hydrophilic molecule that readily incorporates into cell membranes (Sun and Sun, 1976). Current research has shown that <u>in vitro</u> exposure of membranes to low concentrations (0.1-0.5%) of ethanol enhances synaptosomal ($Na^+ + K^+$) - ATPase and mitochondrial transport activity; whereas, high concentrations (1-5%) of ethanol inhibit both activities (Sun, et al, 1977; Burke, et al, 1977). Sun and Sun (1979) have hypothesized that "small amounts of ethanol entering the membrane may result in expansion of the molecular structure with corresponding increase in membrane fluidity.

At higher concentrations (1-5%), however, ethanol may interfere with the transmembrane process by interacting with the hydrophobic area of the membranes and subsequently altering the fine arrangement of the membrane structure." This proposal correlates with the initial stimulatory effect that moderate amounts of ethanol consumption have on the CNS followed by a sedative depressant effect that occurs with excessive ethanol consumption.

Israel (1965) and Kalant (1967) demonstrated that ethanol affected ion movement in a variety of organs and proposed that the inhibition of active transport of ions by ethanol could be a universal membrane phenomenon. Many studies have since demonstrated the ability of ethanol to alter transport, thereby impairing peptide synthesis (Chang, et al, 1967) and altering neural transmission, muscular contraction, respiration, excretion, secretion, hormonal control, and vision.

Alcohol is a central nervous system depressant. It acts as a depolarizing agent on individual nerves. Ethanol can affect baroreceptors and chemoreceptors thereby causing a rise in blood pressure. Ethanol has been shown to alter the hypothalamic-pituitary-target organ axis thereby producing inhibition of vasopressin and oxytocin, increase uptake of triiodothyronine, increase catecholamine secretion of the adrenal medulla as well as increase glucocorticoid release from the adrenal cortex (both probably related to

stress). Other systemic effects of alcohol are alteration of electrolyte balance due to increased retention of sodium, potassium, and chloride with increased magnesium excretion, alteration of acid-base balance producing a slight metabolic acidosis, peripherial vasodilitation, hypoglycemia, induction of hepatic microsomal enzyme systems, and impairment of temperature control by suppressing neuronal activity in thermoregulatory cells (Biochemistry and Pharmacology of Ethanol, 1979). The oxidation of ethanol results in the reduction of nicotinamide adenine dinucleotide (NAD) to $NADH_2$. The "increase of the $NADH_2$/NAD ratio leads to several important biochemical changes: (a) blood lactate is increased leading to acidosis; (b) citric acid cycle activity is decreased; (c) gluconeogenesis is inhibited; and (d) lipogenesis is increased and lipolysis is decreased" (Shepard and Rose, 1973).

Nicotine is an alkaloid that is lipid soluble when in free base form. Its absorption is pH dependent having little or no effect when given in acid solution, but producing severe, toxic and even fatal results when identical doses are given in alkaline solution (Travell, 1960). Nicotine, classified as a CNS stimulant, has widespread and varied pharmacological effects on every organ in the body.

Nicotine has been shown to have both central and peripheral nervous system effects. The stimulation of preganglionic synapses (nicotine receptor sites) by

nicotine has been shown to elicit the release of acetylcholine at postganglionic parasympathetic synapses (muscarinic sites) and norepinephrine at postganglionic sympathetic synapses (adrenergic sites). Also, the release of acetylcholine at preganglionic synapses has been observed to produce a nicotine-like response, therefore, indicating nicotine as a cholinometic compound (Hirschhorn and Rosecrans, 1974). This is thought to be one of the two mechanisms of nicotine action. The second type of nicotine action is its ability to cause the release of neurotransmitter amines (Goodman and Weiss, 1974). Thus, the ability of nicotine to affect norepinephrine, serotonin, and possibly dopamine release along with cholinergic and adrenergic receptor stimulation illustrates its phenomonal control over synaptic transmission in the brain and spinal cord, in autonomic ganglia, at postganglionic autonomic effector sites, and at neuromuscular junctions. The effect of nicotine at all of these sites is dose dependent. Nicotine administration produces a transient excitation followed by depression, paralysis, and death at effective doses (prolonged blockade). Additional systemic effects of nicotine include the release of epinephrine from the adrenal medulla, thereby producing cardiovascular changes, the stimulation of chemoreceptors of the carotid and aortic bodies, causing reflex hypertension, and the release of vasopression (The Health Consequences of Smoking for Women:

a report of the Surgeon General).

The mode of drug interaction between nicotine and ethanol has not been fully established. Studies have not thoroughly investigated whether or not the joint usage of ethanol and nicotine produce an additive, synergistic, or antagonistic effect. From 1974 to the present time, few studies have been published on the interactions of ethanol and nicotine in adult humans and animals. The majority of publications deal with increased mutagenicity of joint usage in reference to head and neck cancers. Adir et al (1980) examined the effect of ethanol pretreatment on nicotine pharmacokinetics in the rat. Rats were exposed to two different consecutive doses of ethanol (4 g/kg/day for 7 days followed by 8 g/kg/day for 5.5 days) via gastric isometric sucrose. On the fourteenth day a 0.4 mg/kg dose of methyl - ^{14}C nicotine was injected IV to both groups, and blood samples were collected at timed intervals for 30 hours. Plasma nicotine and its metabolites were separated by thin-layer chromatography and quantitatied by liquid scintillation counting. The plasma levels of total radioactivity of nicotine, cotinine (the major metabolite of nicotine), and other metabolites were significantly lower in the ethanol rats as compared to the control rats. Even though ethanol pretreatment produced no changes in the hybrid rate constants describing the biphasic decrease of plasma nicotine, it did significantly increase the rate of

cotinine production. Ehtanol pretreatment also increased the volume of nicotine and cotinine distribution and significantly increased total plasma nicotine clearance by 45% ($P < .05$). The indications are that ethanol might alter the distribution of nicotine and act as an inducer for cotinine.

In a human study by Myrsten and Andersson (1973), psychophysiological measurements were recorded after ingestion of alcohol (0.72 g/kg: peak blood alcohol levels - 65 mg c/c) and nicotine in the form of cigarette smoking (5 cigarettes - 1.6 mg nicotine/cigarette). Hand-steadiness impairment was potentiated by each drug while heart rate was increased by nicotine alone. Combined alcohol and nicotine had an antagonistic effect on each other on simple and complex reaction time tasks.

While research is exploding in the area of developmental toxicology to prenatal exposure of ethanol and nicotine, placental transfer of both drugs is a prerequisite for consideration of their direct embryofetotoxicity. Both nicotine and ethanol have been shown to readily cross the placenta. Altshuler et al (1979) demonstrated the transplacental passage of ethanol in the rat model, whereas, Suzuki et al (1974) reported placental transfer and distribution of nicotine in the pregnant rhesus monkey.

Since adults rapidly metabolize alcohol to acetaldehyde, and then to acetate (via hepatic system) it seems reasonable

to ask if alcohol is metabolized *in utero* and whether or not alcohol itself or its metabolites are responsible for reported embryofetotoxicity. Pikkarainen et al (1967) reported that human embryos are deficient in alcohol dehydrogenase and are, therefore, unable to metabolize alcohol into acetaldehyde. Also, in the fetus, the enzyme is very weak and not fully functionable until five years of age. Sjoblum et al (1978) have reported opposite findings in rat fetus. If the maternal metabolism of alcohol to acetaldehyde has already occured, does acetaldehyde also cross the placenta and is it metabolized *in utero*? Kesanimei et al (1975) have reported that acetaldehyde readily passes across the decidua and is metabolized by the placenta in a rat model. While many studies have demonstrated the embryofetotoxicity of alcohol (Brown, et al, 1979; Sandor and Elias, 1968; Chernoff, 1977, etc.) only one study has attempted to directly assess the effect of acetaldehyde on the fetus. O'Shea and Kaufman administered 0.1 ml of 1% and 2% acetaldehyde IV to pregnant mice on days 7, 8, and 9 of gestation. Results were a significant increase in resorptions of mice given acetaldehyde as well as a decrease in length, weight, and protein content of subsequent embryos. Acetaldehyde may contribute to the FAS, but a lack of knowledge persists concerning its placental metabolism and transfer as well as effective doses needed to produce fetal damage.

Stalhandske et al (1969) studied the metabolism of nicotine in the livers of fetal, young, and adult mice *in vitro*. The metabolism of nicotine to its major constituent cotinine was seen to occur late in fetal life. While it is probable that cotinine crosses the placenta, there are no reported studies indicating such. Also, there are no reported studies concerning *in utero* placental metabolism of nicotine.

In the past decade, many studies have been published investigating the *in utero* exposure to alcohol. There is now considerable literature on The Fetal Alcohol Syndrome (FAS) in both human and animal studies involving everything from morphological, developmental, physiological, and behavorial measurements on exposure to a wide range of doses as well as treatment periods.

The observation that maternal drinking may have harmful effects on subsequent offspring dates back to ancient times. Aristotle observed that, "foolish and drunken and hare-brained women most often bring forth children like unto themselves, morose and languid." However, it was not until the independent studies of Lemoine et al in France in 1968, and Jones and Smith in the United States in 1973, that the teratogenicity of maternal alcoholism was firmly established.

Women who drink during pregnancy run a significantly higher risk of spontaneous abortions, abruptio placentae, and neonatal mortality (Harlap and Shiono, 1980; Goujard,

et al, 1975).

Anomalies produced by FAS generally fall into four categories: central nervous system dysfunctions, growth deficiency, typical facial dysmorphism, and a variety of associated malformations. The most commonly observed CNS abnormalities include mild to moderate retardation, microcephaly, poor coordination, hypotemia, irritability, and hyperactive behavior. Both body weight and length growth retardation have been observed in the fetus, neonate, and infant. The most frequently observed facial anomalies are short palpebral fissures often associated with myopic small eyes, ptosis and strabismus, a hypoplastic maxilla and mandible, and a short upturned nose (Chernoff in Currents in Alcoholism, 1980). Other frequently observed anomalies are presented in Table I.

Great intraspecies variation to the susceptibility of alcohol teratogenesis has been reported for both human and mice studies (Chernoff, 1980). In humans, this finding was based on the observation that less than half of the chronic alcoholic women who continue to drink heavily throughout pregnancy produce offspring with the fetal alcohol syndrome (Jones, et al, 1974). In carefully controlled mouse experiments different strains given equal amounts of alcohol display different rates of malformation among their offspring (Chernoff, 1977). It is probable that phenotypic variability primarily results from

differences in ethanol dosage, genetic background, and time and duration of exposure.

While it is evident that fetal exposure to alcohol affects the developing organism, the teratogenic mechanisms remain essentially unknown. Possible explanations for the FAS are alcohol's (and maybe acetaldehyde's) direct teratogenicity, alcohol's indirect affect on maternal and fetal physiological systems (especially neuroendocrine system), and alcohol's direct affect on placental transport function (Taylor, et al, 1981; Henderson, et al, 1981).

Women who smoke during pregnancy have a higher incidence of spontaneous abortions, intrauterine bleeding, prolonged rupture of amniotic membranes, abrupto placentae and placenta previa. More perinatal deaths have been reported in smoking mothers than in nonsmokers (The Health Consequences of Smoking for Women, 1979). Also, a relationship between maternal smoking and Sudden Infant Death Syndrome has been reported (Steele, 1966).

In 1957, Simpson reported that babies born to women who smoke during pregnancy average 200 grams lighter than babies born to women who do not smoke. Since then, numerous studies have been published confirming Simpson's observation (U.S. Department of Health, Education, and Welfare, 1979). It was first hypothesized that smoking mothers produced lower birth weight offspring because smoking reduced gestation; hence, offspring were slightly

premature. However, carefully controlled human and animal studies have since demonstrated that prenatal exposure to smoke (nicotine in animal studies) does not reduce the length of gestation (Andrews and McGary, 1972; Hamosh, 1979). In fact, several publications on prenatal nicotine exposure in animal studies have shown that gestation is prolonged due to delayed implantation caused by a delay in progesterone secretion (Yoshinaga, et al, 1979; Ferry, et al, 1974). Meyer (1978) and Butler et al (1969) have demonstrated that smoke (nicotine) exposure in utero does retard fetal growth at each gestational age.

Conflicting reports have been published as to congenital anomalies produced by in utero exposure to smoke. Some studies have reported no relationship between maternal smoking and offspring anomalies (Borlee and Lechat, 1979), whereas, other studies report a high related incidence (Ericson, et al, 1979; Yerushalmy, 1973; Himmelberger, et al, 1978). This discrepancy is most likely caused by differences in nicotine dosage.

The most commonly reported anomalies associated with maternal smoking are neural tube defects, pyloric stenosis, congenital heart disease, cleft lip and palate, strabismus, and inguinal hernia. Christianson (1980) obtained information from women enrolled in a longitudinal study of pregnancy and offspring development (Kaiser Foundation Health Plan). Data involving 14,735 offspring were analyzed

for congenital anomalies as related to maternal smoking habits. "There were no significant differences in the incidence of congenital anomalies when smokers (all dose levels combined) were compared with those who had never smoked." However, a significantly higher incidence of congenital anomalies was observed when nonsmokers offspring were compared to the offspring of women who smoked 20 or more cigarettes a day. "The increase in incidence associated with heavy smoking occurred predominantly among male offspring and was attributed to anomalies classified as moderate." The incidence of inguinal hernias and strabismus was increased for offspring of both light and heavy smokers with the risk being considerably higher in the offspring of heavy smokers.

Several studies have suggested unfavorable effects of prenatal exposure to smoke on offspring long term growth, intellectual development and behavioral characteristics. Hyperkinesis, irritability, decreased ability for self control and short attention span have been reported to occur more frequently in infants born to smokers when compared to infants born to nonsmokers (Saxton, 1978; Denson, 1975).

In utero exposure to nicotine may produce pregnancy complications and offspring abnormalities by several different mechanisms. Nicotine may have a direct adverse effect as a teratogen on the developing fetus, or it may have an indirect adverse effect by altering maternal-fetal

metabolism (Weathersbee and Lodge, 1979). Sastry et al (1977) have postulated that the deleterious effects of nicotine on the unborn may be due to the decrease in placental amino acid transport produced by nicotine-mediated cholinergic blockade. Sastry's hypothesis is supported by a series of studies on the effects of nicotine on human placenta in vitro. Suzuki et al (1974) demonstrated that nicotine causes intrauterine hypoxia similar to that reported for tobacco smoke.

Several studies have now confirmed a direct correlation between maternal drinking and smoking (Kuzma and Kissinger, 1981; Little, et al, 1976). Accumulating evidence in both human and animal studies indicates that prenatal exposure to the combination of ethanol and nicotine produces synergistic exacerbating effects on subsequent offspring. Human studies illustrating this synergistic interaction have been reported for abruptio placentae, for atypical sleeping positions of newborns, and for the neonatal operant learning tasks of head turning and sucking response as well as an increased incidence of stillbirths and decreased birth weights (Martin, et al, 1977; Goujard, et al, 1975; and Landesman-Dwyer, et al, 1977).

Abel et al (1979) treated pregnant rats from day one of gestation throughout delivery with either 6.0 g/kg/day ethanol (oral intubation), 1.5 mg/kg/day nicotine (subcutaneous injection), or a combination of the two drugs.

Each of the three treatment groups had corresponding pair-fed control groups that were treated with placebos (sucrose or saline). The principle findings of Abel's study were: "(1) prenatal exposure to nicotine plus alcohol did not have a significantly greater effect than alcohol alone on any of the measured parameters except on food consumption during pregnancy and on female liver: body weight ratio at 12 weeks of age; (2) the effects of prenatal exposure to alcohol extend into adulthood causing overall reduction in body weight and increased size of various organs, especially the liver and spleen; (3) prenatal exposure to nicotine alone had minimal effects relative to pair-fed controls". The combination drug group showed no potentiated effects in fecundity, postnatal mortality, litter size, birth weight, or weights at 11 weeks of age.

The discrepancy between Abel's results and the potentiating effects of prenatal ethanol plus nicotine exposure reported in previous human studies was explained by Abel as differences in species, route of drug administration, and the possibility that the dose of nicotine used in his study (1.5 mg/kg) was less than that absorbed by mothers who smoke and below that which produced effects on growth.

Lindenschmidt and Persaud (1980) administered a single dose of both ethanol (two different groups with different doses: one group received 0.02 ml/g of a 25% ethanol

solution IP, and the other group received 0.02 mg/g of a 12.5% ethanol solution IP) and nicotine (5 mg/kg SC) to pregnant rats on day 9 of gestation. Their results showed that the single exposure to both ethanol and nicotine produced no significant deleterious effects on pregnancy outcome, or subsequent neonatal offspring parameters.

Due to the conflicting reports as to whether prenatal exposure to both ethanol and nicotine results in a synergistic or potentiated deleterious effect on the ensuing pregnancy and subsequent offspring development, we observed and reported maternal, neonatal, and postnatal parameters following a prenatal dose of ethanol or nicotine and a prenatal combined dose of both drugs.

The present study used a rodent animal model that has been previously accepted for FAS studies (Chernoff, 1977). Unfortunately, a good animal model imitating human nicotine administration has not been devised. Part of the problem is inherent in the route of administration. An aerosol of nicotine resembling tobacco smoke with only nicotine in it has not been developed. Because of this problem, various models for nicotine administration have been devised. Nicotine has been administered to animals by smoking chambers, by injections, orally, and by implanting nicotine capsules.

Another obstacle in nicotine studies is dosage. Only

recently has an accurate method been developed for determining nicotine blood levels in humans (Jacob and Benowitz, 1981). At the present time there are no methods available for determining longitudinal nicotine blood levels in animal models. Another problem is the pharmacology of nicotine. Further research is needed in both human and animal models to establish dose response curves, nicotine tolerance, nicotine titration effect, and the ability of different tissues to accumulate nicotine when compared to each other as well as exposure at different ages (Stalhandske and Slanina, 1971).

The present study analyzed the effects of prenatal exposure to ethanol, nicotine, and ethanol plus nicotine during the middle two thirds of gestation on maternal weight gain and consumption, length of gestation, birth parameters, perinatal and postnatal mortalities, and postnatal development and function. Measurements indicating postnatal development and function included time of bilateral eye openings and establishment of an auditory-startle reflex, basal metabolic rates, liver function, organ-body weight ratios, bi-weekly weight gains, and behavioral tests.

CHAPTER II

MATERIALS AND METHODS

Study I

Long Evans rats (Charles Rivers) weighing approximately 200 grams each were received on day three of gestation. Animals were caged individually in a temperature controlled room with free access to water and rat chow (Ralston-Purina Chow Co.). A 12 hour light/dark cycle was maintained. Animals were weighed and divided into four groups (Table A).

Table A

GROUP I CONTROLS	GROUP II ETHANOL
1. Subgroup A (n=2) ANESTHETIC CONTROLS	27% calories as ethanol (n=6)*
2. Subgroup B (n=3) SALINE-IMPLANTED CONTROLS	
GROUP III NICOTINE**	GROUP IV ETHANOL + NICOTINE
0.2 mg/kg (n=4)	0.2 mg/kg nicotine (n=6) 27% calories ethanol

*From day 5 through day 19 of gestation the ethanol group was given a modified Leiber-DeCarli diet with 27% total calories as ethanol.

**From day 5 through day 19 of gestation the nicotine group received 0.2 mg/kg of nicotine by implanted Alza osmotic mini pumps.

Animals were given water and rat chow ad libitum until day 5 of gestation. Drug administration began on day 5 of gestation and ended on day 19 of gestation. On these days, all animals were fed Leiber DeCarli ethanol or isocaloric control liquid diets. Diets were fortified with Dutch Chocolate Sego to increase consumption. A concentration of 27% total calories as ethanol was administered to produce mild CNS impairment without gross teratological effects (Lancaster et al, 1982). To insure an equivalent nutritional status across groups, food intake of controls, nicotine, and alcohol groups was restricted to that consumed by the combination nicotine plus ethanol group throughout the 14 day drug regime.

Nicotine (Sigma Corporation, St. Louis, Mo.). was administered by continually infusing Alza osmotic mini pumps. A dose of 0.2 mg/kg was selected as a low enough dose to cause behavioral differences (Morrison, 1967), but not high enough to produce toxic effects in the mother. Alza pumps have a delivery rate of 0.5µl/hr. continually for 14 days (gestation days 5 through 19), except for a 4 hour start up delay following implantation. To control for surgical, anesthetic, and tissue rejection variables, Alza pumps were subscapularily implanted in all animals except the anesthetic controls (subgroup A). Alza pumps of the control subgroup B and the alcohol group were filled with

0.9% sterile saline instead of nicotine.

Diethyl ether was used as an anesthetic for pump implantation because it was easy to administer, was quickly metabolized, and has been successfully used in previous studies for Alza pump implantation in pregnant rats (Hamosh, 1979). Anoxic effects produced by ether in the fetus and mother may be less damaging than the effects of other commonly used anesthetics (Bergmen et al, 1980). The mean time down under ether anesthesia for pump implantation was 45 seconds.

Even though drug administration ceased on day 19 of gestation, pumps were left in situ until day 6 postpartum. Surgical removal of the pumps was necessary because the animals had developed abcesses (78% were sterile). Rats containing pumps were anesthesized with 25 mg/kg pentobarbital IP. After pump removal, wounds were flooded with hydrogen peroxide, sponged with sterile gauze, and squirted with 0.5 cc penicillin G. Wound clips were applied and Bacitracin was used as a topical antiseptic. After surgery, all mothers were returned to their cages with their offspring. Mean time down was 5 hours.

Dams in the ethanol plus nicotine group determined the daily consumption of the other groups. Each of the six ethanol plus nicotine dams were models for pair-feeding the six ethanol dams. Four of the ethanol plus nicotine dams were

paired with four nicotine dams. Daily consumptions of the ethanol dams determined the daily consumptions of the four nicotine dams. The three saline-implanted controls and the two anesthetic controls were also pair-fed with their corresponding ethanol plus nicotine dams. Three days after drug administration began, dams in the ethanol plus nicotine group and dams in the nicotine group died from a nicotine overdos due to miscalculations. The six ethanol dams then became models for pair-feeding the control groups. Drug administration and pair-feeding ceased on day 19 of gestation. All animals were then given water and rat chow ad libitum throughout gestation and weaning.

Maternal weight gains were recorded on days 3, 8, 11, 14, 17, and 19 of gestation. Litter size, birth weights, and sex ratios were all noted within 24 hours following delivery. Neonatal mortalities to day 20 postpartum were recorded. Within 48 hours after birth, each litter was adjusted to 9-10 pups. Offspring weight gains were recorded on days 7, 12, 15, and 20. All pups were weaned and placed on water and rat chow ad libitum on day 21.

Auditory startle reflex was measured in all pups on days 12 through 14. The CNS vestibular-cochlear pathways might have been damaged or delayed by the in utero exposure to either ethanol, nicotine, or both. The startle reflex test was conducted by isolating one pup at a time in a

seperate box and clapping two 13 cm x .64 cm wooden boards together approximately 30 cm above the pup. The process was repeated three times. A positive response was noted if the pup displayed a startle reflex on at least one trial. Group statistics were compared by Chi Square.

Bilateral eye openings and single eye openings were recorded in all pups on days 14 through 16. Because eye openings indicate level of CNS development, delayed eye openings in the drug group's offspring may show evidence of abnormal CNS development due to prenatal exposure to ethanol, nicotine, and ethanol plus nicotine. Group statistics were compared by Chi Square.

It has not been reported that chronic nicotine use affects basal metabolic rates (BMR) as measured by oxygen consumption. Samorajski et al (1978) demonstrated that chronic ethanol consumption increases oxygen consumption. It had not been established that <u>in utero</u> exposure to nicotine, ethanol, or a combination of ethanol and nicotine in the quantities given could permanently effect the BMR's of the subsequent offspring. Oxygen consumptions were determined by a modified Warburg method (Perez and Samorajski, 1980). All pups were seperated from their nursing mothers at least 2 - 3 hours before being tested. For each testing series, groups of 12 pups were brought out from their mothers (6 controls and 6 drug offspring). Six

Warburg apparati were set up so that at any one time three animals from each group (control offspring versus drug offspring) could be tested. Triplicate values were recorded; oxygen consumptions were calculated and averaged for each pup.

Liver is the major organ responsible for ethanol and nicotine metabolism. Chronic in utero exposure to these drugs impairs fetal liver development. Since hepatic metabolizing activity is an indirect measurement of liver function, the ability of the hepatic enzymes to detoxify drugs can be used as an indication of liver function. Therefore, hexobarbitol clearance as measured by sleeping time was used to ascertain offspring liver function.

On day 20 all pups were injected with 80 mg/kg hexobarbitol IP. The time between injection of hexobarbitol and narcosis (determined by absence of righting reflex), and the time between narcosis and returned presence of righting reflex were recorded. The time span between injection and narcosis is an indication of brain function, whereas the time interval between narcosis and the return of the righting reflex is indicative of liver clearance (Shah and Lal, 1971).

Since behavioral changes are the most sensitive assessment of CNS abnormalities, a conventional open field exploration (baseline) and a modified open field exploration

(experimental) test were designed. Pups from all groups were observed in a conventional open field test first. A pup was placed in a box (13 cm x 14 cm x 13 cm) that contained no bottom but possessed a hinged lid. The box was placed on the middle square of a 46 cm x 76 cm grid of equal sized squares (15 total). After a two minute confinement isolation period, the box was lifted off the pup, thereby exposing the grid. The test then consisted of a two minute period in which two parameters were measured. Latency, the time taken to cross all four paws out of the first square, was the first parameter measured. The second parameter was the number of squares traversed in this 120 second test. The pup was then injected with 25 mg/kg IP hexobarbitol and the procedure repeated for the experimental open field exploration. Both the conventional and modified open field tests were performed on day 22 and repeated on day 23.

Organ-body weight ratios of the brain, pituitary, heart, spleen, liver, and adrenals were recorded on day 21 for three of the anesthetic control offspring. Wet tissue weights were determined immediately after dissection and compared to the pup's total body weight.

Study II

Twelve Long Evans rats weighing approximately 200 grams each were received on day three of gestation. Animal

husbandry was exactly as in Study I. After weighing, rats were divided into four groups (as in Study I) with the following numbers; anesthetic control (n=2), saline-implanted control (n=2), ethanol (n=3), nicotine (n=2), and ethanol plus nicotine (n=4). Drug administration was exactly as in Study I. Pair-feedings were as in the first study with the ethanol plus nicotine group as models.

Maternal weight gains were recorded on days 3, 5, 9, 12, 15, and 22 of gestation. Neonatal parameters were recorded exactly as in Study I. Offspring weight gains were recorded on days 5, 9, 12, 15, and 20. Pups were weaned on day 21 and placed on water and rat chow ad libitum.

The methodology for the auditory startle reflex and bilateral eye openings were exactly as in Study I. Both oxygen and hexobarbitol sleeping time test procedures were identical to Study I. The open field exploration protocol was exactly as in Study I. However, these offspring were tested on day 23 (all pups) and on day 27 (only control and prenatally treated nicotine offspring). With the exception of the age and groups, organ-body weight ratio methods were exactly as in Study I. Three saline control offspring and three prenatally treated nicotine offspring were sacrificed for this test on day 24 of gestation.

Study III

Seven Long Evans rats weighing approximately 200 grams arrived on day three of gestation. After weights were recorded, the rats were divided into two groups which consisted of controls (n=2) and nicotine treated (n=5) animals. Nicotine was administered as in Study I, except with delivery of drug on gestation days 4 through 18 instead of on days 5 through 19. Alza pumps were removed on day three postpartum using the exact procedures as in Study I.

Instead of being individually pair-fed, the two controls were "group fed" according to mean consumption of the five nicotine dams on the previous day. All animals were fed the leiber-DeCarli isocaloric control diet fortified with Dutch Chocolate Sego. On day eighteen of gestation "group feedings" were stopped and animals were given water and rat chow ad libitum until day 21 postpartum when the offspring were weaned and their mothers sacrificed.

Maternal prenatal weights were recorded on days 3, 4, 6, 8, 11, 14, 17, and 21 of gestation. Neonatal parameters measured were exactly as in Study I with the exception of litter size adjustments. Each dam nursed 11-12 offspring instead of 9-10. Postpartum maternal weight gains were recorded on days 3, 6, 12, and 14. Offspring weight gains were recorded on days 1, 3, 7, 10, 12, 15, 17, and 20.

Developmental measurements (auditory startle reflex and

eye openings) and oxygen consumption were measured exactly as in Study I. Liver function was measured exactly as in Study I with one exception: in Study III, the test was repeated on day 27 to see if the observed differences on day 20 persisted. Behavioral tests performed included open field exploration, locomotor activity (as measured by wheel running), and one trial learning passive avoidance. The open field behavior was measured as in Study I with the exception of date. Testing was conducted on days 16 and 17 instead of days 23 and 30. In the locomotor activity tests, eighteen offspring from each group were placed in activity wheels, and the number of rotations made in one hour was recorded. The test was repeated for four consecutive days (days 28-31) until the young rats could no longer comfortably fit into the activity wheels.

On day thirty, twelve animals from each group were randomly selected for the one trial shock and passive avoidance test as described by Bartus et al (1980). The apparatus consisted of a box 46 cm x 23 cm with two chambers seperated by a partition that could be raised allowing access from one chamber to the other. A bright light source was concentrated in the first chamber. The test consisted of placing an animal in the first chamber with the partition down and with the light directly overhead. The pup was allowed to adjust to the environment for fifteen seconds. The partition was then lifted and latency to enter the dark

chamber was recorded. As soon as all four paws were into the second chamber, the partition was lowered (preventing reentry into the first chamber) and a 0.2 mamp, 2 second shock was applied. The animal was then returned to its cage. The next day animals were tested for latency alone. A maximum of 90 seconds was allowed for past shocking memory. Passive avoidance memory was again tested on day 38 (8 days post shock treatment).

Since in utero exposure to nicotine may affect the development of nicotine receptors, a postnatal behavior test stimulating nicotine receptors was devised. The test consisted of a baseline and experimental (nicotine injected) measure of horizonal motor activity. Two horizonal motor activity apparati (Motron, Stockholm) were set up to record singly caged pups (one control offspring versus one nicotine offspring) with an electronic motility meter. Infared photocell interruptions were accumulated and printed out at 5 minute intervals. From day 41 through day 43, eleven pups from each of the two groups were tested for 15 minutes as a baseline for horizontal activity. Animals were then injected with 0.2 mg/kg IP nicotine and horizontal motor activity was measured for 60 seconds.

CHAPTER III

RESULTS

Study I

Only 8 out of 21 dams obtained for the study delivered offspring. The 4 nicotine dams and the 6 ethanol plus nicotine dams were mistakenly given lethal overdoses of nicotine. One of the saline-implanted control animals died unexpectedly during the pump implantation surgery. Only 4 out of the 6 ethanol dams delivered. The two nongravid dams may not have been pregnant upon arrival, or they possibly could have aborted their fetuses. Therefore, the dams that delivered in each group were as follows: 2 anesthetic control dams (100% delivered pups), 2 saline-implanted control dams (100% delivered pups), and 4 ethanol dams (66% delivered pups).

Maternal weight gains from day 3 through day 19 of gestation showed no significant differences among any of the dams (Figure I). Maternal liquid diet consumption from day 5 through day 19 of gestation showed no significant differences among any of the dams (Figure II).

All 8 dams delivered on day 23 of gestation. Within 24 hours after birth, all pups were weighed, toe clipped, and checked for anomalies. Litter size , sex ratios, and the percent mortality to day 20 were recorded along with birth weights and anomalies (Table II). Evaluation by

nonparametric multiple comparisons (NPMC) showed that the ethanol offspring had lower birth weights ($Z < .05$) than both the anesthetic control offspring and the saline-implanted control offspring. The only anomaly noted was crepey skin in one of the 36 ethanol pups. There were no signifi ant differences in sex ratios and litter sizes among any of the groups. The percent mortality to day 20 was significantly higher ($X^2 < .001$) for the ethanol offspring. Seventeen of the 36 ethanol offspring were cannibalized within the first 3 days of life. One ethanol dam cannibalized all of her 9 pups and later died during her Alza pump removal. A severe non-sterile abcess was formed around her implanted pump.

Offspring weight gains are shown in Figure III. On days 7, 12, 15, and 20, weights of the anesthetic control offspring were significantly higher ($Z < .05$, NPMC) than the weights of the saline-implanted control pups and the ethanol pups. Since the birth weights of the two control groups were not significantly different from each other, the stress factor due to ether anesthesia during Alza pump implantation was ruled out. However, both saline-implanted control dams and ethanol dams were anesthesized on day 6 postpartum for surgical pump removals. Possibly the 5 hour narcosis time due to pentobarbital anesthesia was responsible for decreased lactation and subsequent lower offspring weight

gains.

Ethanol offspring had significantly fewer ($X^2 < .005$) auditory startle reflexes on day 12 (Table III). On day 13, the ethanol offspring still had lower numbers of positive responses to the auditory startle stimulus. Delayed bilateral eye openings on day 14 and day 15 indicated slower rates of development in the ethanol offspring.

There were no significant differences recorded among any of the groups on basal metabolic rates (day 15), organ-body ratios (day 21), hexobarbitol sleeping time test (day 20), and open field exploration (days 22 and 23). All data were evaluated by nonparametric multiple comparisons.

Study II

None of the anesthetic control dams (n=2) or the ethanol dams (n=3) delivered. The two saline implanted control dams delivered and the two dams in the nicotine group delivered; however, one dam lost her nicotine pump within a few days following implantation. Hence, she and her offspring were excluded from the study. Only one of the four ethanol plus nicotine dams delivered.

There were no significant differences in total maternal weight gains from day 3 to day 22 of gestation (Figure IV). One of the saline-implanted control dams and the one nicotine dam consumed the same amount of liquid diet (62 ml/day: ±3). The other saline control dam consumed a mean of

46 ml/day (±3.4 ml); and the ethanol plus nicotine consumed a mean of 52 ml/day (±2.4 ml) (Figure V).

All dams delivered on day 23 of gestation. Within 24 hours following delivery, all pups were weighed, sexed, inspected for anomalies, and toe clipped. The litter size of the ethanol plus nicotine dam was greatly reduced when compared to the control and nicotine dams. Three of the six ethanol plus nicotine offspring had crepey skin and were cyanotic. Birth weights were significantly lower ($Z < .05$, NPMC) in the ethanol plus nicotine pups. The nicotine dam had unexpected significantly higher number of male offspring (66%). The neonatal mortality rate was significantly higher ($X^2 < .005$) for the ethanol plus nicotine offspring (Table IV).

Offspring weights are illustrated in Figure VI. The ethanol plus nicotine offspring had significantly lower ($Z < .05$, NPMC) weights on days 5, 9, 12, 15, and 20.

The results of the developmental measurements of auditory startle reflex and bilateral eye openings are depicted in Table V. The ethanol plus nicotine offspring were slower to develop the auditory startle reflex ($X^2 < .001$) on day 12 and slower in bilateral eye openings ($X^2 < .001$) on both day 14 and day 15.

The hexobarbitol sleeping test revealed significant differences ($Z < .05$, NPMC) between the saline-implanted

control offspring and the nicotine offspring on day 20 (Figure VII). The nicotine offspring had a faster injection to narcosis time, indicating a difference in brain function. There were no significant differences in liver function measurement of narcosis time.

Open field exploration on day 23 showed significant differences ($Z < .05$, NPMC) between the nicotine and control offspring on both experimental latencies and experimental squares traversed. Two minutes after injection with 25 mg/kg IP hexobarbitol, the nicotine offspring displayed a hyperactivity effect instead of the normal sedative effect (Figure VIII). To verify the longevity of the hyperactivity recorded in the nicotine pups, the test was repeated on day 30 (Figure IX). A Welch's test gave a "p" value of $< .06$ for experimental latency. The experimental latency time on day 30 was a reversal of latency measured on day 23. On day 30, the nicotine offspring appeared sedated after exposure to a low dose (25 mg/kg IP) of hexobarbitol; whereas, the control offspring were hyperactive.

No significant differences were found between any of the three groups in oxygen consumption tests (day 15). Only nicotine and saline control offspring were sacrificed on day 24 for organ-body ratios. No significant differences in organ-body ratios were found.

Study III

One hundred percent of the saline-implanted control dams (n=2) delivered, but only 40% of the nicotine dams (n=5) delivered. Both control dams and one nicotine dam delivered on day 22. The other nicotine dam delivered early the next day with an approximate 12 to 14 hour lag time from the other three dams partuition.

Neither prenatal maternal weights (Figure X) nor postnatal maternal weights showed significant differences between the two groups (Figure XI).

There were no significant differences in birth weight between the control offspring and the nicotine offspring when compared by a time analysis of variance (ANOVA). A time ANOVA was the statistical method of choice since not all dams delivered at the same time. One nicotine dam delivered 12-14 hours later than the other nicotine dam and the two control dams. Neither sex ratio, litter size, nor mortality showed significant differences between the control offspring and the nicotine offspring.

Figure XII illustrates offspring weight gains. On day 20 the nicotine offspring had weights significantly higher ($p < .005$; Welch's t) than those of the control offspring. By day 27 the significance increased to $p < .001$ evaluated by Welch's t.

The only developmental measurement that was

significantly different between pups prenatally treated with nicotine and pups of saline-implanted controls was the auditory startle reflex on day 12 ($X^2 < .001$). The delay in the development of the auditory startle reflex persisted until day 13 but the delay was not significantly different between prenatally treated nicotine pups and control pups (Table VII). No significant differences were noted in bilateral eye openings.

The hexobarbitol sleeping time test (days 20 and 21) showed a significant difference in narcosis time ($p < .003$; Welch's t). The renatally treated nicotine offspring had shorter narcosis times (Figure XIII) than did control offsprings. Locomotor activity scores on day 29 and day 30 were significant to the $p < .002$ and $p < .006$ values respectively when evaluated by the Welch's t (Figure XIV). The nicotine offspring were less active than the control offspring on all days tested.

There were no significant differences recorded in basal metabolic rates (day 15), open field exploration (days 16 and 17), and on hexobarbitol sleeping time test repeated on day 30. No significant differences were recorded between the two groups for horizontal motor activity (day 41 through day 43) in either baseline activity or experimental (0.2 mg/kg IP nicotine) activity.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Comparisons between drug treated groups cannot be made because at least one dam in each group did not deliver. Study I only produced anesthetic control offspring, saline-implanted control offspring, and ethanol offspring. Results from Study I confirm the already numerous publications on the deleterious effects of *in utero* exposure to ethanol. Ethanol dams had reduced litter sizes, significantly lower offspring birth weights, and significantly higher perinatal mortality rates than either the anesthetic control dams or the saline-implanted control dams. The high mortality rate for ethanol offspring was entirely due to maternal cannibalization. Maternal cannibalization of young is not an unusual occurance during times of stress. Maternal gestational exposure to ethanol and subsequent cannibalization of young is thought to be due to stress rather than due to maternal malnutrition since dams were fed isocaloric diets with identical amounts of nutrients, trace elements, and vitamins. Another possible explanation for maternal cannibalization of offspring is the reported findings of alcohol induced trace element malabsorptions (Schroeder and Nason, 1971). The only anomaly noted was crepey skin in one of the 36 ethanol offspring.

Offspring weight gains recorded from day 7 to day 20 showed an unexpected significantly higher weight gain for the anesthetic control pups when compared to either the ethanol offspring or the saline-implanted control offspring. It is believed that the observed significant difference in weight gain between the anesthetic control offspring and saline-implanted control offspring was due to maternal postnatal exposure to pentobarbital. Studies by Dyball (1975) and Prilusky and Deis (1975) reported that pentobarbital significantly inhibited the release of oxytocin, thereby reducing the amount of milk available for nursing pups.

Prenatal exposure to ethanol has been shown to cause delayed development of offspring (Randall and Taylor, 1979; Tze and Lee, 1975). In this study, development of an auditory startle reflex on day 12 as well as delay in bilateral eye opinings on days 14 and 15 may reflect delayed myelination of CNS neurons as reported in previous studies (Lancaster, et al, 1982). There were no significant differences observed between prenatally treated ethanol offspring, anesthetic control offspring, and saline-implanted control offspring on basal metabolic rates, hexobarbitol sleeping time test, organ-body weight ratios, and open field exploration.

Study II produced offspring from two saline-implanted control dams, one nicotine treated dam, and one ethanol

plus nicotine treated dam. Zero percent of the anesthetic control dams (n=2), zero percent of the ethanol treated dams (n=3), and twenty-five percent of the ethanol plus nicotine dams (n=4) delivered. It is not known if these dams aborted their fetuses or if they never conceived. Evidence for the exacerbating affects of prenatal exposure to both ethanol and nicotine is reflected in the abnormalities produced in the ethanol plus nicotine treated dams with regard to reduced litter size, and offspring neonatal parameters, weight gains, and developmental parameters. Fifty percent (3 out of 6) of the exposed prenatally ethanol plus nicotine offspring manifested the crepey skin anomaly and were also cyanotic. In the previous study, only one out of 36 exposed prenatally ethanol offspring expressed crepey skin. The cyanosis of the exposed prenatally ethanol plus nicotine offspring was probably due to the hypoxic effect of nicotine (Suzuki, 1974). Birth weights and weight gains were significantly lower for the offspring of the ethanol plus nicotine treated dam when compared to either control offspring or nicotine exposed offspring. This observation supports both human and animal studies concerning greatly reduced birth weights and long term retarded weight gains for offspring exposed prenatally to ethanol and nicotine. This phenomenon may reflect primarily an ethanol effect since fetal exposure to ethanol has been shown to decrease serum growth hormone (GH)

levels and impair ornithine decarboxylase (ODC) activity in the brain and heart of rats (Thadani, et al, 1977 and 1979). It is hypothesized that a decrease in growth hormone may cause a decrease in ODC activity (which is partially regulated by GH) and this in turn may diminish polyamine synthesis, ultimately affecting protein synthesis (Henderson, et al, 1981).

The nicotine dam had an unexpected significantly higher number of male offspring than either the control dams or the ethanol plus nicotine dam. This observation is the opposite of that reported by Peters et al (1979).

Results from developmental parameters showed that the prenatally treated ethanol plus nicotine offspring were significantly slower than the control offspring in developing the auditory startle reflex at day 12 and bilateral eye openings at days 14 and 15. It was noted that even though prenatally treated pups gave a positive response in the auditory startle reflex test, it was a very weak response when compared to the control offspring.

Results from the day 20 hexobarbitol sleeping time test indicate a difference in the brain function of prenatally treated nicotine offspring when compared to control offspring. The pups prenatally treated with nicotine had significantly faster injection to narcosis times than did the control pups. The pups prenatally treated with ethanol plus nicotine displayed even faster injection to narcosis

times than did the prenatally treated nicotine offspring; however, significance was lost due to a low number of animals. Day 23 open field parameters of experimental latency and experimental number of squares traversed showed significant differences in behavior between pups prenatally treated with nicotine and control pups. Nicotine offspring were still active 2 minutes ofter a 25 mg/kg hexobarbitol IP injection, whereas, control offspring were sedated. Normally, when animals are injected with hexobarbitol, a hyperactivity stage is experienced before sedation. Therefore, the shorter latency time and the higher number of squares traversed for the injected nicotine pups represent a longer drug induced hyperactivity period. Since hexobarbitol induced hyperactivity-sedation is a measure of brain function, the offspring prenatally treated with nicotine appear to have abnormal brain function when compared to control offspring.

The open field test was repeated on day 30 to determine if differences observed on day 23 persisted. The unexpected results were a significant difference in experimental latency that was a reversal of the day 23 findings. When injected with 25 mg/kg hexobarbitol on day 30, control offspring displayed a more hyperactive response than did prenatally treated nicotine offspring. Since the experimental open field test is a novice one, no information was available for "normal" response comparisons of 23 and 30

day old rats.

Study III was designed to determine the effects of *in utero* exposure to nicotine on gestational days 4 through 18 instead of days 5 through 19 as in the previous study. Study III produced offspring from two saline-implanted control dams and two nicotine treated dams. No significant differences were observed for maternal parameters or for the neonatal parameters of litter size, birth weight, sex ratios, or anomalies. However, the nicotine offspring had higher mortality rates than did the control offspring.

Pups prenatally treated with nicotine showed significantly higher weight gains than control offspring on day 20 and on day 27. It can be postulated that *in utero* exposure to nicotine may affect future hypothalamic function of the developing offspring.

Pups exposed prenatally to nicotine showed convincing evidence for the ability of nicotine to delay CNS development. On day 12, significantly fewer offspring of nicotine treated dams responded to the auditory startle stimulus. This delay persisted through day 13. Saxton (1978) reported a similar finding in the offspring of smoking mothers. Saxton hypothesized that the auditory decrement observed in smokers offspring was due to an adverse hypoxic effect of carbon monoxide on the cochlear organ during development. The present study indicates that nicotine, not carbon monoxide, produced the auditory decrements possibly through

a hypoxic mechanism. Absence of an auditory startle reflex may be due to the impaired development of motor pathways as well as sensory pathways.

Results from the hexobarbitol sleeping time test indicated a difference in liver function between control offspring and *in utero* exposed nicotine offspring. Pups treated prenatally with nicotine demonstrated significantly shorter narcosis times than did control pups. Since hexobarbitol narcosis time is a direct measurement of liver metabolism of hexobarbitol, the prenatally exposed nicotine pups either had larger livers or more enzymes than the control pups, therefore, metabolizing the drug faster.

Offspring of nicotine treated dams were significantly less active than the control pups on days 29 and 30 of the locomotor wheel running test. It is not known if pups exposed to nicotine prenatally were slower than control pups because their weights were larger and they were not able to fit into the wheels as comfortably as the control offspring, or if the results indicate a behavioral alteration of cerebral motor pathways.

In conclusion, the three separately performed studies do indicate that the *in utero* exposure to ethanol, nicotine, and ethanol plus nicotine adversely affects offspring development. Future studies in this area should involve the establishment of nicotine and ethanol pharmacokinetics along with maternal, fetal, and neonate blood levels of

drugs. Some important questions that need to be answered are: (1) the role of acetaldehyde in FAS: its placental transfer and metabolism, (2) *in utero* nicotine metabolism, and (3) ethanol and nicotine interactions in response to species variation, dosage, and route of administration.

CHAPTER V

SUMMARY

Both ethanol and nicotine *in utero* exposure produced differences in subsequent developing offspring. While prenatal exposure to ethanol demonstrated more dramatic abnormalities in neonatal parameters, it appeared that prenatal exposure to nicotine produced more significant differences in postnatal development and behavioral measurements. Results from the combination ethanol plus nicotine *in utero* exposure suggested synergistic exacerbating effects on developing offspring compared to single drug *in utero* affects. Neither single drug (ethanol or nicotine) produced differences in any of the maternal gestation parameters of weight gain, consumption or length of gestation.

TABLE I
Malformations of Fetal Alcohol Syndrome*

Frequent (80% of patients)	Occasional (26-50% of patients)	Uncommon (1-25% of patients)
Microcephaly	Ptosis	Blepharophimosis
Short palpebral fissures	Strabismus	Auricular conchal maldevelopment
Short upturned nose	Epicanthal folds	Cleft lip or palate
Hypoplastic filtrum	Posterior auricular rotation	Small teeth
Hypoplastic maxilla	Prominent lateral palatine ridges	Pulmonary artery stenosis
Thin upper vermilion	Atrial septal defect	Ventricular septal defect
Infantile micrognathia	Genitolabial hypoplasia	Atrioventricular canal
Prenatal and postnatal growth deficiency	Cutaneous hemangiomas	Right aortic arch
	Abnormal palmar creases	Tetraolgy of Fallot
	Pectus excavatum	Horseshoe kidney
		Hypronephrosis
		Renal crossed ectopia
		Renal hypoplasia
		Renal dysplasia
		Renal pelvicalyectasis
		Ureteropelvic obstruction
		Joint contractures
		Nail hypoplasia
		Polydactyly
		Klippel-Feil anomaly
		Scoliosis
		Hernias
		Diastasis recti

*From Krous. Pathol. Annuals 16: 295-311

TABLE II

Study I: Neonatal Parameters

		Anesthetic	Saline-Implanted	Ethanol
Litter Size	$\bar{X}$	11.5	12.0	9
	SEM	± .5	± 0	±1.5
	(n)	(23)	(24)	(36)
Sex Ratio		9♂/14♀	10♂/14♀	16♂/20♀
Birth Weights	$\bar{X}$	7.20	6.94	5.89 *
	SEM	± .104	± .117	± .151
	(n)	(23)	(24)	(36)
% Mortality		0%	0%	47%**

* Z < .05, nonparametric multiple comparisons; Prenatal treated ethanol offspring had significantly smaller birth weights than either offspring of anesthetic control dams or saline-implanted control dams.

**X^2 < .001, Chi Square; Prenatally treated ethanol had a significantly higher percent mortality than either one of the control offspring.

TABLE III

Study I: Developmental Parameters

Test	Day Postnatal	Group		
		Anesthetic Control	Saline-Implanted Control	Ethanol
Auditory Startle Reflex (% positive response)	Day 12	100%	100%	74%*
	Day 13	100%	100%	89%
	Day 14	100%	100%	100%
Bilateral Eye Openings (% positive response)	Day 14	39%	55%	21%
	Day 15	100%	100%	89%
	Day 16	100%	100%	100%

*X^2 < .005, Chi Square; Prenatally treated ethanol offspring were significantly slower than either anesthetic control offspring or saline-implanted control offspring to develop an auditory startle reflex on day 21.

TABLE IV

Study II: Neonatal Parameters

		Saline-Implanted Controls	Nicotine	Ethanol + Nicotine
Litter Size	$\bar{X}$	9.5	12	6
	SEM	± .5		
	(n)	(21)	(12)	(6)
Sex Ratio		9♂/10♀	9♂/3♀ *	2♂/4♀
Birth Weights	$\bar{X}$	7.46	6.84	5.32**
	SEM	± .095	± .108	± .240
	(n)	(19)	(12)	(6)
% Mortality		0%	0%	33%***
Crepey Skin Anomaly		0%	0%	50%****

*X^2 < .05, Chi Square; Prenatally treated nicotine offspring had a significantly higher male-to-female sex ratio than saline-implanted control offspring or prenatally treated ethanol plus nicotine offspring.

**Z < .05, Nonparametric multiple comparisons; Prenatally treated ethanol plus nicotine offspring had significantly lower birth weights than either saline-implanted control offspring or prenatally treated nicotine offspring.

***X^2 < .005, Chi Square; Prenatally treated ethanol plus nicotine offspring had significantly higher mortality rates than offspring from saline-implanted control dams and nicotine treated dams.

****X^2 < .001, Chi Square; Prenatally treated ethanol plus nicotine offspring had a significantly higher incidence of crepey skin anomaly than either offspring from saline-implanted control dams or nicotine treated dams.

Table V

Study II: Developmental Parameters

Test	Day Postnatal	Group		
		Saline-Implanted Control	Nicotine	Ethanol + Nicotine
Auditory Startle Reflex (% positive response)	12	100%	100%	50%*
	13	100%	100%	100%
Bilateral Eye Openings (% positive response)	14	53%	42%	0%**
	15	100%	100%	50%***
	16	100%	100%	100%

*X^2 < .001, Chi Square; Prenatally treated ethanol plus nicotine offspring were significantly slower than control offspring or prenatally treated nicotine offspring to develop the auditory startle reflex on day 12 as well as bilateral eye openings on days 14 and 15.

**X^2 < .001, Chi Square.

***X^2 < .001, Chi Square.

TABLE VI

Study III: Neonatal Parameters

		Saline-Implanted Controls	Nicotine
Litter Size	$\bar{X}$ SEM (n)	11 ± 1 (22)	11 ± 0 (22)
Sex Ratio		17♂/5♀	19♂/4♀
Birth Weights	$\bar{X}$ SEM (n)	5.35 ± .081 (22)	5.38 ± .091 (22)
% Mortality		0%	13%

TABLE VII

Study III: Developmental Parameters

Test	Day Postnatal	Group	
		Saline-Implanted Controls	Ethanol + Nicotine
Auditory Startle Reflex (% positive response)	12	91%	20%*
	13	100%	85%
	14	100%	100%
Bilateral Eye Openings (% positive response)	14	4.5%	5%
	15	77%	84%
	16	100%	100%

*X^2 < .001, Chi Square; Prenatally treated nicotine offspring showed a significantly slower development of the auditory startle reflex than did offspring of saline-implanted control dams.

Figure I. Study I: Maternal Weights from Day 3 through Day 19 of Gestation.

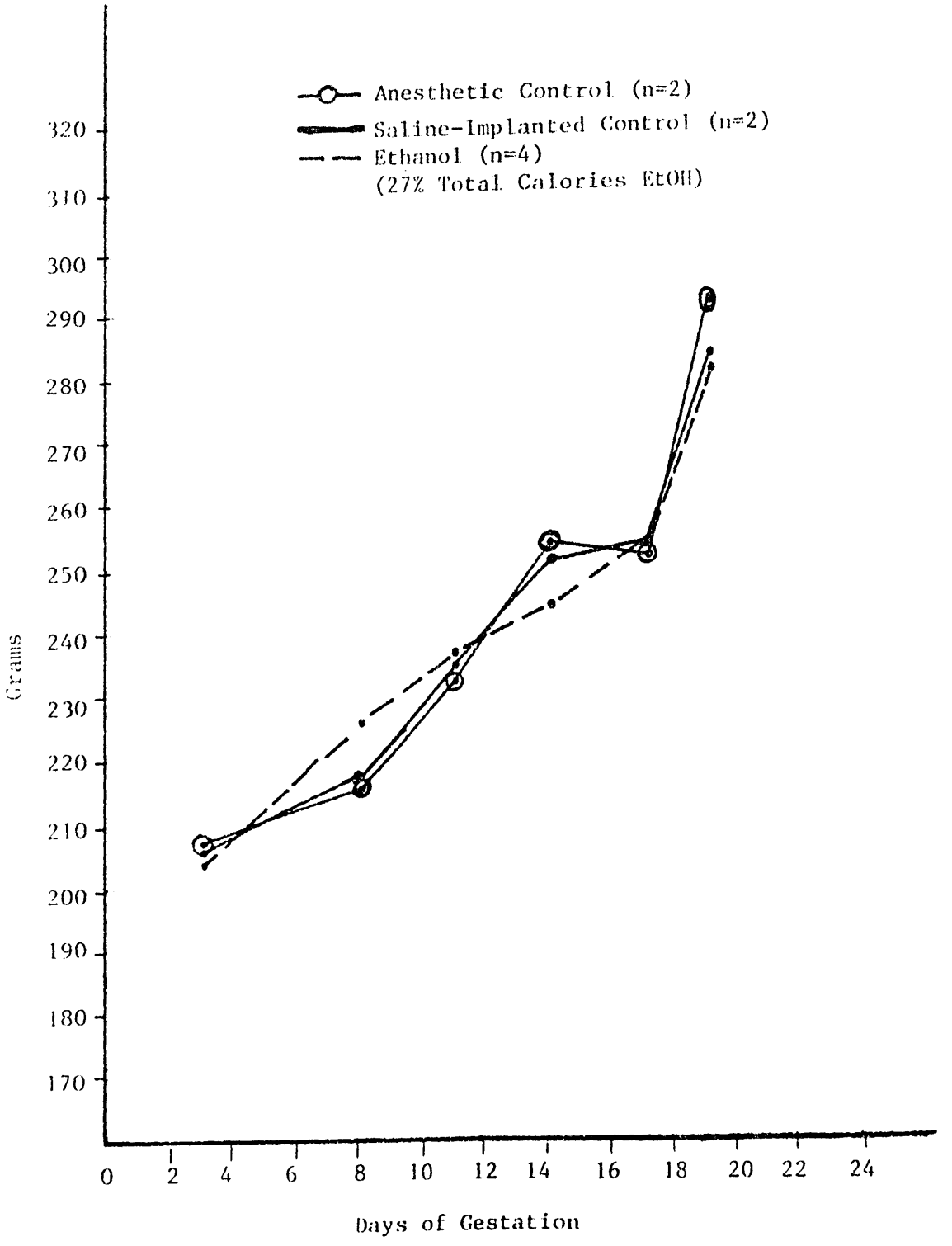

Anesthetic Control (n=2)
Saline-Implanted Control (n=2)
Ethanol (n=4)
(27% Total Calories EtOH)
320
310
300
290
280
270
260
250
240
230
220
210
200
190
180
170
Grams
0
2
4
6
8
10
12
14
16
18
20
22
24
Days of Gestation

Figure II. Study I: Maternal Liquid Diet Consumption from Day 5 through Day 19 of Gestation (Mean ± S.E.M. Consumption for 14 Days per Rat).

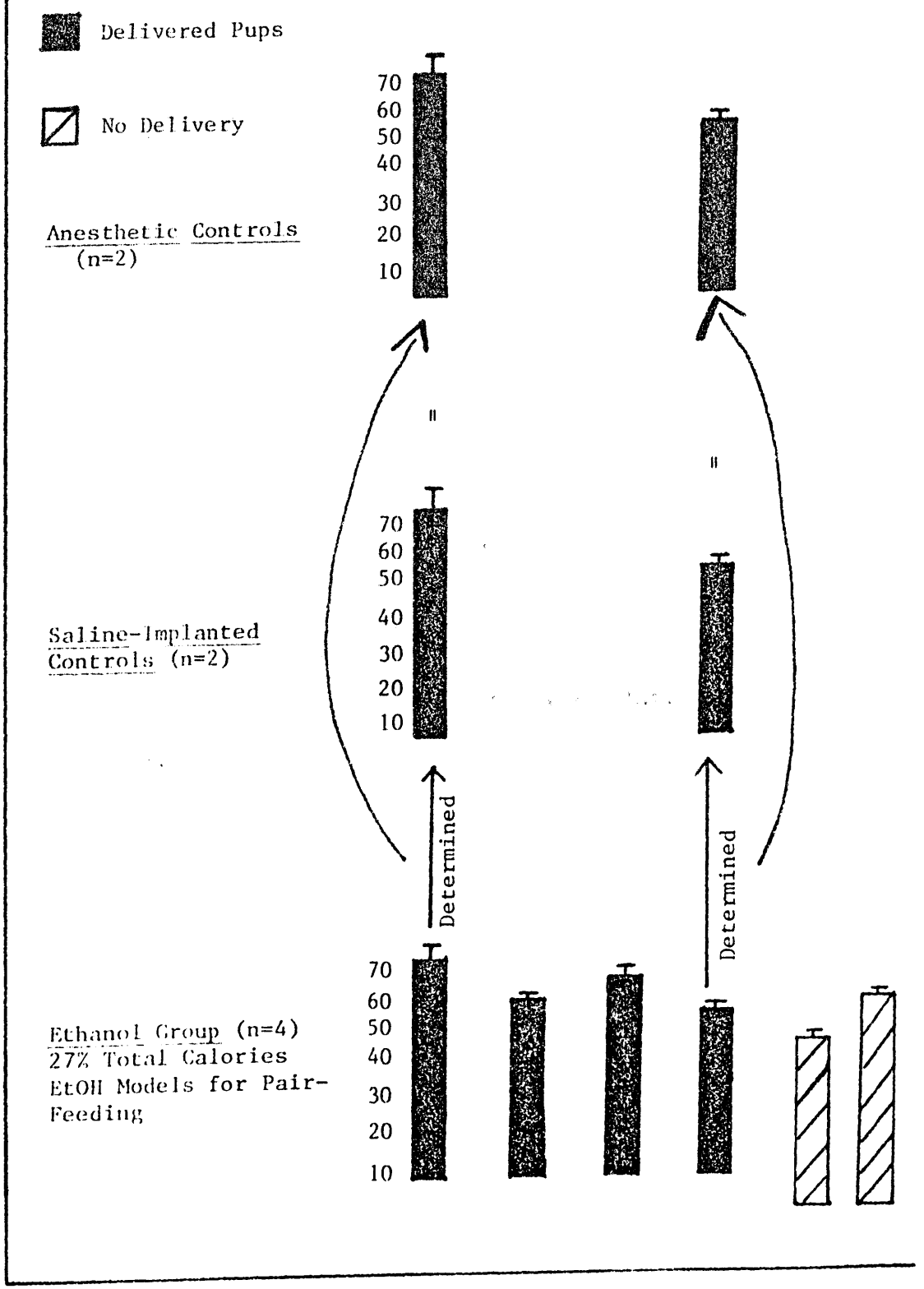

Delivered Pups
No Delivery
Anesthetic Controls (n=2)
Saline-Implanted Controls (n=2)
Ethanol Group (n=4) 27% Total Calories EtOH Models for Pair-Feeding
70
60
50
40
30
20
10
Determined
Determined

Figure III. Study I: Offspring Weight Gains.
*Z < .05, nonparametric multiple comparisons; Offspring of anesthetic control dams compared to offspring of both saline-implanted control dams and ethanol treated dams.

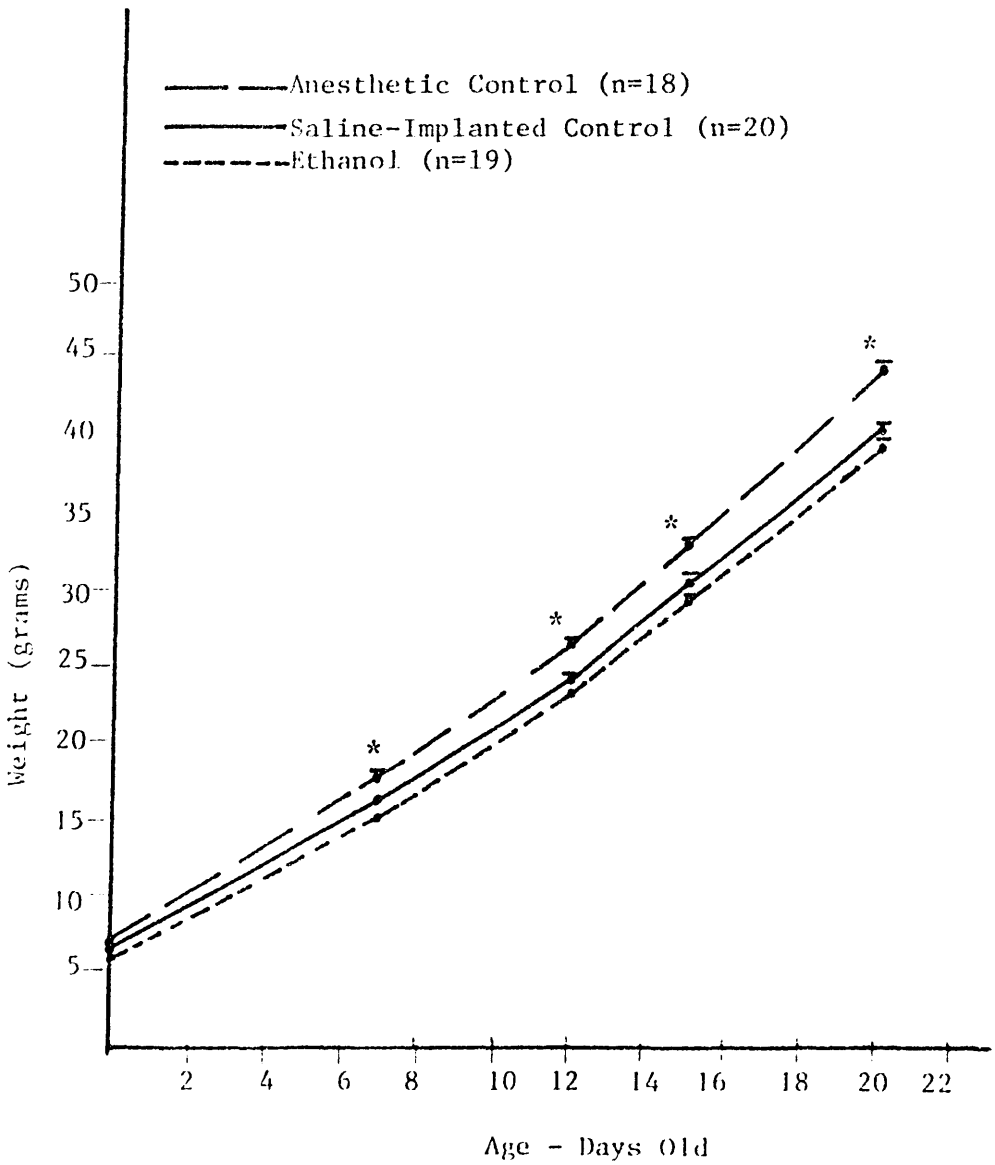

Anesthetic Control (n=18)
Saline-Implanted Control (n=20)
Ethanol (n=19)
Weight (grams)
50
45
40
35
30
25
20
15
10
5
2
4
6
8
10
12
14
16
18
20
22
Age - Days Old
*
*
*
*

Figure IV. Study II: Material Weights from Day 3 through Day 22 of Gestation.

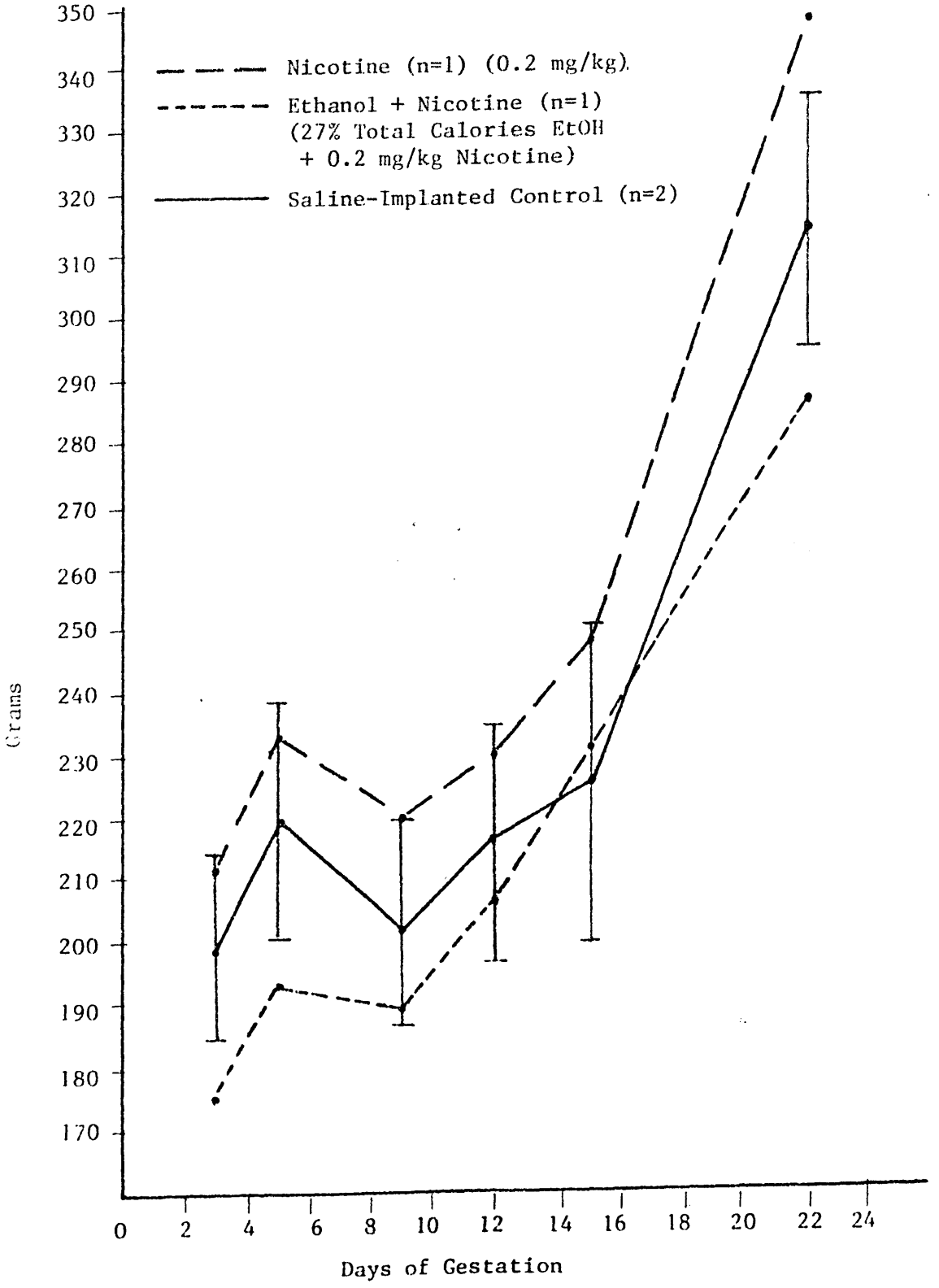

Nicotine (n=1) (0.2 mg/kg)
Ethanol + Nicotine (n=1)
(27% Total Calories EtOH
+ 0.2 mg/kg Nicotine)
Saline-Implanted Control (n=2)
Grams
350
340
330
320
310
300
290
280
270
260
250
240
230
220
210
200
190
180
170
0
2
4
6
8
10
12
14
16
18
20
22
24
Days of Gestation

Figure V. Study II: Material Liquid Diet Consumptions from Day 5 through Day 19 of Gestation (Mean ± S.E.M. Consumption for 14 Days Per Rat).

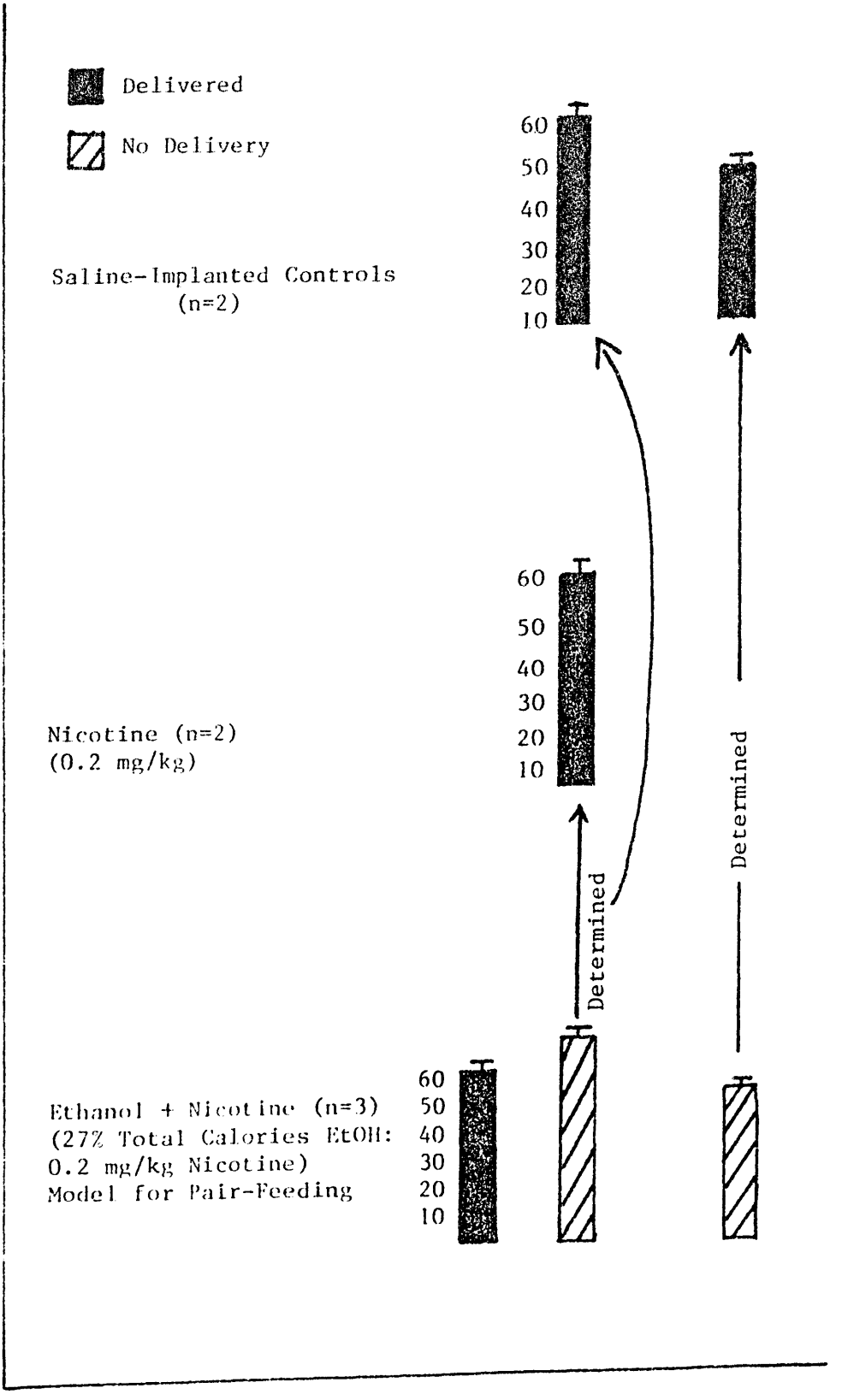

Delivered
No Delivery
Saline-Implanted Controls
(n=2)
Nicotine (n=2)
(0.2 mg/kg)
Ethanol + Nicotine (n=3)
(27% Total Calories EtOH:
0.2 mg/kg Nicotine)
Model for Pair-Feeding
60
50
40
30
20
10
Determined
Determined

Figure VI. Study II: Offspring Weight Gains.
*Z < .05, Nonparametric multiple comparisons;
Pups exposed prenatally to ethanol and nicotine
weighed significantly less than either control
pups or pups exposed prenatally to nicotine.

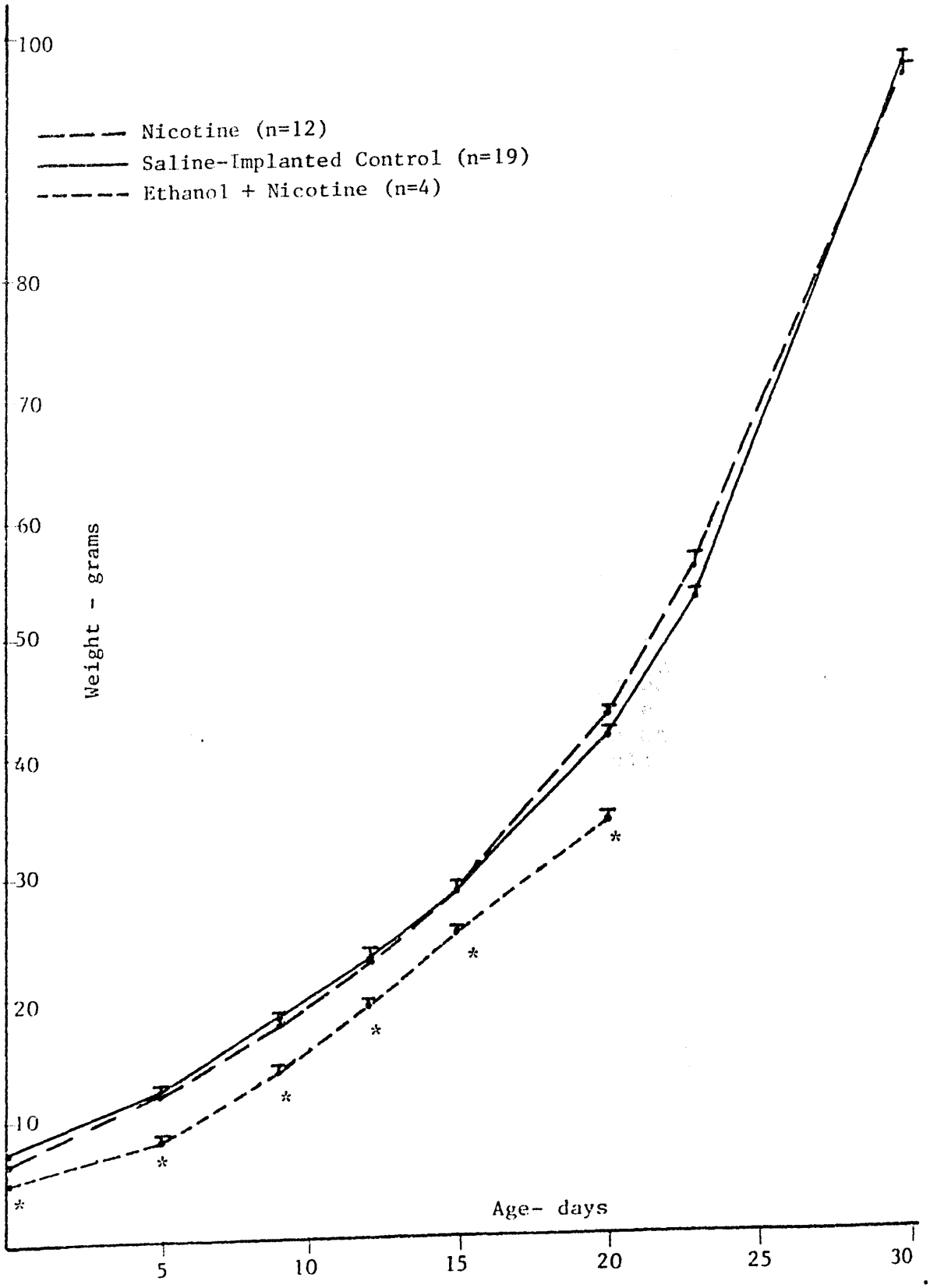

Nicotine (n=12)
Saline-Implanted Control (n=19)
Ethanol + Nicotine (n=4)
Weight - grams
100
80
70
60
50
40
30
20
10
Age- days
5
10
15
20
25
30
*

Figure VII. Study II: Hexobarbitol Sleeping Test (80 mg/kg IP) at day 20.
*Z < .05, nonparametric multiple comparisons; Pups exposed to nicotine in utero displayed significantly faster narcosis times than did control offspring.(Because of the low number of animals, significance was not obtained for the pups exposed prenatally to ethanol and nicotine).

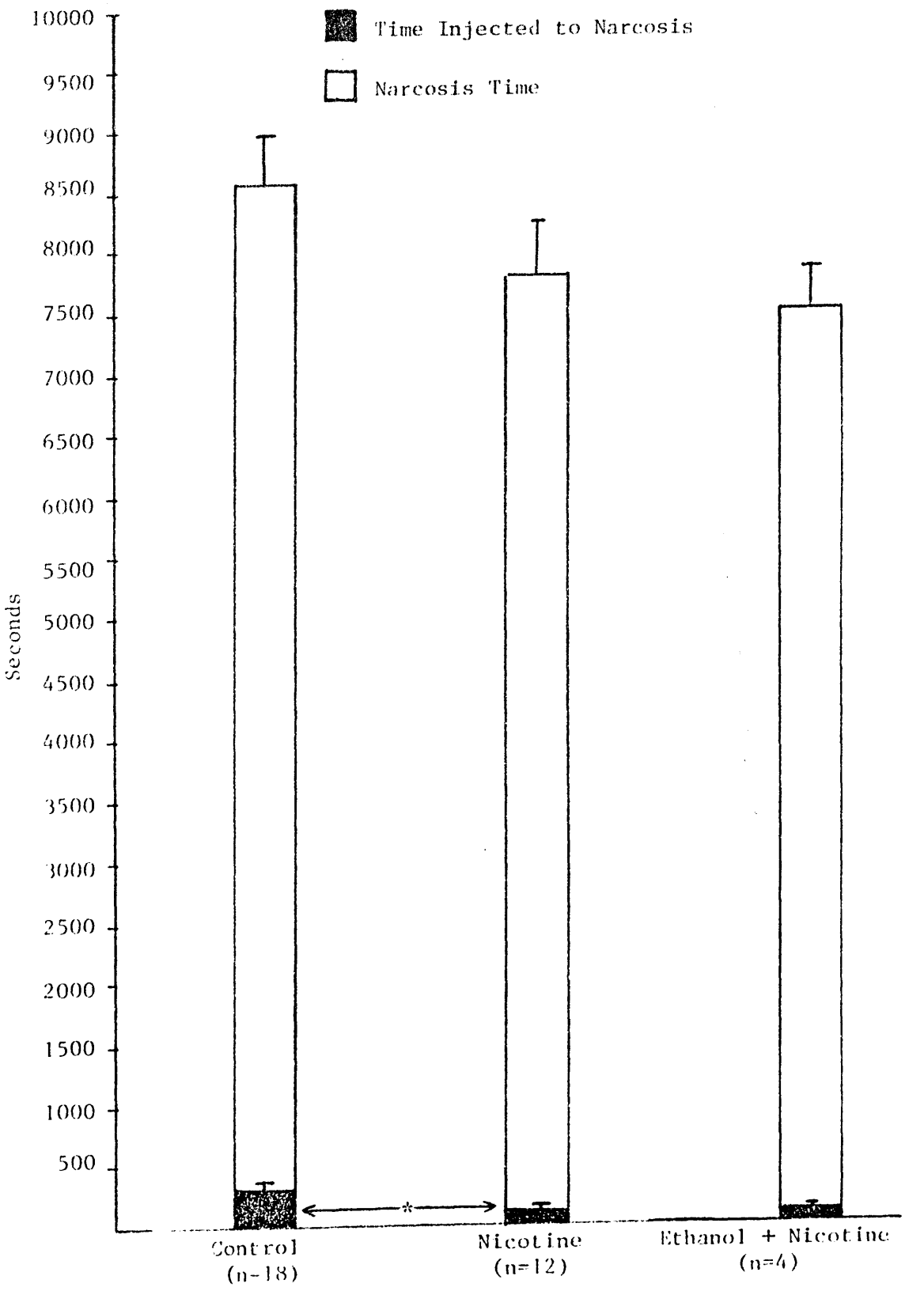

Time Injected to Narcosis
Narcosis Time
Seconds
10000
9500
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
*
Control
(n-18)
Nicotine
(n=12)
Ethanol + Nicotine
(n=4)

Figure VIII. Study II: Open Field Latency and Number of Squares Traversed on Day 24.

*Z < .05, Nonparametric multiple compatisons between control offspring and prenatally treated nicotine offspring in experimental latency.

**Z < .05, Nonparametric multiple comparisons between control offspring and prenatally treated nicotine offspring in experimental squares traversed.

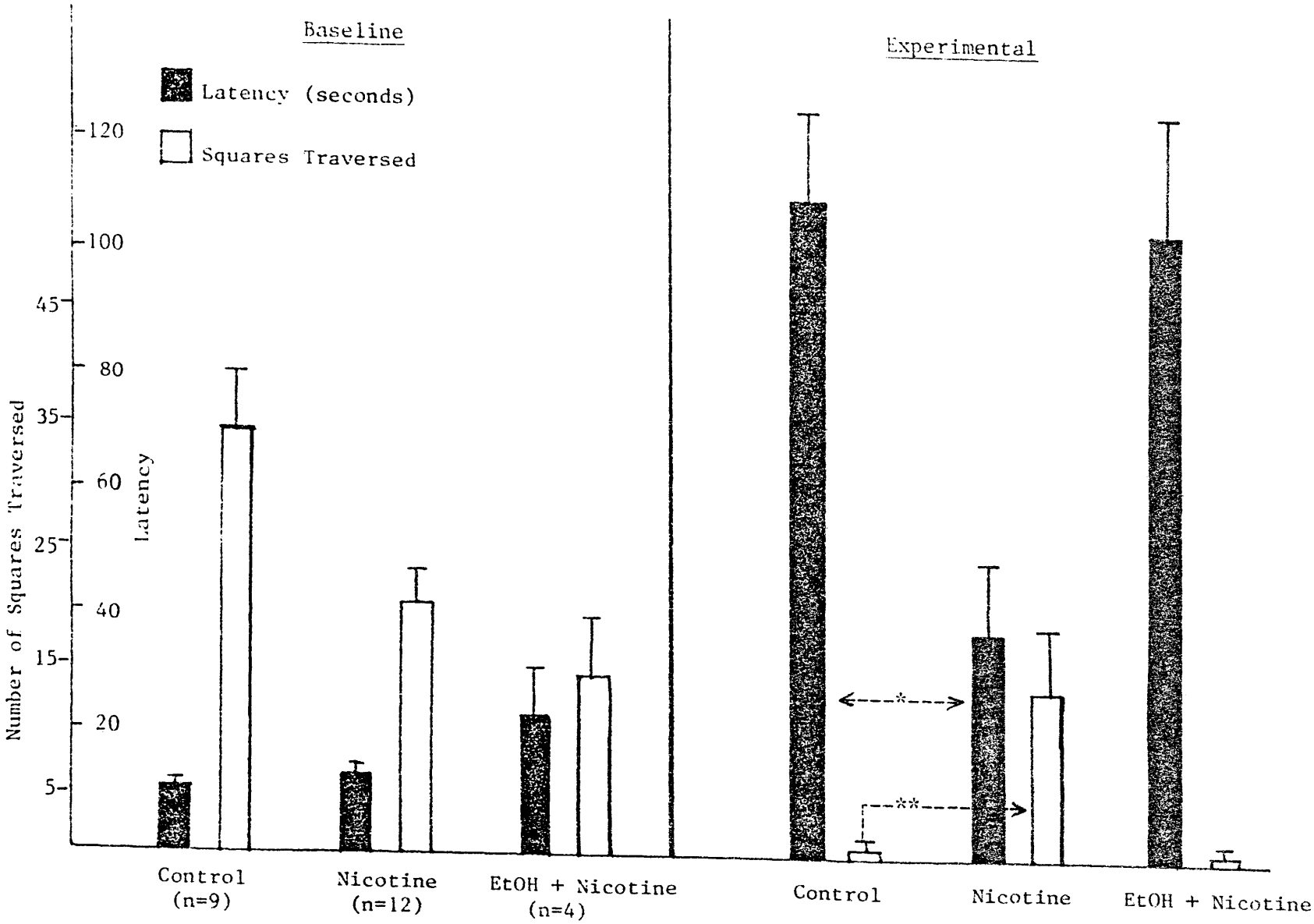

Baseline
Experimental
Latency (seconds)
Squares Traversed
Number of Squares Traversed
Latency
120
100
80
60
40
20
45
35
25
15
5
*
**
Control (n=9)
Nicotine (n=12)
EtOH + Nicotine (n=4)
Control
Nicotine
EtOH + Nicotine

Figure IX. Study II: Open Field Latency and Number of Squares Traversed on Day 30.
*p < .06; Welch's t Experimental latency between control offspring and offspring treated prenatally with nicotine.

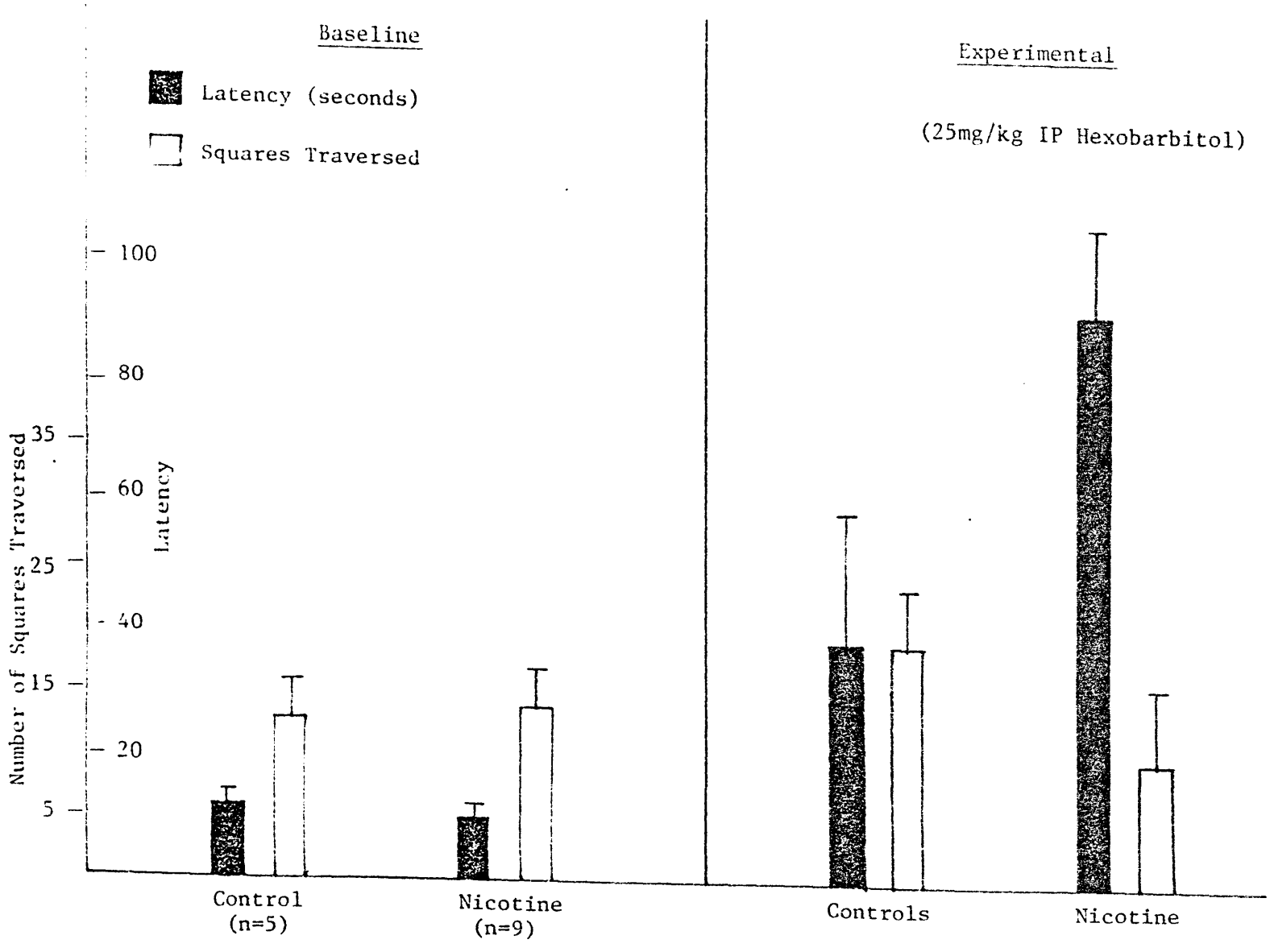

Baseline
Latency (seconds)
Squares Traversed
Experimental
(25mg/kg IP Hexobarbitol)
Number of Squares Traversed
100
80
60
40
20
35
25
15
5
Latency
Control
(n=5)
Nicotine
(n=9)
Controls
Nicotine

Figure X. Study III: Maternal Weights from Day 3 through Day 21 of Gestation.

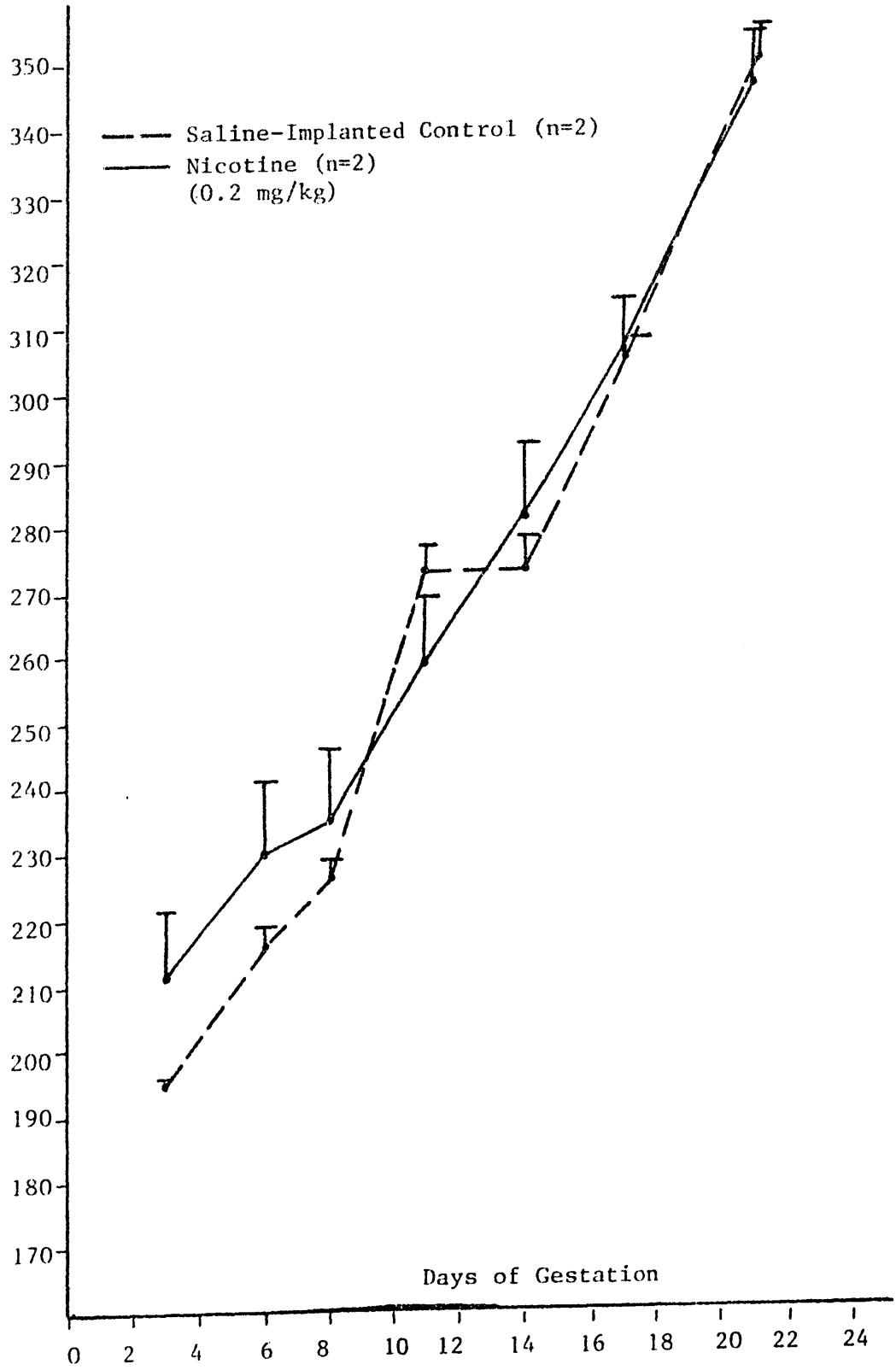

Saline-Implanted Control (n=2)
Nicotine (n=2)
(0.2 mg/kg)
350
340
330
320
310
300
290
280
270
260
250
240
230
220
210
200
190
180
170
Days of Gestation
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure .I. Study III: Maternal Liquid Diet Consumption from Day 4 through Day 18 of Gestation (Mean ± S.E.M. Consumption for 14 days per Rat).

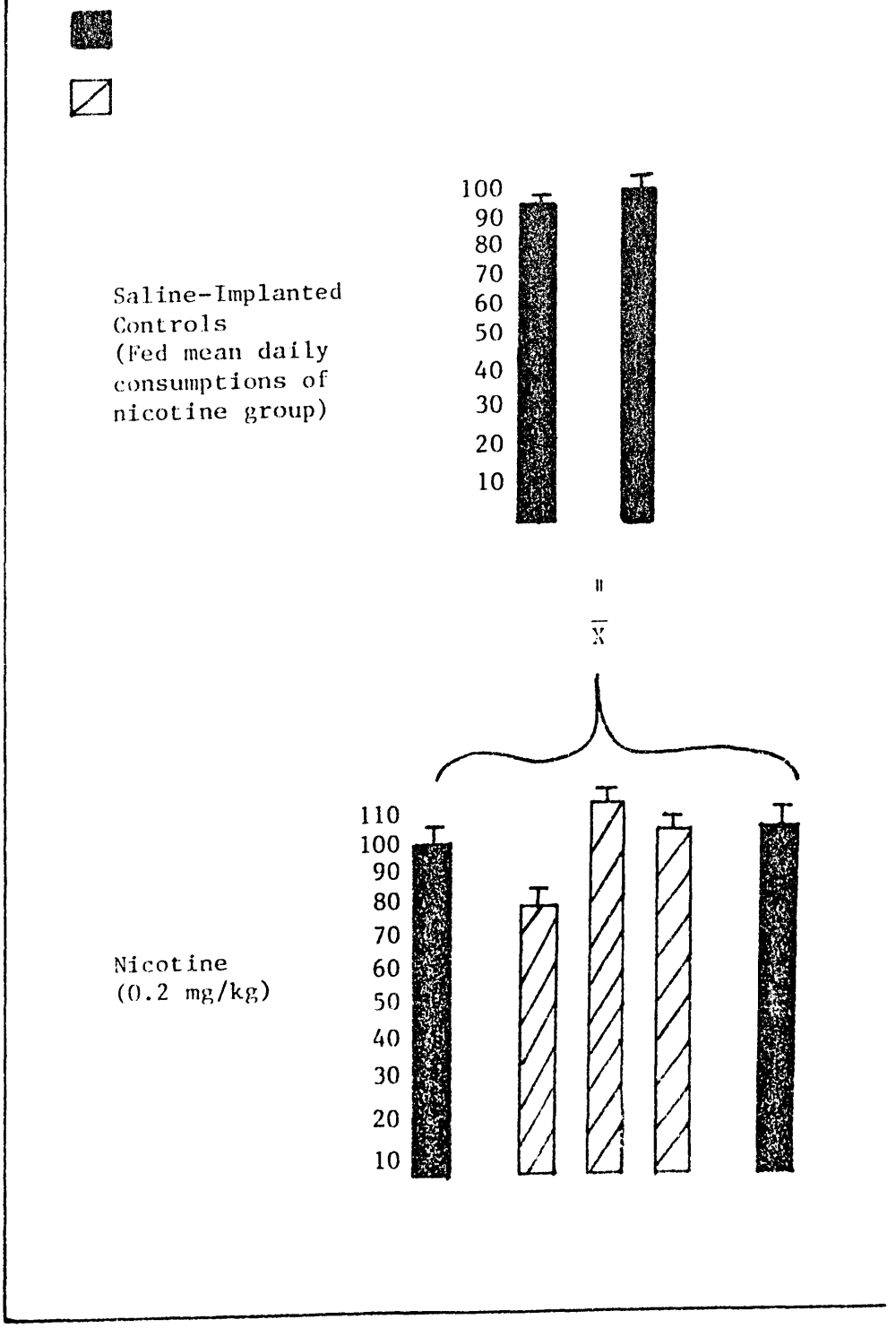

Saline-Implanted Controls (Fed mean daily consumptions of nicotine group)
100
90
80
70
60
50
40
30
20
10
X
Nicotine (0.2 mg/kg)
110
100
90
80
70
60
50
40
30
20
10

Figure XII. Study III: Weight Gains of Offspring
*p < .005, Welch's t.
**p < .001, Welch's t.

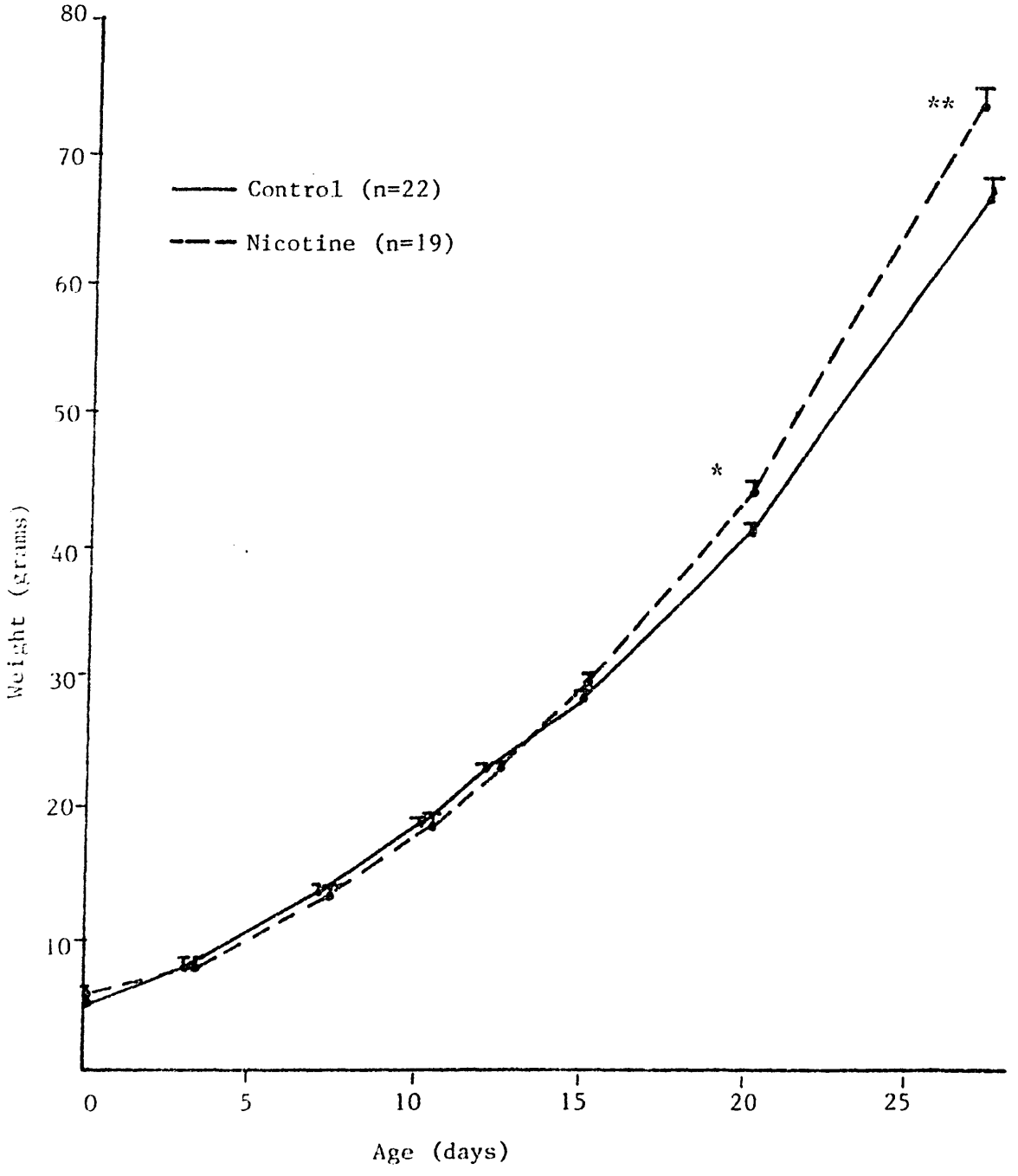

80
70
60
50
40
30
20
10
Weight (grams)
Control (n=22)
Nicotine (n=19)
*
**
0
5
10
15
20
25
Age (days)

Figure XIII. Study III: Hexobarbitol Sleeping Time Test (80 mg/kg IP) at Day 20 and 21.
*p < .003, Welch's t; Comparison of narcosis time for control offspring versus prenatally treated nicotine offspring.

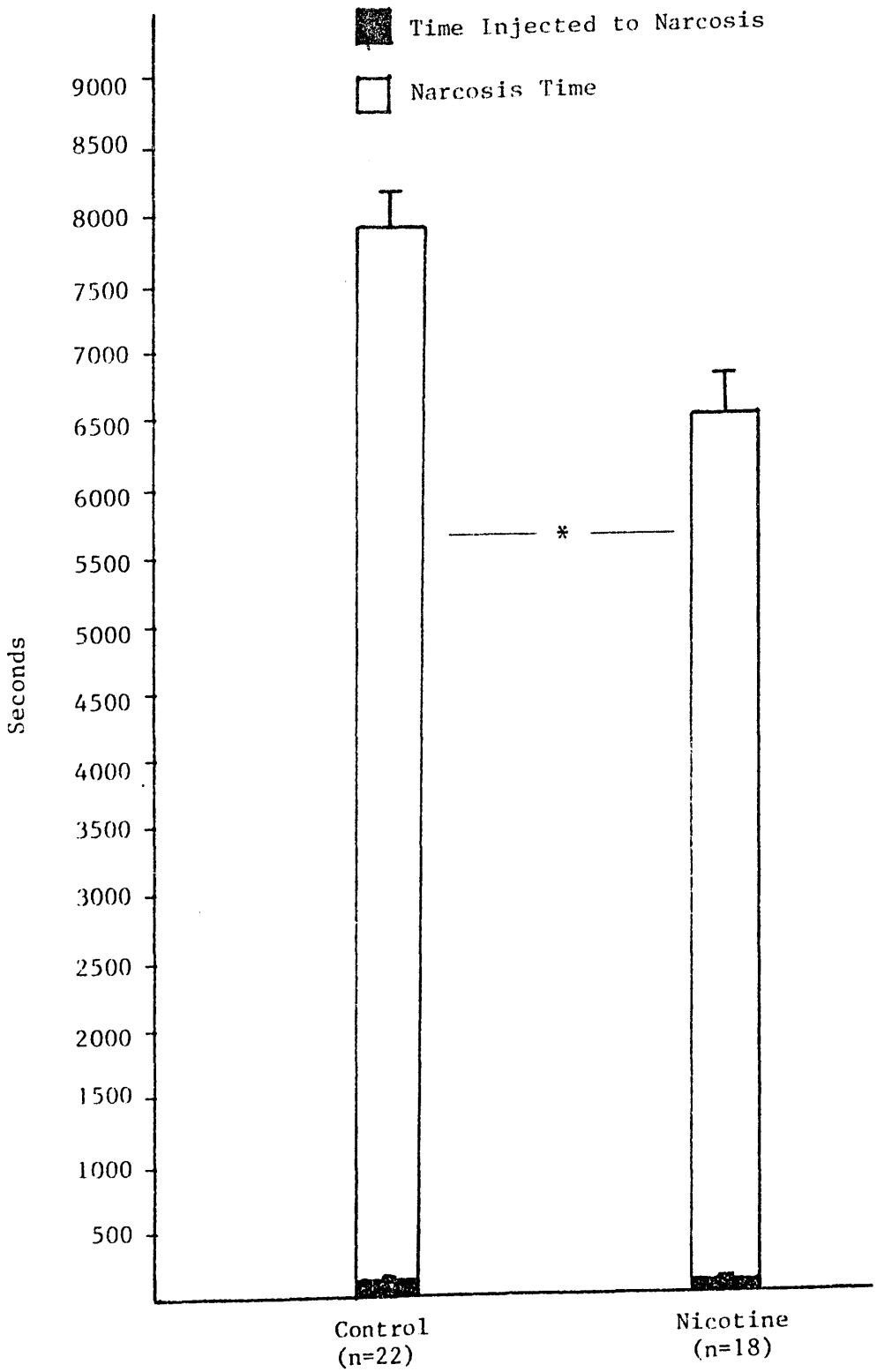
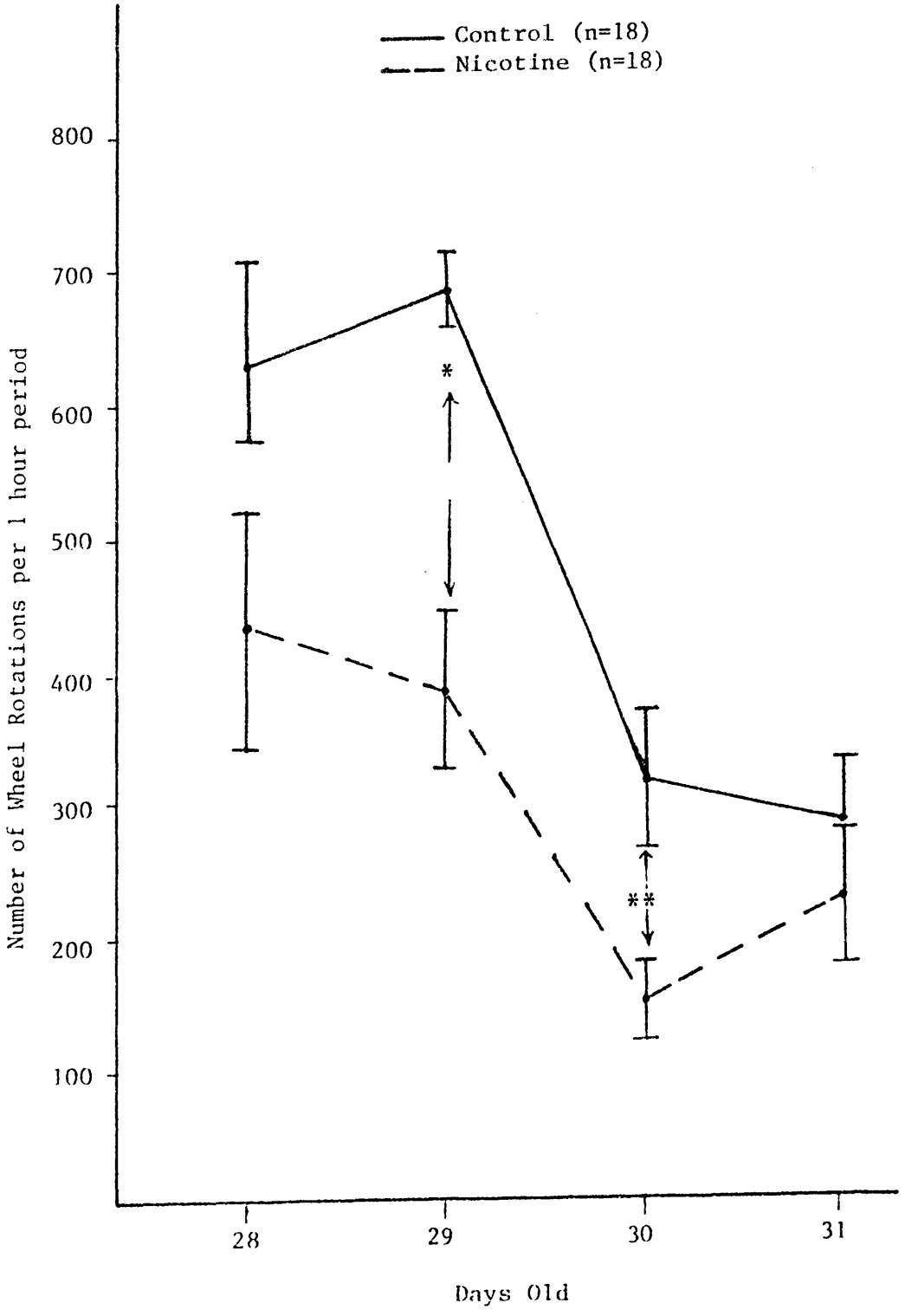

Time Injected to Narcosis
Narcosis Time
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
Seconds
*
Control
(n=22)
Nicotine
(n=18)

Figure XIV. Study III: Locomotor Activity of Control Offspring versus Nicotine Offspring on Day 28 through Day 31.

*p < .002, Welch's t; Day 29 comparisons of control offspring versus prenatally exposed nicotine offspring.

**p < .006, Welch's t; Day 30 comparisons of control offspring versus prenatally exposed nicotine offspring.


Control (n=18)
Nicotine (n=18)
800
700
600
500
400
300
200
100
Number of Wheel Rotations per 1 hour period
*
**
28
29
30
31
Days Old

CHAPTER VI

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