LIVER MALDEVELOPMENT IN THE FETAL ALCOHOL SYNDROME

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics
Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba

August, 1998

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Previous studies have documented that liver/body weight ratios and rates of hepatic DNA synthesis are decreased in a rat model of fetal alcohol syndrome (FAS). In an attempt to determine the mechanism(s) responsible for these findings, rates of liver maturation, liver histology, and the status of the growth hormone (GH) / insulin – like growth factor (IGF) / insulin - like growth factor binding protein (IGFBP) axis were documented in FAS pups during gestation and the post – partum period and compared with control animals. Pregnant Sprague – Dawley rats were fed a liquid diet containing ethanol as 36% of the total calories, an isocaloric control liquid diet, or had *ad lib* access to the control liquid diet throughout pregnancy. Dams were continued on their respective diets until weaning. Upon weaning, pups were fed control liquid diet until the time of sacrifice. Sacrifices were performed at gestational days 16 and 20 and post – partum days 1, 7, and 40. Albumin, alpha fetoprotein, growth hormone receptor, IGF - 1, IGF - II, and IGFBP - 1, -2, -3, - 4 mRNA were documented at each time point by Northern Blot Analyses. In 40 day old pups, serum glucose, insulin, glucagon and insulin sensitivity were also determined by commercial assays and a rapid insulin sensitivity test (RIST) respectively. The results of the study revealed that there was no consistent finding of premature maturation of the liver in FAS pups compared with controls. In terms of the GH/IGF/IGFBP axis, growth hormone receptor mRNA expression was decreased at post - partum day 40 (53%) while IGF – I mRNA expression was

decreased, IGFBP-3 mRNA expression increased, and there were no significant changes in IGFBP – I, - 2, or - 4 mRNA expression throughout pregnancy. In addition, FAS pups were hyperglycemic and displayed a 3.5 - fold decrease in serum insulin levels. The same rats also demonstrated severe insulin resistance (glucose clearance in FAS pups; 104 ± 11 mg/kg vs 320 ± 29 mg/kg in controls, mean SD, $p < 0.0001$). In conclusion, the results of this study do not provide an explanation for why liver development is impaired in FAS. However, the results do indicate that the hepatic GH/IGF/IGFBP axis and glucose homeostasis are significantly altered in FAS and these alterations are in keeping with a diabetic profile.

ACKNOWLEDGEMENTS

To begin, I'd like to thank my advisor, Dr. Gerald Y. Minuk. His endless patience, guidance, and support were invaluable, but what meant the most was his aid in cultivating my interest in a career in science. My experience with Dr. Minuk was especially rewarding in that he provided much insight into the clinical aspects of research in addition to my laboratory training, and as a result my education in the Liver Diseases Unit has bell very well rounded.

I would also like to thank Dr. YueWen Gong. Dr. Gong has provided me with a substantial background in molecular biology and I thoroughly enjoyed my time in the lab working with him. In addition, I greatly appreciate his time in evaluation of my thesis and his helpful comments.

I would like to thank Dr. Frank Burczynski for his time as a committee member on this thesis. Dr. Burczynski's careful criticism was very useful, and it was always presented in a unique manner - he helped to lighten the pressure near the end of this work.

I also thank the department of Pharmacology and Therapeutics at the University of Manitoba, in particular Dr. Ratna Bose. Dr. Bose's encouragement, advice, and motivation will always remain with me, and I look forward to some of the challenges put forward by her as a result of my time spent in the department. In addition, I would like to acknowledge Dr. Wayne Lautt and his laboratory for their assistance in the glucose and insulin estimations.

I must not forget my colleagues in the LDU. To my fellow students (past and present) - GanLu, Ari, Mike, and Ian - the experience of learning together has been very fulfilling. To the clinical staff Elaine and Rachel - I thank you for

hours of conversation and education, in particular the late nights of challenging each other over a variety of drug classifications. Finally, to my friend Manna - the experience of working with you and especially learning from you has been incredible - but perhaps even more rewarding has been your friendship and support.

Of course, to the Medical Research Council of Canada I give thanks for the financial support they have provided for the duration of this program and I look forward to further endeavors with the council.

To my parents, without whom none of this would be possible, I thank you. Your endless love and encouragement were always there and they helped me to pull through the rough times and celebrate the wonderful findings. Straggling home in the middle of the night after an experiment gone wrong was incredibly comforting, especially with Misty waiting patiently at the door.

Last but certainly not least, to my husband Brian. To think we both started our Master's together unengaged and here we are two years later, finished and married. The challenges we've faced were tough at times, but definitely not insurmountable and the fact that we've been through all of it together shows how dedicated we both are - both to each other, and to our craft. Without your support and understanding, the life of science would not have been half as enjoyable. Here's to our PhD's in Edmonton, future post doc's, and everything else G-d puts in our path.

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1. INTRODUCTION

1.0 ALCOHOL CONSUMPTION DURING PREGNANCY

There exist a number of historical references to the cautionary use of alcohol during pregnancy. "*Behold, thou shalt conceive, and bear a son; and now drink no wine or strong drink...*" (Judges 13:7). Alcohol consumption by a couple was forbidden on their wedding night in ancient Rome in fear of conception of imperfect children (Streissguth *et al.*, 1980). In 1726, the College of Physicians and Surgeons in London reported that consumption of alcohol during pregnancy will result in "weak, feeble and distempered children".

These early concerns regarding alcohol consumption during pregnancy have been echoed more recently as moderate consumption of alcohol becomes more socially acceptable and advocated as being cardioprotective. Indeed, recent population studies indicate that approximately 62% of the general population consume alcohol daily and 39% of these in child bearing ages (Statistics Canada, 1995).

Despite the longstanding awareness of the dangers of alcohol consumption during pregnancy, and the increasing research in the area, there is still much knowledge to be gained. For example, neither a safe amount nor

a safe time for consumption of alcohol during pregnancy has been established (Streissguth *et al.*, 1980; Persaud, 1988). Other unknown variables include the impact (if any) of differences in the ability of the mother to metabolize alcohol, rates of gastric emptying, paternal alcohol consumption prior to conception, contributions of the metabolites of alcohol to development, and functionality of the fetal hepatic metabolizing enzymes.

Typically, the fetus is dependent upon the mother's ability to metabolize alcohol for elimination of ethanol from it's own circulation. Therefore, if maternal metabolic function is altered, and ethanol rather than its metabolites is responsible for adversely affecting the fetus, fetal problems would be expected to ensue.

There is evidence to suggest that ethanol can adversely affect the fetus as early as the pre-implantation phase, almost immediately following fertilization (Kaufman, 1983). The mechanism appears to involve alcohol - induced chromosomal non-dysjunction in the female pronucleus, thus leading to aneuploidy of the fetus and resulting in spontaneous abortion.

There is a common misconception that alcohol consumption during later stages of pregnancy is safe because most phases of fetal development are completed, however studies have shown that alcohol consumption by a pregnant rat during late pregnancy (i.e. the equivalent of the second and third

trimesters in humans) results in altered neural development and CNS dysfunction (West and Hamre, 1985). In addition, there are further risks involved with the consumption of alcohol during lactation. For example, there is a vulnerable period of brain growth that occurs post-natally (Dobbing, 1976). As alcohol is present in the breast milk of women who consume alcohol during lactation, based on animal data, these infants may be at risk of acquiring problems in growth and development in the neonatal period.

Ethanol is capable of crossing the placental barrier between mother and fetus in approximately equal concentrations to those seen in the mother (Dilts, 1970; Jones *et al.*, 1973; Streissguth *et al.*, 1980; Abel, 1982). Of particular interest are the high levels of ethanol seen in the amniotic fluid surrounding the fetus. It is possible that the fetus is at additional risk of teratogenesis due to continuous exposure to the ethanol resulting from the constant exchange between the fetus and amniotic fluid (Persaud, 1988).

Despite a plethora of studies dealing with the effects of ethanol on fetal brain and skeletal growth, there is a relative paucity of data regarding the effects of alcohol consumption by a mother during pregnancy on the fetal liver. While some reports exist regarding liver weights, liver enzyme abnormalities, and the effects of ethanol on fetal hepatocyte cell cultures, there is a lack of information concerning the long term effects of alcohol consumption by a pregnant female on liver development and function in offspring exposed to

ethanol. In addition, although many attempts have been made to identify the mechanism of action that is responsible for altered brain and skeletal growth patterns in these offspring, there remains a lack of insight into the mechanism(s) that might apply to impaired liver development if it truly exists in the fetal alcohol syndrome.

1.1 FETAL ALCOHOL SYNDROME

In 1968 a group of French researchers under the guidance of Dr. Pierre Lemoine identified a specific constellation of birth defects in infants born to alcoholic mothers. This pattern of defects was reported soon thereafter in the United States and it was at this time the term Fetal Alcohol Syndrome (FAS) was applied.

1.1.0 Diagnosis

The diagnosis of FAS requires the combination of verification that the mother was consuming alcohol throughout her pregnancy along with a characteristic triad of malformations. This triad involves facial anomalies, brain damage and growth deficits. Facial anomalies typically involve the eyes (i.e. short palpebral fissures), nose (i.e. shortened/flattened bridge) and mouth (i.e. smooth philtrum, thin vermillion, cleft lip/palate). Brain damage is usually

indicated by the presence of microcephaly, irritability in the infant, hyperactivity, learning disabilities as the child ages, and mild to moderate mental retardation. The growth deficiencies affect both weight and length of the neonate. Often these deficiencies are present throughout the life of the individual (Moore and Persaud, 1993). Involvement of other organ systems in FAS has not been extensively studied.

Over the years there has been some crossover in identification of the complete FAS syndrome versus FAE - Fetal Alcohol Effects. Essentially, the absence of one of the features of FAS indicates the diagnosis of FAE. Regardless of the diagnosis - FAS vs. FAE, it is apparent that consumption of alcohol by a pregnant woman has potentially serious and irreversible effects on the unborn child. Some of the figures discussed below - i.e. incidence rates for the Yukon, British Columbia and Saskatchewan, are thought to reflect the occurrence of both FAS and FAE. With a more defined outline of the criteria for each condition, one can expect a clearer idea of the magnitude of the FAS problem. This is not to say that FAE is less serious than FAS, however the differentiation may serve to better indicate the various ways that alcohol consumption during pregnancy can impact on the fetus.

In short, the effects of FAS are widespread and, as indicated below, constitute a significant portion of the population in Canada. Efforts to better define the syndrome and consequences of the condition are essential to

improving our understanding and treatment of this disorder.

1.1.1 Prevalence

FAS is a leading cause of birth defects and mental retardation in children. On a global level, the incidence of FAS is approximately 1 case per 1,000 live births. In North America, the incidence of FAS is 1.9 cases per 1000 live births, with incidences as high as 27/1000 in Saskatchewan, 25/1000 in British Columbia and 46/1000 in the Yukon (Figures courtesy of Z. Lisakowski, Addictions Foundation of Manitoba). It has been suggested that the unequal distribution is due to differences in the alcohol consumption of the populations studied at the various sites. However, recent evidence from the Addictions Foundation of Manitoba has suggested otherwise in that the figures from Saskatchewan, British Columbia and the Yukon were random urban samples, without a cultural bias present. A yet unpublished study by Chudley and Moffat at the University of Manitoba has identified the presence of FAS in 10% of a study population - those examined were between the ages of 5 and 15.

1.1.2 Natural History of FAS

Reflecting the neurological damage in individuals born with FAS, newborns typically demonstrate irritability, tremulousness, seizure activity, poor sucking ability, and hyperacusity (Clarren and Smith, 1978). These

characteristics may persist for months after birth. As the child ages, signs of altered cerebellar function appear as do hypotonicity and hyperactivity. Abnormal electroencephalograms have been reported in children with FAS. Impaired intellectual performance appears to intensify as the child ages (Jones et al., 1974).

The growth deficits seen at birth are often present throughout the life of the individual and catch - up growth is infrequent. Those rare instances where children display normal prenatal growth patterns are followed by deficiencies in growth as time progresses. As FAS children develop, decreases in adipose tissue become prominent.

The facial dysmorphology produced by exposure to ethanol *in utero* is evident in the newborn and persists thereafter.

1.1.3 Treatment

Perhaps reflecting the lack of understanding of the mechanisms involved, there are no known treatments for FAS other than discouraging alcohol consumption by pregnant females. Public educational awareness groups and advertisements are promoted. For established FAS cases, attempts are made in the school systems to manage the attention deficits and hyperactivity that are associated with FAS. Corrective surgery has been used to

correct some of the facial anomalies (i.e. cleft lip/cleft palate).

1.2 LIVER DEVELOPMENT

In humans, liver development begins very early in the first trimester from the caudal portion of the foregut. The liver then undergoes a rapid growth phase. Haematopoiesis begins during the sixth week of gestation and accounts for the large size of the liver relative to body mass at this stage of fetal development (Moore and Persaud, 1993, pg. 243). By the ninth week of gestation, the liver represents approximately ten percent of the total fetal weight - however this ratio is reduced by the time of birth to approximately two percent of the baby's birth weight. In the rodent, liver development begins at approximately 9 days of gestation and undergoes rapid growth between days 11 and 15.

A number of factors are involved in liver development - hormonal, autocrine and paracrine. Although much effort has been expended in the past decade in an effort to identify these factors, a clear understanding of the regulatory mechanisms involved in liver development has yet to be developed. More recently, efforts have focused on which genes are being expressed during development and the precise stage of their expression (Zaret, 1996). Although there is a substantial amount of information concerning anatomical changes during development and inductive events (i.e. resulting from homeobox genes),

little is known about the primary events in the genetic control of liver development.

Polyamines are polyvalent cations that are considered to be essential to liver development (Tabor and Tabor, 1984). The principle polyamines include putrescine, spermidine, and spermine. These and other polyamines are formed as the result of decarboxylation of ornithine. In their absence, DNA and protein synthesis following partial hepatectomy is markedly impaired (Luk, 1986). Ornithine decarboxylase (ODC) is the rate - limiting enzyme in the biosynthetic pathway of polyamines and levels of this enzyme are known to increase following stimulation of liver growth/regeneration. Previous studies have identified elevated levels of polyamines and ODC in fetal rat livers when compared to levels in adults (Eguchi et al., 1992). The effects of ethanol consumption during pregnancy on fetal liver polyamines has only recently been addressed (Sessa and Perin, 1997). Diamine oxidase, an enzyme responsible for regulating tissue concentrations of polyamines, is decreased in fetal rats exposed to ethanol in utero.

Another system that is thought to be of particular importance in fetal liver development is the growth hormone (GH) / insulin - like growth factor (IGF) / insulin - like growth factor binding protein (IGFBP) axis. This axis serves to mediate somatic growth and development in general and as such, may also be involved in liver development.

GH is the primary regulator of IGF-I synthesis, and this regulation is mediated by the binding of GH to its specific receptor - the growth hormone receptor (GHR) which is a transmembrane receptor in the liver.

The IGFs are important for the regulation of DNA synthesis, cell proliferation, protein synthesis and glucose uptake/metabolism in most organs (Le Roith, 1997; Baxter, 1995; Sara and Carlsson-Skwirut, 1986). Both IGF-I and IGF-II play a role in embryonic development. In the case of IGF - I, the effect on development is largely in the post - natal period, while IGF - II's main developmental effects are seen during gestation (Evain-Brion, 1994). Both are peptide hormones with approximately 50% of their amino acid sequence in common with each other and with insulin. Both have insulin-like actions (Le Roith, 1997; Baxter, 1995).

Aside from the various similarities that are shared by IGF - I and IGF - II, there are also well established, distinct roles played by each growth factor. IGF - I is regulated by GH, insulin, and nutrition. In addition to its different mode of regulation, IGF - I has been shown to have a prominent function in glucose homeostasis (Baxter, 1995). For example, studies have reported the inhibition of hepatic glucose output as a result of IGF - I infusion, however, the mechanism for this blockade remains to be identified. Interestingly, reduced levels of IGF - I have been reported in patients with cirrhosis of the liver and/or those who have been subjected to nutritional deprivation - conditions

associated with insulin resistance and hyperglycemia.

There have been reports describing the activity of IGF - I in the rat during liver regeneration (Russell, 1985). Specifically, a drop in IGF - I concentration was seen, largely as a consequence of decreased consumption of food rather than the decreased liver mass resulting from partial hepatectomy.

IGF-II is regulated by placental lactogen and nutrition and is important for prenatal development. IGF-II mRNA is more abundant during embryonic development than IGF-I. In the liver, mRNA transcripts of IGF-II have been identified - band sizes of 4.0, 2.4, 1.75 and 1.25 kb were detected in 4 day old rats. Levels of mRNA expression then decreased as the rat aged. Many investigators have reported a distinct drop in expression of IGF - II in the adult rat (Beck et al., 1988). It has a been suggested that the specific presence of IGF - II in the liver may reflect synthesis and release of IGF - II during embryogenesis. This may therefore contribute an endocrine function to IGF - II in addition to its established autocrine/paracrine functions during development (Bondy *et al.*, 1990; Stylianopoulou *et al*, 1988).

IGF-I and IGF-II circulate at higher concentrations than insulin (i.e. in nanomolar concentrations). They primarily circulate bound to one of eight identified IGFBPs known to modulate the activity of the IGFs (Le Roith, 1997). IGFs and their binding proteins are synthesized in the liver, and to a lesser

extent in other tissues.

IGFBPs bind to the growth factors and influence their ability to exert their tissue - specific effects. IGFBPs 1 and 3 have a higher affinity for IGF-I and IGFBPs 2 and 4 for IGF-II. In some instances their binding augments the effect of the IGF and in others it is attenuated. How this differential effect occurs is unclear.

1.3 ETHANOL AND LIVER DEVELOPMENT

Liver growth is significantly decreased following consumption of alcohol (Sessa and Perin, 1997; Minuk *et al.*, 1995; Diehl *et al.*, 1988; Luk 1986; Duguay *et al.*, 1982). In the 70% partial hepatectomy model, the regeneration that occurs in the ethanol - consuming rats is significantly lower than that seen in controls. From these and other studies, it is evident that alcohol seriously inhibits the ability of the liver to recover from injury/trauma.

The inhibitory effects that ethanol has on the regenerative activity of the liver raises the question of whether alcohol consumption by a pregnant female would impair the development of fetal liver and if so, by what mechanism(s).

As mentioned previously, there are numerous studies reporting the effects of ethanol consumption during pregnancy on fetal body growth patterns

and brain development, however, there is limited information concerning the effects of ethanol on other organ systems. Those studies that have been published include reports of decreased liver weights in the fetus and newborn as a result of chronic ethanol consumption by the mother during gestation (Henderson *et al.*, 1979; Pullen *et al.*, 1988; Buts *et al.*, 1992). There have also been reports identifying the effects of maternal ethanol consumption on fetal microsomal enzymes (Rawat 1976; Buts *et al.*, 1992). Finally, there have been studies documenting decreased replication of hepatocytes in tissue culture as a result of ethanol treatment (Devi *et al.*, 1993). However, important aspects and / or inconsistencies in these studies remain to be resolved, for example, in the majority of these studies ethanol administration was restricted to the gestational period of life and therefore, the experimental design did not reflect the human situation where consumption during the weaning period also occurs. An additional factor is that the potential long term effects of ethanol on the liver were not addressed. Finally, the *in vivo* effects of ethanol on hepatocyte replication were not described.

Recent data from our own laboratory has established that liver development is indeed altered in rat pups with FAS (submitted). Specifically, in those pups exposed to ethanol from the time of conception (in maternal blood) until the time of weaning (in maternal breast milk), liver weights and rates of hepatic DNA synthesis were significantly lower than in pups born of dams fed isocaloric control diets (Figures 1 and 2). Although the mechanism(s)

responsible for these effects was not identified, it did not appear to involve disturbances in hepatic polyamine levels as ODC activity was not significantly altered in FAS pups when compared to controls.

Regarding the effects of ethanol on IGF expression, previous studies have shown that chronic exposure to ethanol not only results in decreased expression and plasma concentrations of IGF-I in adult rats (Sonntag and Boyd, 1988), but also in rats exposed to ethanol in utero. The latter indicating the sensitivity of these growth factors prenatally. In addition, pregnant rats exposed chronically to low levels of ethanol have produced offspring with reduced hepatic mRNA expression for IGF-I (Breese and Sonntag, 1995). Low levels of plasma IGF-I appear to occur concurrently with deficits in somatic growth as a result of ethanol exposure in utero. There has also been a study identifying specific changes in hepatic IGF-I and IGF-II mRNA expression in rats exposed to ethanol during gestation when compared with controls (Singh *et al.*, 1994). In this study IGF-I and IGF-II mRNA expression was decreased in the livers of ethanol exposed fetuses at gestational day 22 of age.

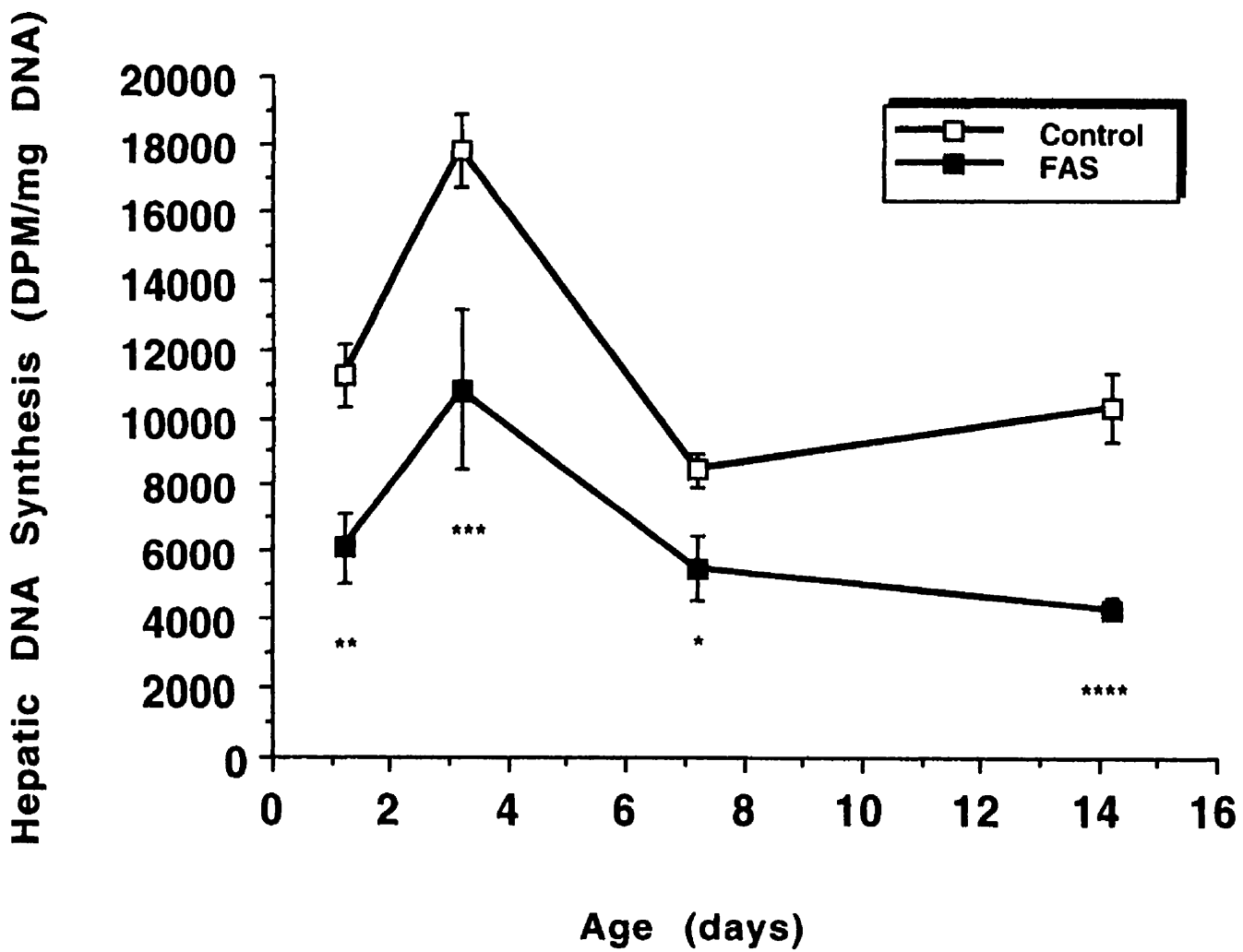
Hepatic DNA Synthesis (DPM/mg DNA)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
16
Age (days)
Control
FAS
**
***
*
****

Figure 1: Hepatic DNA synthesis in FAS pups and *Ad lib* controls at various times following birth. Data points represent the mean and error bars the standard error of the means.

* $p<0.05$ ** $p<0.005$ ***$p<0.001$ **** $p<0.0005$ versus controls as determined by ANOVA.

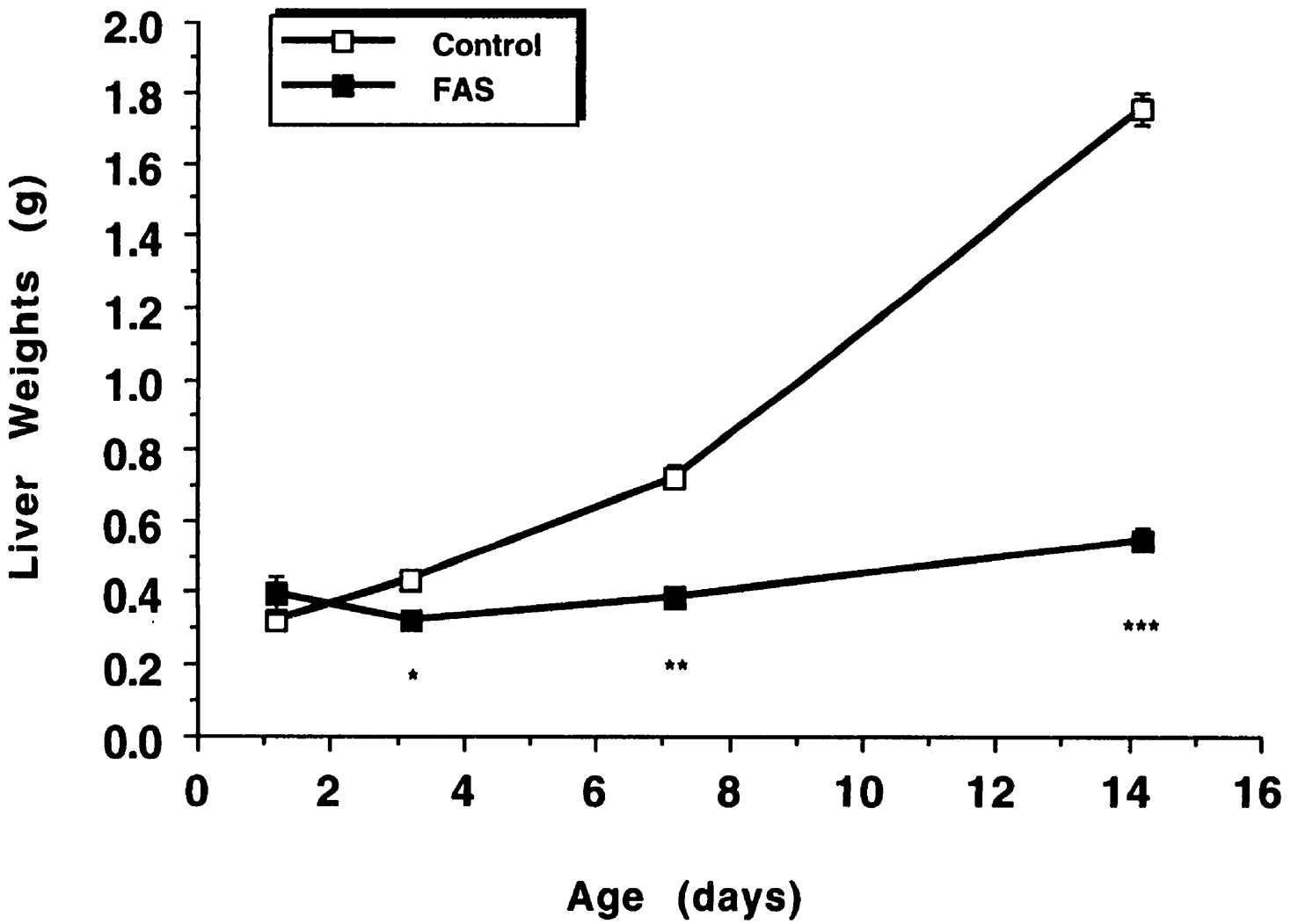
Control
FAS
Liver Weights (g)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
*
**
***
Age (days)

Figure 2: Liver weights measured at various times after birth in FAS pup and *Ad lib* controls. Data points represent the mean liver weights of pups and error bars the standard error of the means.
* $p<0.05$ ** $p<0.005$ *** $p<0.001$
versus controls as determined by ANOVA.

The principle objective of this thesis was to identify the mechanism(s) whereby liver development is impaired in FAS. Plausible mechanisms that were considered included; a) premature maturation of the developing liver, and b) modification in the GH / IGF / IGFBP axis.

1.4 HYPOTHESIS

It is hypothesized that, in an animal model of FAS, the impaired liver development that occurs reflects ethanol-induced premature maturation of the liver and/or disturbances in the GH / IGF / IGFBP axis.

2. APPROACH TO TESTING THE HYPOTHESIS

Prior to testing this hypothesis, it is important to review the validity of the animal model of FAS to be employed and the markers of liver development and maturation that presently exist.

2.0 ANIMAL MODEL OF FAS

An appropriate animal model facilitates the regulation of many factors that may interfere with identifying true consequences of the treatment - for example one is capable of controlling the amount of ethanol provided, the nutritional status of the animal, and the use of other drugs that may potentially mimic effects that are due to ethanol (Streissguth *et al.*, 1980). Additional benefits of an appropriate animal model include the ability to evaluate conditions in a much shorter period of time - for example the use of rats where gestation is approximately 21 days versus 9 months in humans. Rodents have been established as valid animal models of development owing to their correlation in developmental patterns with the human situation.

Specific factors and conditions must be present to accurately develop a suitable model for studying the effects of maternal ethanol consumption on the liver in the developing fetus. Authorities in the field of teratogenesis relating to ethanol exposure identify the need for the following measures: a) animals should voluntarily consume the ethanol, b) it should be available to them at all times to maintain consistent ingestion, c) to mimic the human condition the caloric intake must consist of at least 25% ethanol, d) the animal should display physical addiction to the ethanol, and e) the maintenance of nutrition and caloric intake is essential (Persaud, 1988). Also important in

evaluating the consequences of FAS is an accurate duplication of the human condition. Thus, maintenance of ethanol provision following birth is important - i.e. during lactation.

2.1 MARKERS OF LIVER DEVELOPMENT AND MATURATION

Two important liver specific genes that are associated with stages of liver development are albumin and α-fetoprotein (AFP) (Panduro *et al.*, 1987).

Albumin mRNA expression in the liver is apparent early in gestational life - approximately day 10 in the rodent, transcription gradually increases throughout gestation and after birth, reaching maximal levels between weeks 8-12 of postnatal life (Arias *et al.*, 1994, p.88).

The pattern of expression for AFP differs somewhat from that of albumin. AFP mRNA is first expressed at the early stage of liver development where the liver bud/hepatic diverticulum arises from the foregut (i.e. approximately the 9th day of gestation), (Arias *et al.*, 1994, p.4). Unlike albumin, levels of AFP mRNA expression decrease as the time of birth approaches, then increase slightly in the newborn and subsequently decline further in postnatal life until they are virtually undetectable in the adult (Gil *et al.*, 1996; Moorman *et al.*, 1990).

3. MATERIALS AND METHODS

3.0 MATERIALS

The following are a list of the materials used in the study and the source from which they were obtained: [^{32}P]deoxycytidine triphosphate ([^{32}P]-dCTP) from DuPont (Boston, Massachusetts). Diets: Control Liquid Rat Diet Preg-LD '82 High Protein F1264 SP and Ethanol Liquid Rat Diet Preg-LD '82 High Protein F1265 SP obtained from BioServ (Frenchtown, NJ). Adult Sprague - Dawley rats from Central Animal Care Services, University of Manitoba (Winnipeg, Canada). GT-Zeta probe nylon membranes and UV cross - linker Gene Linker UV Chamber from Bio-Rad (Mississauga, Ontario, Canada and Hercules, California, USA respectively). DH5α *E. coli* bacteria, Lenox L Broth Base (LB broth), λ HindIII DNA standard, the restriction enzymes HindIII, PstI, EcoRI, the restriction enzyme buffers React 2, React 3, and olb, klenow fragment (large fragment DNA polymerase I) (Random Primers DNA Labelling System) from GIBCO BRL (Burlington, Ontario, Canada). pZ523 G25 Sepahdex Columns from 5-3 PRIME Inc. (Boulder, Co.). The restriction enzyme SacI, and buffer One Phor - All, and Sephadex G-50 DNA grade nick columns from Pharmacia Biotech (Oakville, Ontario, Canada). GeneClean II from Bio/Can Scientific (Mississauga, Ontario). IGFBP-2 from Matt Rechler (NIH, Bethesda, Maryland, USA). Rat growth hormone receptor cDNA from Genentech (San

Francisco, California). IGF-II cDNA from Dr. L. Murphy (University of Manitoba, Winnipeg, Canada) (band sizes correlated with those reported by Beck *et al.*, 1988), Albumin, IGF-I and α-fetoprotein cDNA from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). Glucagon RIA kit from Linco (St. Charles, Mo., USA). Insulin RIA kit from INCSTAR (Stillwater, Minnesota, USA). National Institutes of Health Image 1.6 Densitometric Analysis Program from the National Institutes of Health (Bethesda, Maryland).

3.1 ANIMAL CARE AND TREATMENT

This study was approved by the Central Animal Care Services Review Committee at the University of Manitoba.

Timed pregnant Sprague Dawley female rats (180 - 220 grams) were received at Central Animal Care Services in the Basic Medical Sciences Building at the University of Manitoba on day 1 of pregnancy. The selection of rats for this study was based on the fact that pervious studies in the area were performed using this animal, and the preliminary data, oweing to the large number of animals required and the correlation in terms of development with the human situation, made the rat the most feasible choice. The animals were maintained in a temperature controlled environment (24°C) with a 12 hour

light/dark cycle. Rat gestation is on average 21 days in length from the time of fertilization to the time of birth. Based on an average litter size of twelve pups, dams were randomly assigned to the following groups: ethanol liquid diet throughout pregnancy, pair fed isocaloric control liquid diet throughout pregnancy, and *ad libitum* control liquid diet throughout pregnancy (Tables I, II). Pups born to dams who consumed the ethanol liquid diet are referred to as FAS pups/animals, those born to dams who consumed the control formulation *ad libitum* are Ad lib animals, and those born to dams who consumed the diet isocalorically are referred to as isocal animals. The diets were prepared fresh daily and were the only source of calories for the animals. An adaptation period of 2-3 days was used to introduce the ethanol into the diet in increasing concentrations until the full proportion of ethanol to diet was achieved. The ethanol liquid diet contained ethanol as 36% of the total calories - which was replaced by carbohydrate in the control formulation. All dams were continued on the diets being administered at the time of delivery in the post-partum period until weaning which occurs at approximately day 22 of age. The pregnant dams were housed in individual cages with their corresponding diet available in addition to tap water *ad libitum*.

Rat fetuses were removed at gestational days 16 and 20 via abdominal incision in the mother following ether anaesthesia. The fetuses were examined for malformations and then weighed. Following decapitation, fetal trunk blood was collected, centrifuged and stored at -20°C for future analyses. Fetal livers

were rapidly excised, weighed, snap frozen in liquid nitrogen and stored at -70°C. Pups were also sacrificed at post-partum days 1, 7, and 40 via decapitation. Once again, blood was collected, centrifuged and stored at -20°C. Livers were rapidly excised, weighed, frozen in liquid nitrogen and stored at -70°C. In all pups, visual examination of craniofacial features, and measurements of brain weights and crown - rump lengths were performed.

AMINO ACID PROFILE (gm/L)

Alanine	1.5	Lysine	4.09
Arginine	2.07	Methionine	1.78
Aspartic Acid	3.57	Phenylalanine	2.53
Cystine	0.82	Proline	5.64
Glutamic Acid	11.23	Serine	3.17
Glycine	1.38	Threonine	2.48
Histidine	1.55	Tryptophan	0.86
Isoleucine	3.05	Tyrosine	3.17
Leucine	4.61	Valine	3.63

CARBOHYDRATE PROFILE (gm/L)

Monosaccharides	31	Trisaccharides	17
Disaccharides	22	Polysaccharides	89

FATTY ACID PROFILE (gm/L)

C_4	Butanoic	-	C_{17}	Heptadecanoic	-
C_6	Hexanoic	-	$C_{17:1}$	Heptadecaenoic	-
C_8	Octanoic	-	C_{18}	Stearic	0.32
C_{10}	Decanoic	-	$C_{18:1}$	Oleic	6.18
$C_{10:1}$	Decenoic	-	$C_{18:2}$	Linoleic	4.76
C_{12}	Lauric	-	$C_{18:3}$	Linolenic	0.07
$C_{12:1}$	cis-9-dodecenoic	-	C_{20}	Eicosanoic	0.02
C_{14}	Myristic	2.70*	$C_{20:1}$	cis-11-Eicosenoic	0.01
$C_{14:1}$	cis-9-tetradecanoic	-	$C_{20:4}$	Arachidonic	-
C_{15}	Pentadecanoic	-	C_{22}	Docosanoic	-
$C_{15:1}$	cis-9-Pentadecenoic	-	$C_{22:1}$	cis-13-Docosenoic	-
C_{16}	Palmitic	1.88	$C_{22:2}$	Docosadienoic	-
$C_{16:1}$	cis-9-Hexadecenoic	.15	C_{24}	Tetracosanoic	-

MINERAL PROFILE (gm/L)

Aluminum	-	Manganese	14.60*
Calcium	1.02	Phosphorus	0.81
Chlorine	0.39	Potassium	0.94
Copper	1.50*	Selenium	0.03*
Fluorine	0.25*	Sodium	0.26
Iodine	0.07*	Sulfur	0.25
Iron	8.80*	Zinc	8.34*
Magnesium	0.13	Chromium	0.88*

VITAMIN PROFILE (mg/L)

Ascorbic Acid	-	PABA	10.00
Biotin	0.05	Pyridoxine	1.75
Cal. Pantothenate	4.00	Riboflavin	1.50
Choline	200.00	Thiamine	1.50
Folic Acid	5.0	Vitamin A (IU)	3,000
Inositol	30.00	Vitamin D (IU)	400
Menadione	30.00**	Vitamin E (IU)	30
Niacin	7.50	Vitamin B12	10.00**

PROXIMATE PROFILE (%)

Protein	22.5	Carbohydrates	62.7
Fat	5.30	Fiber	4.04
Ash	3.21	Moisture	4.00

CALORIC PROFILE (kcal/L)

Protein	246
Fat	119
Carbohydrate	635
Total	1000

INGREDIENTS: Maltose Dextrin, Casein, Fiber, Olive Oil, Salt Mixture, Safflower Oil, Corn Oil, Vitamin Mix, Suspending Agent, Choline, L- Cystine, DL-Methionine

Table I: Analysis of Liquid Rat Diet Preg-LD '82 High Protein - Control

* mg

** mcg

AMINO ACID PROFILE (gm/L)

Alanine	1.5	Lysine	4.09
Arginine	2.07	Methionine	1.78
Aspartic Acid	3.57	Phenylalanine	2.53
Cystine	0.82	Proline	5.64
Glutamic Acid	11.23	Serine	3.17
Glycine	1.38	Threonine	2.48
Histidine	1.55	Tryptophan	0.86
Isoleucine	3.05	Tyrosine	3.17
Leucine	4.61	Valine	3.63

CARBOHYDRATE PROFILE (gm/L)

Monosaccharides	29.1	Trisaccharides	-
Disaccharides		Polysaccharides	39.9

FATTY ACID PROFILE (gm/L)

C_4	Butanoic	-	C_{17}	Heptadecanoic	-
C_6	Hexanoic	-	$C_{17:1}$	Heptadecaenoic	-
C_8	Octanoic	-	C_{18}	Stearic	0.32
C_{10}	Decanoic	-	$C_{18:1}$	Oleic	6.18
$C_{10:1}$	Decenoic	-	$C_{18:2}$	Linoleic	4.76
C_{12}	Lauric	-	$C_{18:3}$	Linolenic	0.07
$C_{12:1}$	cis-9-dodecenoic	-	C_{20}	Eicosanoic	0.02
C_{14}	Myristic	2.70*	$C_{20:1}$	cis-11-Eicosenoic	0.01
$C_{14:1}$	cis-9-tetradecanoic	-	$C_{20:4}$	Arachidonic	-
C_{15}	Pentadecanoic	-	C_{22}	Docosanoic	-
$C_{15:1}$	cis-9-Pentadecenoic	-	$C_{22:1}$	cis-13-Docosenoic	-
C_{16}	Palmitic	1.88	$C_{22:2}$	Docosadienoic	-
$C_{16:1}$	cis-9-Hexadecenoic	.15	C_{24}	Tetracosanoic	-

MINERAL PROFILE (gm/L)

Aluminum	-	Manganese	14.60*
Calcium	1.02	Phosphorus	0.81
Chlorine	0.39	Potassium	0.94
Copper	1.50*	Selenium	0.03*
Fluorine	0.25*	Sodium	0.26
Iodine	0.07*	Sulfur	0.25
Iron	8.80*	Zinc	8.34*
Magnesium	0.13	Chromium	0.88*

VITAMIN PROFILE (mg/L)

Ascorbic Acid	-	PABA	10.00
Biotin	0.05	Pyridoxine	1.75
Cal. Pantothenate	4.00	Riboflavin	1.50
Choline	200.00	Thiamine	1.50
Folic Acid	5.0	Vitamin A (IU)	3,000
Inositol	30.00	Vitamin D (IU)	400
Menadione	30.00**	Vitamin E (IU)	30
Niacin	7.50	Vitamin B12	10.00**

PROXIMATE PROFILE (%)

Protein	31.12	Carbohydrates	43.52
Fat	8.32	Fiber	6.16
Ash	4.90	Moisture	4.00

CALORIC PROFILE (kcal/L)

Protein	251
Fat	120
Carbohydrate	274
Ethanol	355
Total	1000

INGREDIENTS: Maltose Dextrin, Casein, Fiber, Olive Oil, Salt Mixture, Safflower Oil Corn Oil, Vitamin Mix, Suspending Agent, Choline, L-Cystine, and DL-Methionine

Table II: Analysis of Liquid Rat Diet, Preg-LD '82 High Protein - Ethanol

* mg

** mcg

3.2 RNA ISOLATION

Total RNA was isolated from liver tissues as previously described using the Lithium Chloride (LiCl) / Urea method (Auffray and Rougeon, 1980). Briefly, 0.5 - 1.0 gram of tissue was homogenized with 3M LiCl/6M Urea for one minute. The homogenates were stored at -20°C for 0.5 hours to allow the RNA and LiCl to form small pellets. Samples were spun at 110,000 x g for 20 minutes at 4°C. The supernatants were removed and discarded, and the pellets air dried. Pellets were then dissolved in 1.0 ml 0.1% Sodium Dodecyl Sulfate (SDS) / 0.2 mM EthyleneDiamineTetraAcetic acid (EDTA) and the solutions were transferred to tubes containing 1.0 ml phenol and 1.0 ml chloroform. After extraction with phenol/chloroform, samples were spun for a further 10 minutes at 3800 x g. The organic phases were removed and discarded and 1.0 ml of chloroform was added to each aqueous phase. This was followed by centrifugation at 3800 x g for 5 minutes. The resultant upper aqueous phases were transferred to new tubes containing 125 µl 3M Sodium Acetate (NaAc) / Acetic Acid (HAC) pH 5.2 and 2.0 ml ice cold 100% ethanol. These were stored overnight at -70°C. Samples were then spun at 3800 x g for 45 minutes. Supernatants were removed and the pellets were dissolved in 300µl sterile double distilled water (d_2H_2O). The optical densities at a wavelength of 260 nm (OD_{260}) were determined and the remainder of the solutions were stored at -70°C overnight with 3M Sodium Acetate (Na/HAC) pH 5.2 and ethanol to precipitate the RNA. Samples were then microfuged at

4°C at 158,000 x g for 20 minutes and placed on ice while the supernatants were removed and discarded. The pellets were dissolved in sterile d_2H_2O to a concentration of 5μg/ul. Based on the OD_{260} measurements of these solutions, the necessary volumes required to load 20μg of total RNA were determined.

3.3 DETERMINATION OF DNA AND RNA CONCENTRATION

DNA and RNA determinations were performed as described previously (Sambrook *et al.*, 1989). Briefly, solutions that contain 50 μg/ml of double stranded DNA will have an absorbance of 1 at an optical density of 260nm, and a solution containing 40μg/ml of RNA has an absorbance of 1 at an OD of 260nm. Thus the concentrations of the nucleic acids are performed as:

For double-stranded DNA,

Concentration of DNA (μg/μl) = $Absorbance_{260nm}$ x 50 x dilution factor ˆ 1000

For RNA,

Concentration of RNA (μg/μl) = $Absorbance_{260nm}$ x 40 x dilution factor ÷ 1000

3.4 AGAROSE GEL ELECTROPHORESIS

Electrophoresis of agarose gels was used to separate DNA fragments

based on size for subsequent purification and preparation of probes.

Solutions:

1 X TAE (Tris/Acetic Acid/EDTA) Buffer: 0.04M Tris base, 0.02M glacial acetic acid, 1mM disodium EDTA, pH to 7.2 with acetic acid

Loading Buffer: 0.125% Bromophenol Blue, 0.125% Xylene cyanole, 12.5% Ficoll

Molecular Weight Marker: λ HindIII DNA standard & 1kb DNA marker

1.0 g Agarose was mixed with 100 ml 1 X TAE to make 1% agarose gels. The solution was boiled to melt the agarose and cooled to approximately 45°C and 10μl of 10 mg/ml ethidium bromide was added. The solution was then poured into a plastic gel tray, sealed and prepared with combs for setting of a gel slab.

Sample and loading buffer were mixed and loaded into the wells. The samples were electrophoresed in 1 X TAE buffer for one hour at 100V for separation of DNA bands. An ultraviolet light transilluminator was used for visualization of the DNA bands to document appropriate separation.

3.5 RECOVERY OF DNA FROM AGAROSE GELS

A Geneclean II kit was used for purification of DNA from the agarose gels. Essentially, following electrophoresis, the DNA bands of interest (determined by known molecular weights) were excised from the agarose gel using a scalpel during visualization with UV light. The gel fragments were then placed in 1.5 ml microfuge tubes and weighed. 6M Sodium iodide at a volume twice that of the gel fragment was added to each tube and the solutions were incubated at 65°C for 5 minutes to melt the agarose. Following this, 10 µl of Glassmilk suspension was added to each tube and the resultant solutions were mixed by inversion. The suspensions were then incubated on ice for 5 minutes allowing time for the Glassmilk to bind to the DNA and this was followed by microfuge at 12,000 x g for 5 seconds. The supernatants were discarded and 300 µl New Wash solution was used to resuspend each pellet. This was repeated twice and following the third microfuge, the pellets were dissolved with 40 µl Tris/EDTA (TE) pH 7.4 and then incubated at 65°C for 5 minutes to remove the Glassmilk from the DNA. The samples were then microfuged for 2 minutes and the supernatants which contained the DNA were transferred to fresh microfuge tubes.

3.6 PHENOL/CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION OF DNA SAMPLES

Purification and concentration of DNA samples were achieved by extraction with phenol/chloroform and precipitation with ethanol. DNA samples were mixed with equal volumes of buffer-saturated phenol, mixed by inversion and microfuged for 5 minutes at 580 x g. The upper aqueous phases were transferred to new tubes containing equal volumes of phenol, and again microfuged for 10 minutes. The supernatants were transferred to new tubes containing equal volumes of chloroform and following inversion, the samples were centrifuged at 580 x g for 10 minutes. The upper aqueous phases were transferred to fresh tubes and combined with 2.5 x volume of 95% ethanol and 0.1 volume of 3M NaAc pH 5.2. These were incubated at -80°C for 30 minutes and the DNA then precipitated by microfuge for 15 minutes at 4°C. The supernatants were discarded and 500µl ice cold 70% ethanol was added to each tube to remove excess salt. This was followed by precipitation of the DNA by microfuge for 5 minutes at 4°C. The DNA pellets were dried for 10 minutes in a Speed Vac and resuspended in 10 µl sterile d_2H_2O.

3.7 BACTERIA CULTURES

E. coli bacteria were used for amplification of plasmid DNA.

3.7.0 TRANSFORMATION OF COMPETENT CELLS

50 µl of competent cells (DH5α *E. coli*) were used and 0.5 µl of whole plasmid was added to the bacteria. The combinations were incubated on ice for 20 minutes and then heat shocked for 2 minutes in a 37°C water bath. 200µl of LB broth was then added to each tube and the mixtures incubated for 1 hour at 37°C. Following this, the solutions were plated out on agar plates with LB and antibiotic and the plates were incubated upside down overnight at 37°C.

3.7.1 BACTERIAL SUBCULTURE

5 ml of LB broth was placed in culture tubes. A steriloop was used to remove 2 medium sized colonies from the plates and these colonies were then suspended in the culture tube containing LB, along with antibiotics. The cultures were grown overnight in a 37°C shaking incubator (225 rpm) and subsequently used as an inoculum for large scale cultures.

3.7.2 LARGE VOLUME BACTERIAL CULTURE

2.5 ml of bacteria from the small bacterial subcultures was added to 250 ml sterile LB broth, along with antibiotics. These cultures were incubated overnight, again at 37°C in a shaking incubator.

3.7.3 STORAGE OF BACTERIA IN GLYCEROL

0.5 ml of sterile 100% glycerol was combined with 0.5 ml of the overnight bacteria cultures and stored at -70°C. For recovery of the stored bacteria, a sterile inoculating loop was used to recover bacteria and for streaking on agar plates with LB and antibiotics for plasmid selection.

3.8 PLASMID DNA ISOLATION

3.8.0 BACTERIAL LYSIS

Solutions:

Solution A = 50 mM Glucose, 25 mM Tris/Cl pH 8.0, 10 mM EDTA pH 8.0 low osmotic pressure to swell bacteria

Solution B = 0.2 N NaOH, 1%SDS; alkaline lysis of bacterial cell wall and membrane

Solution C = 5 M Potassium acetate and glacial acetic acid to precipitate the protein

Large scale cultures were poured into 250 ml tubes for high speed centrifuge and the samples were spun for 10 minutes at 2500 x g in a JA-14 rotor. The supernatants were discarded and the bacterial pellets at the bottom of the flasks were treated to lyse the bacteria and release the plasmids. 10 ml

of solution A was added to each flask and the contents were pipetted up and down to dissolve the pellets. The solutions were transferred to 50 ml Oakridge tubes and 10 ml of solution B was added to each tube. The tubes were capped and the contents were mixed by inversion - the solutions become quite heavy due to the lysis of bacteria. 10 ml of solution C was added to each bottle and the contents were well shaken - becoming cloudy as the protein denatured. Samples were then centrifuged for 30 minutes at 14,000 x g in a JA-17 rotor. The supernatants containing RNA, DNA and plasmids were transferred to 50 ml culture tubes and precipitated with 0.6 volumes of isopropanol. The protein-containing pellets were discarded. The isopropanol/supernatant mixtures were mixed by inversion and incubated at 4°C for one hour.

3.8.1 HARVESTING PLASMID DNA

Samples were removed from the 4°C refrigerator and centrifuged for 10 minutes at 850 x g. The supernatants were discarded and the pellets containing the plasmid DNA were air dried for 30 minutes. 1.5 ml of TE pH 8.0 was added to each sample to resuspend the pellets by pipette action. The solutions were transferred to fresh tubes and 8.0 - 10.0 µl of RNAse was added to each sample and mixed by inversion. Samples were then incubated at 37°C for 15 minutes. An equal volume of phenol was added to each sample and then mixing was achieved by inversion. The samples were microfuged at

850 x g for 5 minutes to separate the upper aqueous phases from the lower phenol phases and the aqueous phases were transferred to fresh tubes containing equal volumes of phenol. Once again, separation was achieved by centrifugation for 10 minutes and the upper aqueous phases were transferred to fresh tubes containing equal volumes of chloroform. The extraction of chloroform was performed by centrifugation for 10 minutes at 850 x g. The supernatants were transferred to new tubes and 360 µl of 5M sodium chloride was added to each sample. Following this, Sephadex G25 columns were prepared and placed on collection tubes. Samples were applied to the columns and centrifuged for 12 minutes at 2200 x g to separate plasmid DNA from genomic DNA with plasmid DNA passing through the columns to aggregate in the collection tubes. Isopropanol was added to the tubes to precipitate the DNA and the solutions were mixed by inversion.

Tubes were centrifuged at 14, 000 x g for 25 minutes at 4°C. The resultant pellets were washed with ice cold 70% ethanol and again spun for 5 minutes at 14, 000 x g. The supernatants were discarded and the pellets were dried by speed vac for 10 minutes. 400 µl of TE pH 7.4 was added to dissolve each pellet and the solutions were transferred to sterile microfuge tubes.

3.9 RESTRICTION ENZYME DIGESTION

Restriction enzyme digests of plasmid DNA were performed to isolate the DNA fragments in preparation of cDNA probes. For a 20 µl volume restriction enzyme digest of plasmid DNA, 1.0 - 2.0 µg of DNA was used, along with 2 µl of 10 X restriction enzyme buffer and 2 U of restriction enzyme(s).

The mixtures were incubated in a 37°C water bath overnight. The samples were mixed with 2 µl of loading buffer and electrophoresed on a 1% agarose gel as described above.

3.10 cDNA PROBES

A plasmid construct (pratghr.2.2) for the rat growth hormone receptor was obtained from Genentech. The 1086 base pair fragment was excised from a pUC119 backbone using the EcoRI restriction enzyme in the presence of React 3 buffer.

The pG3-2-11 plasmid containing the rat IGFBP-2 cDNA clone was obtained from Matt Rechler (NIH) (Brown and Rechler, 1989). The 585 base pair fragment was excised from the pGem3 backbone using the restriction enzymes HindIII and SacI.

The pHA68B12 plasmid containing the albumin cDNA was obtained from ATCC. The 1 kb insert was excised from pBR322 using the restriction enzyme PstI.

Also obtained from ATCC was the pHAF7 clone containing the cDNA for alpha fetoprotein. The 1.2 kb insert was excised from pBR322 using PstI.

The phigf1 construct containing the IGF - I cDNA was obtained from ATCC. The 659 base pair insert was excised from pKT218 using PstI.

IGFBPs 1, 3, and 4, and IGF - II cDNA inserts were obtained from Dr. L. Murphy at the University of Manitoba.

3.11 RADIOLABELLING OF cDNA PROBES

The method of random prime radiolabelling 500 ng of insert or 1000 ng of plasmid was employed as described previously (Sambrook *et al.*, 1989). cDNA samples were boiled for 5 minutes to separate the double helix and rapidly placed on ice to prevent reannealing. The samples were microfuged for one second and placed on ice, and 4.5µl olb solution (2M Tris/HCl pH 8.0, 5 M $MgCl_2$, β-mercaptoethanol, 2M Hepes pH 7.0, dCTP, dTTP, dGTP, and random

primer) and 0.5µl klenow fragment were added to each tube. [^{32}P]dCTP was added to a specific activity of 3,000 Ci/mmol and labeling took place at room temperature for 2 hours. Removal of non-specific label was performed via column separation using nick columns. The pure labels were boiled for 5 minutes, placed on ice and then used for hybridization with membranes in Northern blot analysis.

3.12 NORTHERN BLOT ANALYSIS

Northern blot hybridization was performed according to the method of Sambrook *et al.* (Molecular Cloning, 1989). Briefly, a 1% agarose, 2.2 mol/L formaldehyde, 1X MOPS denaturing gel was prepared. A total of 20 µg of total RNA was incubated with 17µl loading buffer at 65°C for 0.5 hour. Samples were then loaded onto the agarose gel and electorphoresed at 100 V for one to two hours. Gels were transferred to GT-Zeta probe nylon membranes overnight with 10 X SSC. Membranes were cross-linked by exposure to ultraviolet light. Following this, membranes were hybridized for 20 hours at 42°C in 50% Formamide, 0.12M Na_2HPO_4 pH7.2, 0.25M NaCl, 7% sodium dodecyl sulfate for homologous probes.

The following complementary DNA probes were used: albumin, α-fetoprotein, IGF - II, rat growth hormone receptor, IGF - I, IGFBP - 1, 2, 3, 4.

Following hybridization, membranes were washed twice for 15 minutes at room temperature in 2 X SSC and 0.1% SDS and once at 42°C in 0.1% SSC and 0.1% SDS. Autoradiography was performed with Kodak XAR film at -70°C using an intensifying screen. Densities of the bands were obtained by scanning, and analysed with a NIH image 1.6 densitometry program. Expression was normalized relative to the 28S ribosomal transcript.

3.13 GLUCOSE DETERMINATIONS

Blood glucose measurements were performed in collaboration with Dr. W.W. Lautt in the department of Pharmacology and Therapeutics at the University of Manitoba. The methodology consisted of injection of 25 µl serum samples into a glucose analyzer using a syringe/pipette. Enzymology involved the glucose oxidase / peroxidase reaction. The measurements were provided in mg/dl and samples were run in duplicate or triplicate.

3.14 INSULIN AND GLUCAGON DETERMINATIONS

Serum insulin and glucagon levels were determined via radioimmunoassay using commercial kits purchased from the Incstar corporation and Linco Research incorporated, respectively. Standard curves were established for each assay and levels of each hormone were determined by extrapolation from the standards and calculations from maximum binding percentages.

3.15 INSULIN SENSITIVITY DETERMINATIONS

These estimations were also performed in collaboration with Dr. W.W. Lautt. Briefly, tracheotomies were performed on anaesthetized rats and body temperatures were maintained at 38°C. Cannulation of the right jugular vein was performed for infusion of glucose. Cannulation of the left jugular vein was performed for administration of anaesthetic. To prevent intravascular clot formation, heparin was administered to the rats at a dose of 200 IU/kg. The left femoral artery and vein were used for an arterial - venous shunt which allows for the rapid blood sampling required for arterial glucose analysis. The rats were stabilized for 0.5 hour before the first glucose analysis. The rapid insulin

sensitivity test (RIST) consisted of: arterial samplings every 5 minutes until three stable levels were obtained that could be used as a basal glucose level. 50 mU/kg of insulin was provided over a period of 5 minutes, glucose sampling began after the first minute and was continued every 2 minutes during the half hour test period. Glucose infusion was used to maintain blood glucose values at the basal level. The RIST index is the amount of glucose that was infused in mg/kg over the 30 minute test period necessary to maintain a stable blood glucose level.

3.16 DATA AND STATISTICAL ANALYSES

It should be noted that due to the limited size of the livers in the rat fetuses and newborns, the Northern blot analyses display pooled tissues at gestational days 16 and 20 of age and at post – partum day one. As such, confidence intervals could not be generated and applied to these figures. However, the densitometry of blots run with the same mRNA samples for the above mentioned time points were consistent when tested on separate membranes. For the later time points, post – partum days 7 and 40, RNA was extracted from the liver tissues such that a minimum of three membranes was prepared and analyzed, with mRNA from different animals. Therefore, confidence intervals could be applied to these time points for analyses.

Normalization of the northern analyses were established by densitometry using 28S as a standard. Density of the bands was obtained by a Hewlett Packard scanner and analysed with a National Institutes of Health Image 1.6 Densitometric Analysis Program.

4. RESULTS

4.0 MATERNAL AND PUP OUTCOME

Pregnant dams that consumed the ethanol liquid diet throughout pregnancy displayed signs of physical addiction to the alcohol by mid - pregnancy. Specifically, they exhibited signs of anxiety and nervous behavior when compared with controls. In addition, the incidence of stillbirths was higher in the group of pups born to mothers who consumed ethanol throughout pregnancy when compared with controls (19% vs. 2% respectively, $p < 0.05$).

Pups born to mothers who consumed ethanol throughout pregnancy displayed signs of addiction to ethanol including extreme agitation, restlessness and inability to calm unless stroked and rocked in hand for extended periods of time. These pups also demonstrated significantly decreased brain weights and crown – rump lengths (in keeping with FAS) when compared to pups born to dams who consumed the control diets throughout pregnancy ($p < 0.05$).

4.1 LIVER HISTOLOGY

Histological analysis of the liver tissues revealed similar findings in all three groups; mild steatosis and extensive extramedullary haematopoiesis. In no group was there evidence of inflammation or fibrosis.

4.2 LIVER MATURATION

The results of Northern blot analyses for albumin mRNA are provided in Figure 3. In FAS pups albumin mRNA expression was apparent and highest at the earliest time point examined – gestational day 16. It then declined slightly to levels that approximate those in day 40 young adults. In *ad lib* control pups, albumin mRNA was also detected at gestational day 16, however, rather than declining, the levels rose to a peak at post – partum day one before declining to levels seen in young adults. Although samples from isocaloric – fed control pups were not available for analysis at gestational day 16, the pattern somewhat resembled that observed in *ad lib* controls in that albumin mRNA levels increased to a peak at post – partum day one before declining to levels at 40 days of age. Thus, in general, albumin mRNA levels appeared to peak earlier in FAS pups than in *ad lib* or isocaloric - fed control pups By 40 days of age, the levels of expression for albumin mRNA appeared similar in all three groups.

Figure 4 provides the results of Northern blot analyses for AFP mRNA . In FAS animals, AFP mRNA expression was apparent at gestational day 16. It then remained at a fairly consistent, low level as the animals aged, with no apparent peak in expression. Conversely, but in agreement with previous reports, the *ad lib* control animals displayed AFP mRNA at gestational day 16, followed by an abrupt peak at the time of birth. Subsequent to this, levels declined to young adult levels at 40 days of age. The pattern of expression for isocaloric – fed control animals was similar to that seen in FAS animals, with a slight rise in levels of mRNA expression from gestational day 20 to a slight peak at post - partum day one. Thus, the findings with respect to AFP mRNA expression suggest that the differences seen in FAS pups compared to *ad lib* fed controls likely represent differences in maternal caloric consumption rather than the effects of ethanol *per se* on the developing liver.

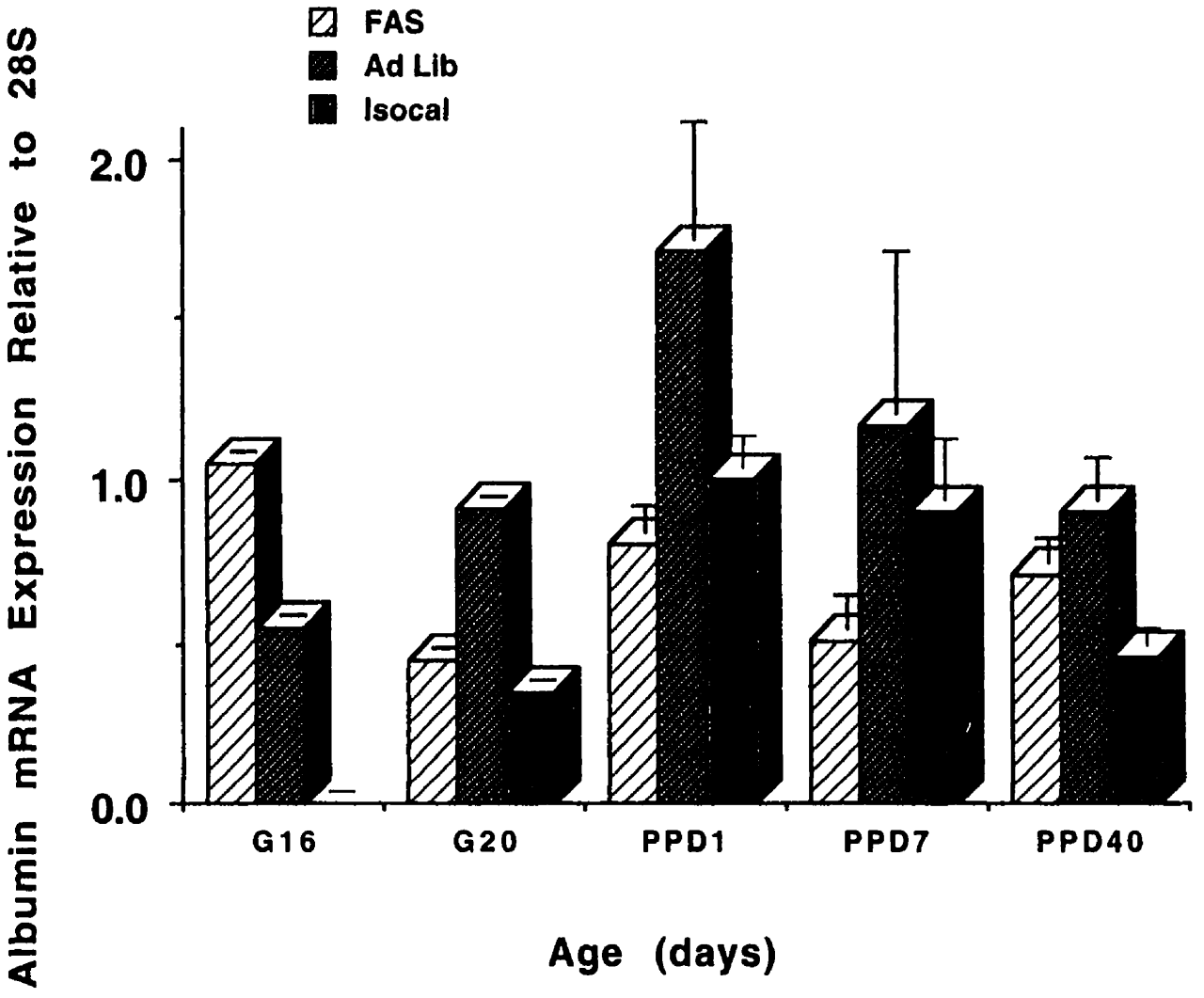
FAS
Ad Lib
Isocal
Albumin mRNA Expression Relative to 28S
2.0
1.0
0.0
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 3: Northern blot analyses of albumin mRNA expression in the livers of rats at gestational days 16 and 20 (G16, G20), and post – partum days 1, 7, and 40 (PPD1, PPD7, PPD40)

FAS = Fetal Alcohol Syndrome pups
Ad lib = pups born of dams fed the control diet ad libitum throughout pregnancy
Isocal = pups born of pair – fed (for FAS) control dams

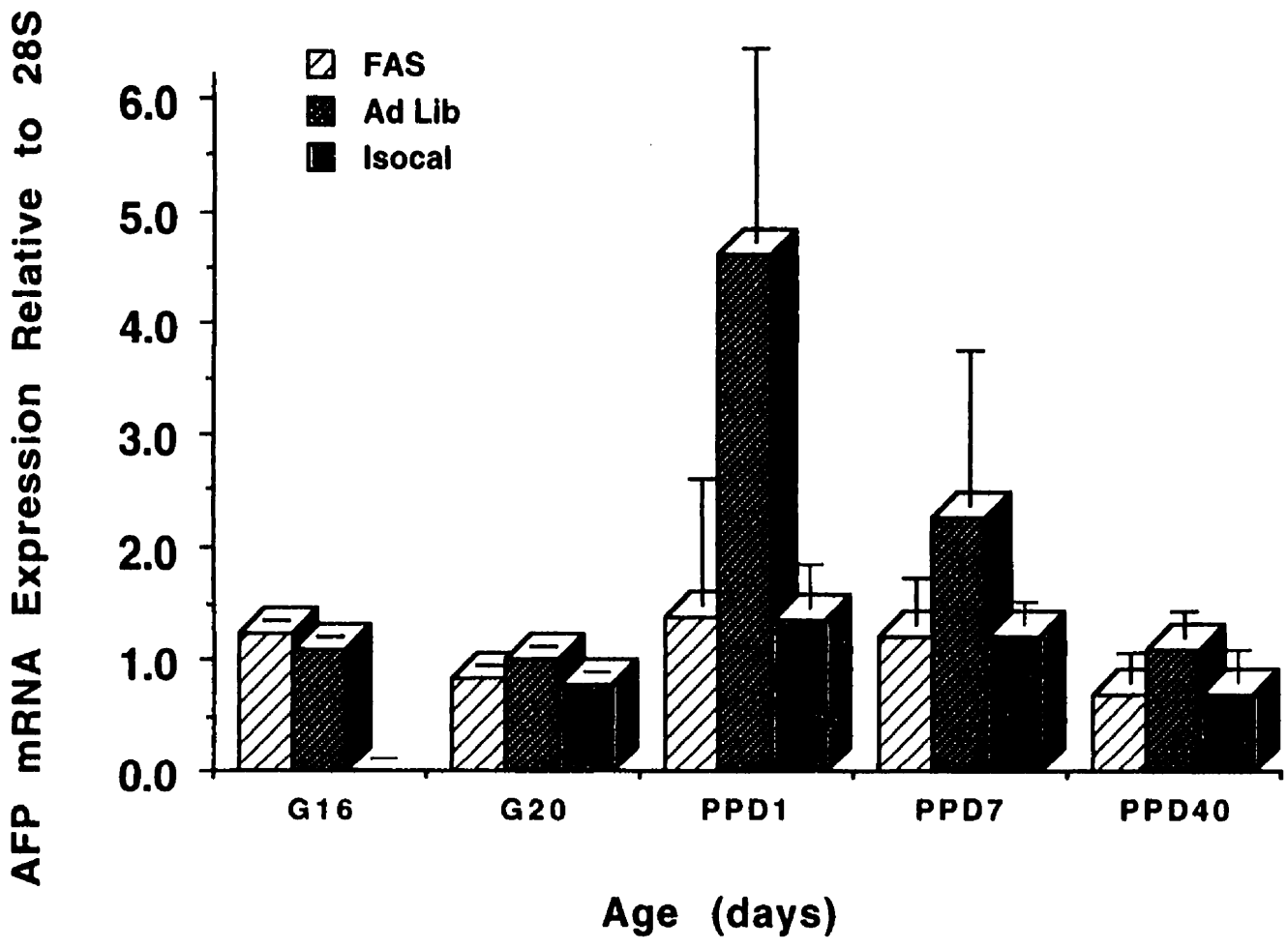
AFP mRNA Expression Relative to 28S
FAS
Ad Lib
Isocal
6.0
5.0
4.0
3.0
2.0
1.0
0.0
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 4: Northern blot analyses of alpha fetoprotein mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, *Ad lib*, and Isocal rats

4.1 STATUS OF THE GH / IGF / IGFBP AXIS

Figure 5 provides the results of GHR mRNA expression in the various study groups at post – partum day 40. During gestation and the early post – partum period, there was no difference in expression between FAS and either group of control pups (data not shown). However, at post – partum day 40, GHR mRNA expression was approximately 55% lower in FAS animals compared with *ad lib* controls, and 65% lower than in isocaloric controls.

The results of IGF – I mRNA expression are shown in Figure 6. In FAS fetuses, expression of IGF – I mRNA was evident at gestational day 16, and declined thereafter with markedly lower levels at gestational day 20, post – partum day one and post – partum day seven. By earlier adulthood, as seen at post - partum day 40, levels had increased slightly. The pattern of mRNA expression for IGF – I in *ad lib* control animals differed from that seen in FAS rats. Although expression was also apparent in this group at gestational day 16, levels increased rather than decreased as the time of birth approached and peaked just after birth at post – partum day one. Subsequent to this, levels declined at post – partum day seven and were decreased further at post – partum day 40. A similar pattern of expression but of lower magnitude was seen in the isocaloric controls.

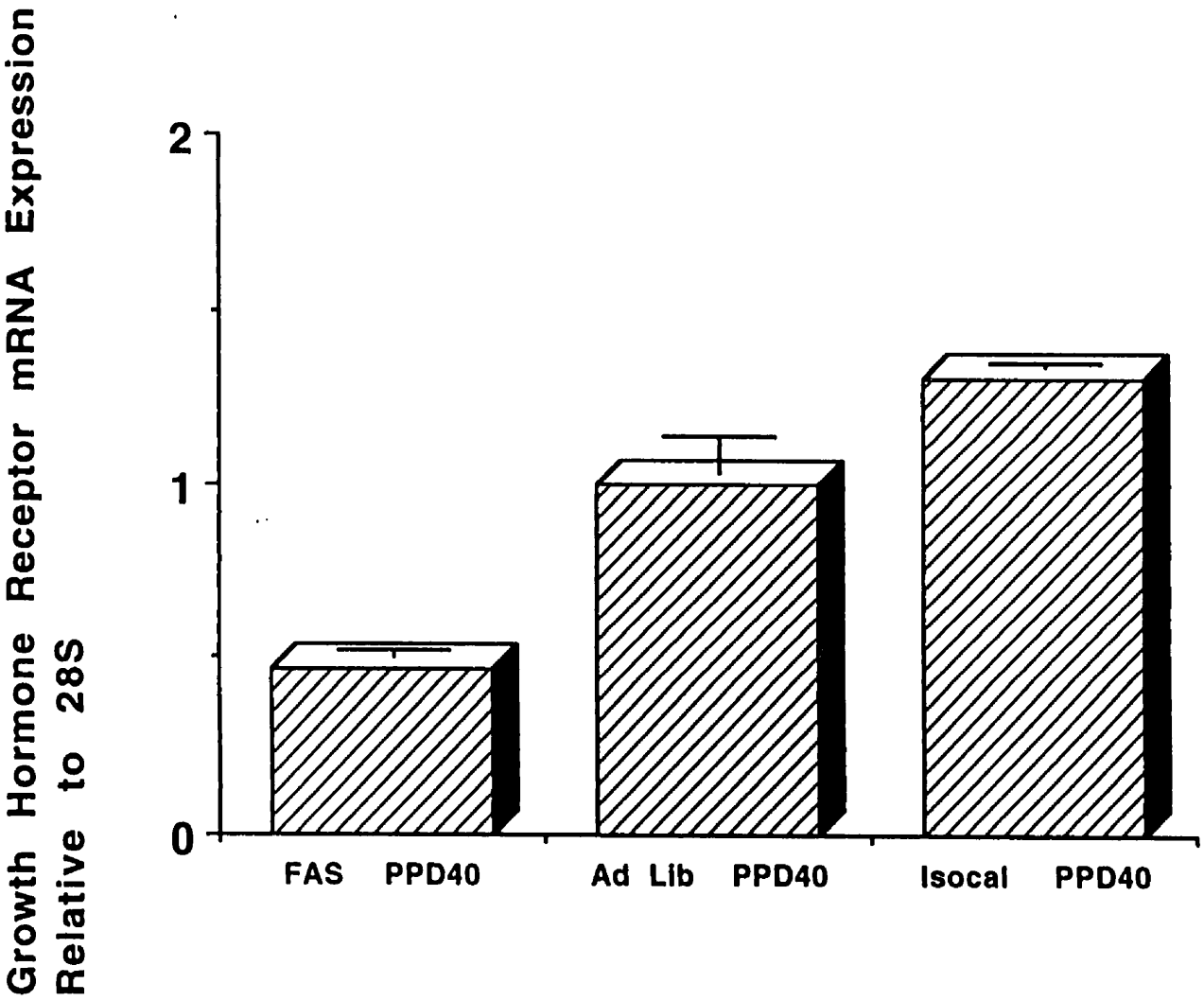
Growth Hormone Receptor mRNA Expression
Relative to 28S
2
1
0
FAS
PPD40
Ad Lib
PPD40
Isocal
PPD40

Figure 5: Northern blot analyses of Growth hormone receptor mRNA expression at PPD40 in FAS, *Ad lib*, and Isocal rats.

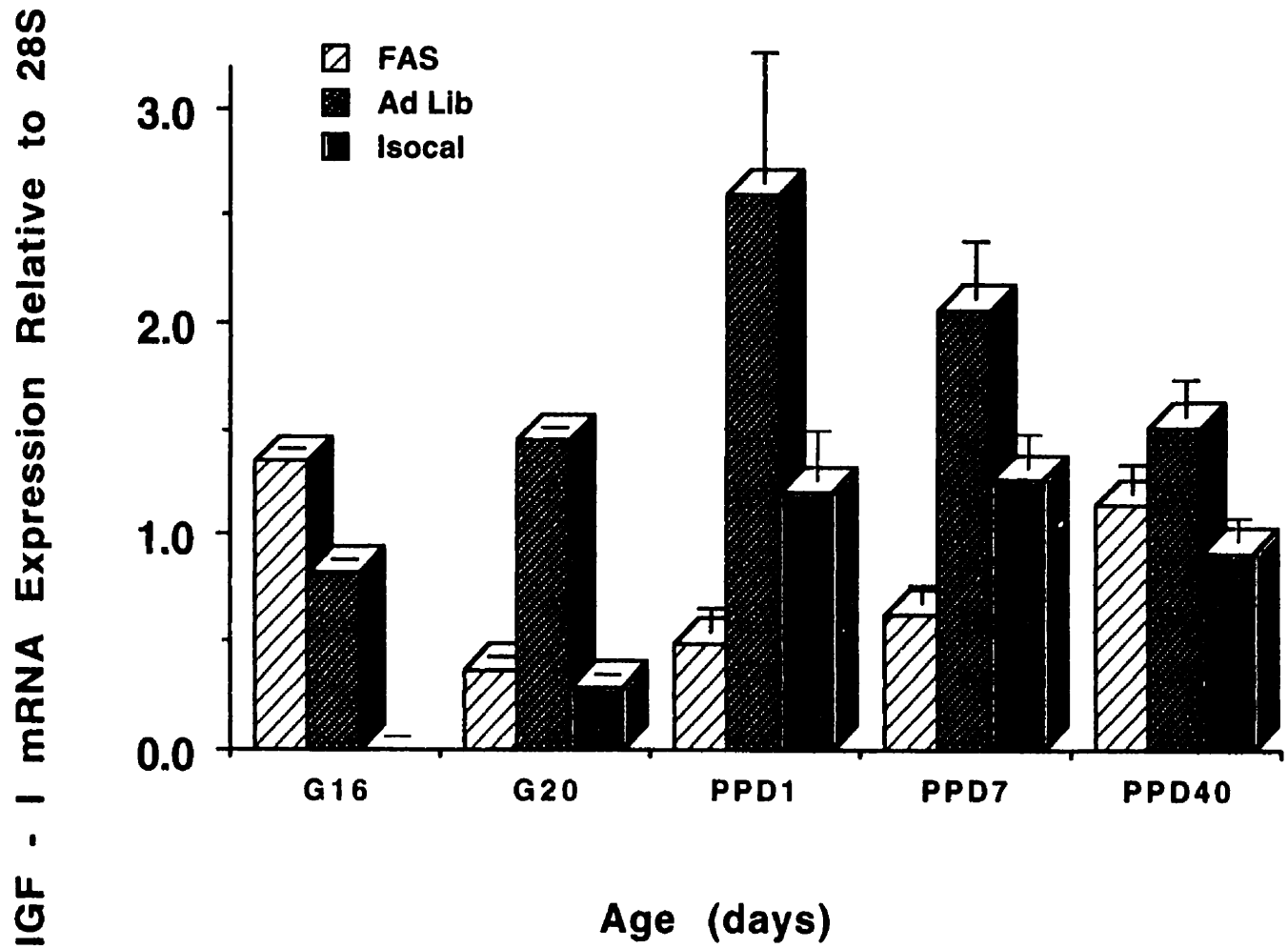
IGF - I mRNA Expression Relative to 28S
FAS
Ad Lib
Isocal
3.0
2.0
1.0
0.0
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 6: Northern blot analyses of Insulin – like growth factor – I mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, *Ad lib*,and Isocal rats.

The results of Northern blot analyses for IGF – II mRNA expression are provided in Figure 7. In FAS animals, expression was apparent on gestational day 16 at levels that remained relatively constant until post - partum day one. As the rat aged and approached adulthood, the levels declined somewhat. In the *ad lib* fetuses, IGF – II mRNA expression did not fluctuate a great deal during gestation and the early post - partum period, but increased at post – partum day seven and subsequently declined to a lower level at post – partum day 40. The pattern of expression for the isocaloric control animals reflected relatively similar levels of mRNA expression throughout the study period aside from a slight peak at day seven.

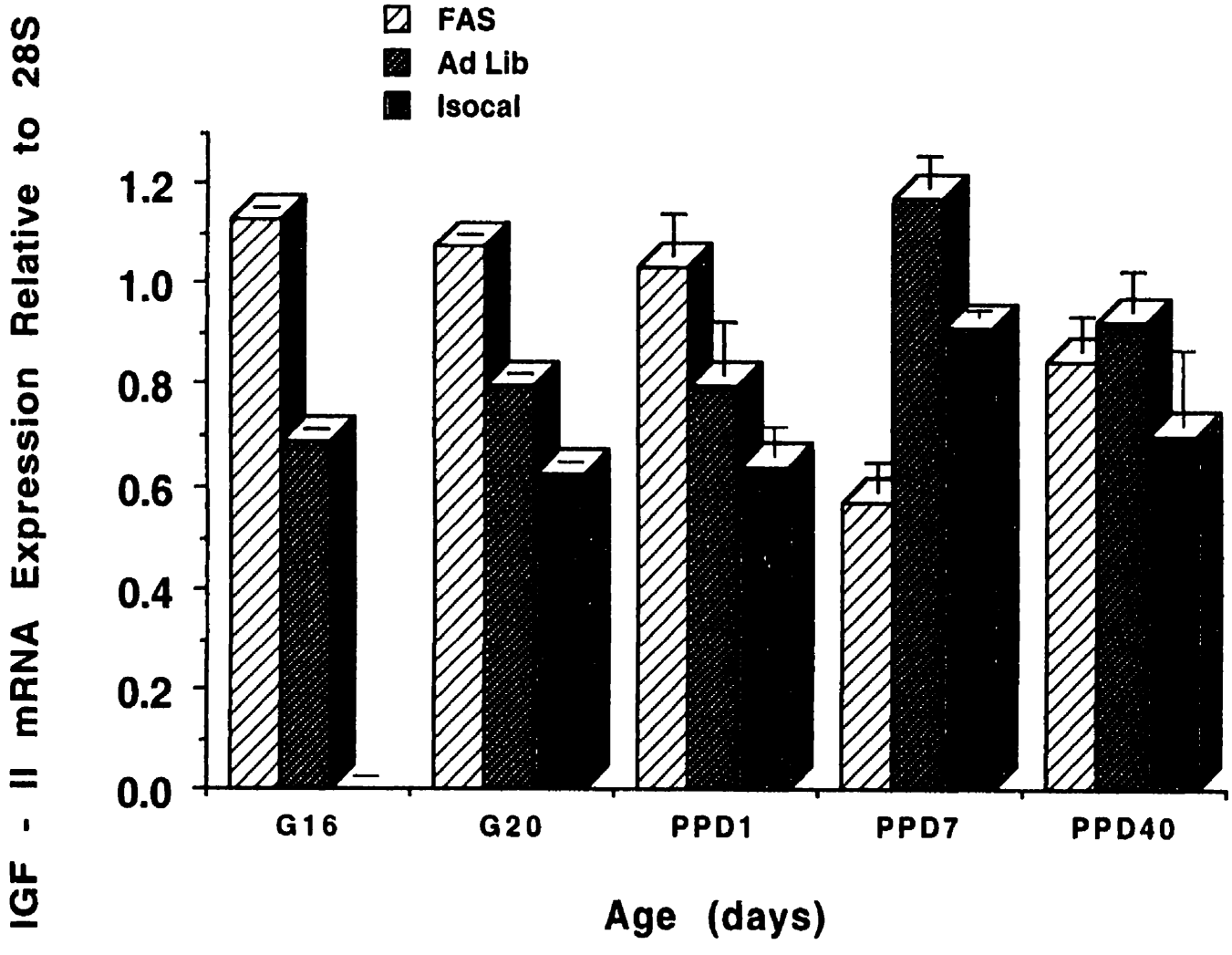
FAS
Ad Lib
Isocal
IGF - II mRNA Expression Relative to 28S
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 7: Northern blot analyses of Insulin – like growth factor – II mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, A*d lib*, and Isocal rats

The results of IGFBP – 1 - 4 mRNA expression are provided in Figures 8 through 11, respectively. As shown in Figure 8, IGFBP - 1 mRNA expression was present at gestational day 16 and increased three fold to levels at gestational day 20 and post - partum day one before falling to levels at post - partum day 40. The pattern for *ad lib* controls, was somewhat similar. However, prior to post - partum day one, levels were consistently lower than in the FAS group but higher on post - partum days seven and 40. In isocaloric controls, IGFBP - 1 mRNA expression peaked on post - partum day one and was consistently lower than the FAS group at all time points.

Figures 9 - 11 provide the results of Northern blot analyses for IGFBPs -2 - 4. In each case, mRNA expression was essentially similar in all three groups, being apparent on gestational day 16 and peaking on the same gestational or post - partum day (IGFBP - 2; PPD1, IGFBP - 3; G20, and IGFBP - 4; PPD1) before falling to young adult levels.

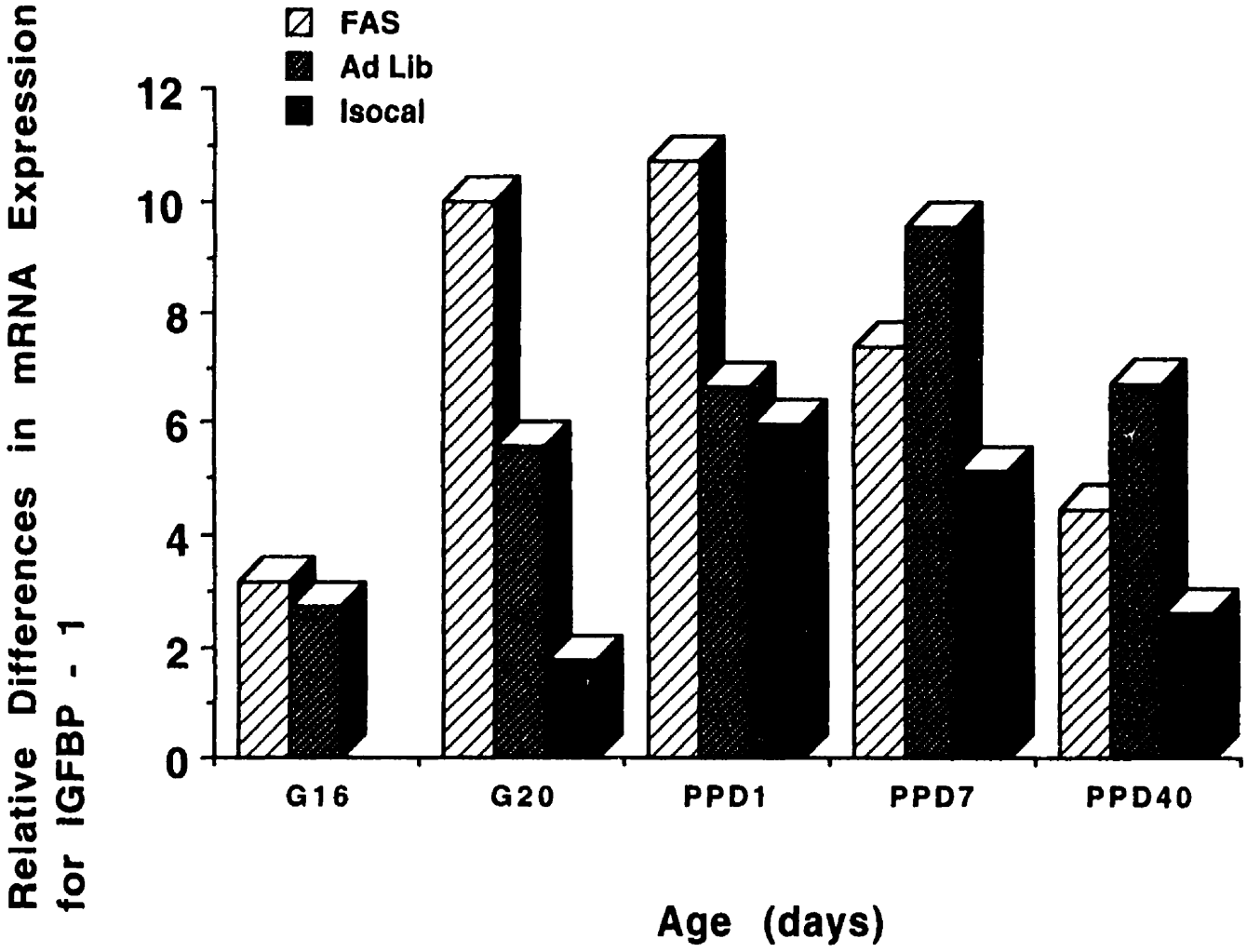
FAS
Ad Lib
Isocal
12
10
8
6
4
2
0
G16
G20
PPD1
PPD7
PPD40
Relative Differences in mRNA Expression for IGFBP - 1
Age (days)

Figure 8: Northern blot analyses of Insulin – like growth factor binding protein - 1 mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, *Ad lib*, and Isocal rats
N = 2 membranes probed with the IGFBP-1 cDNA

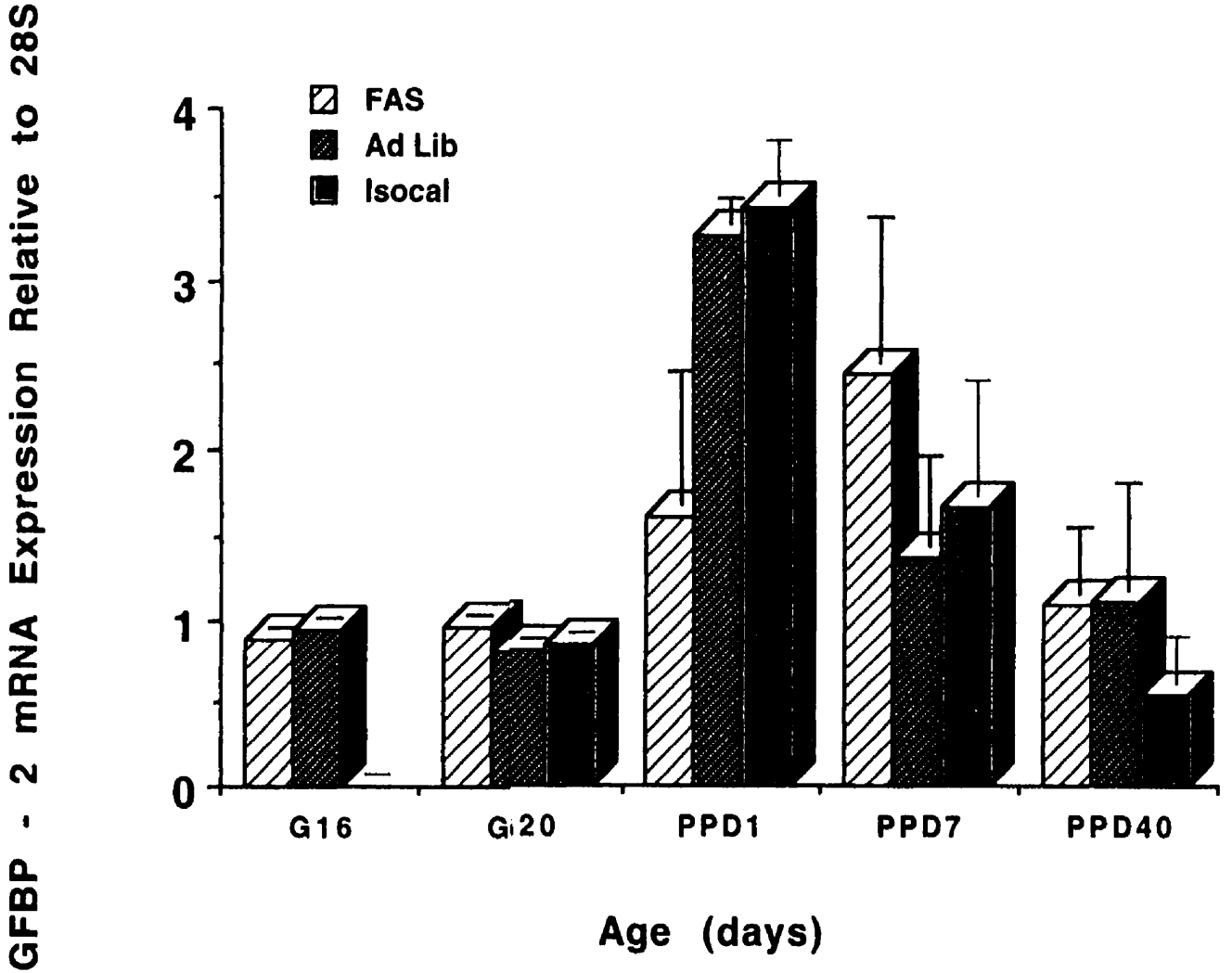
IGFBP - 2 mRNA Expression Relative to 28S
FAS
Ad Lib
Isocal
4
3
2
1
0
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 9: Northern blot analyses of Insulin – like growth factor binding protein - 2 mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, *Ad lib*, and Isocal rats.

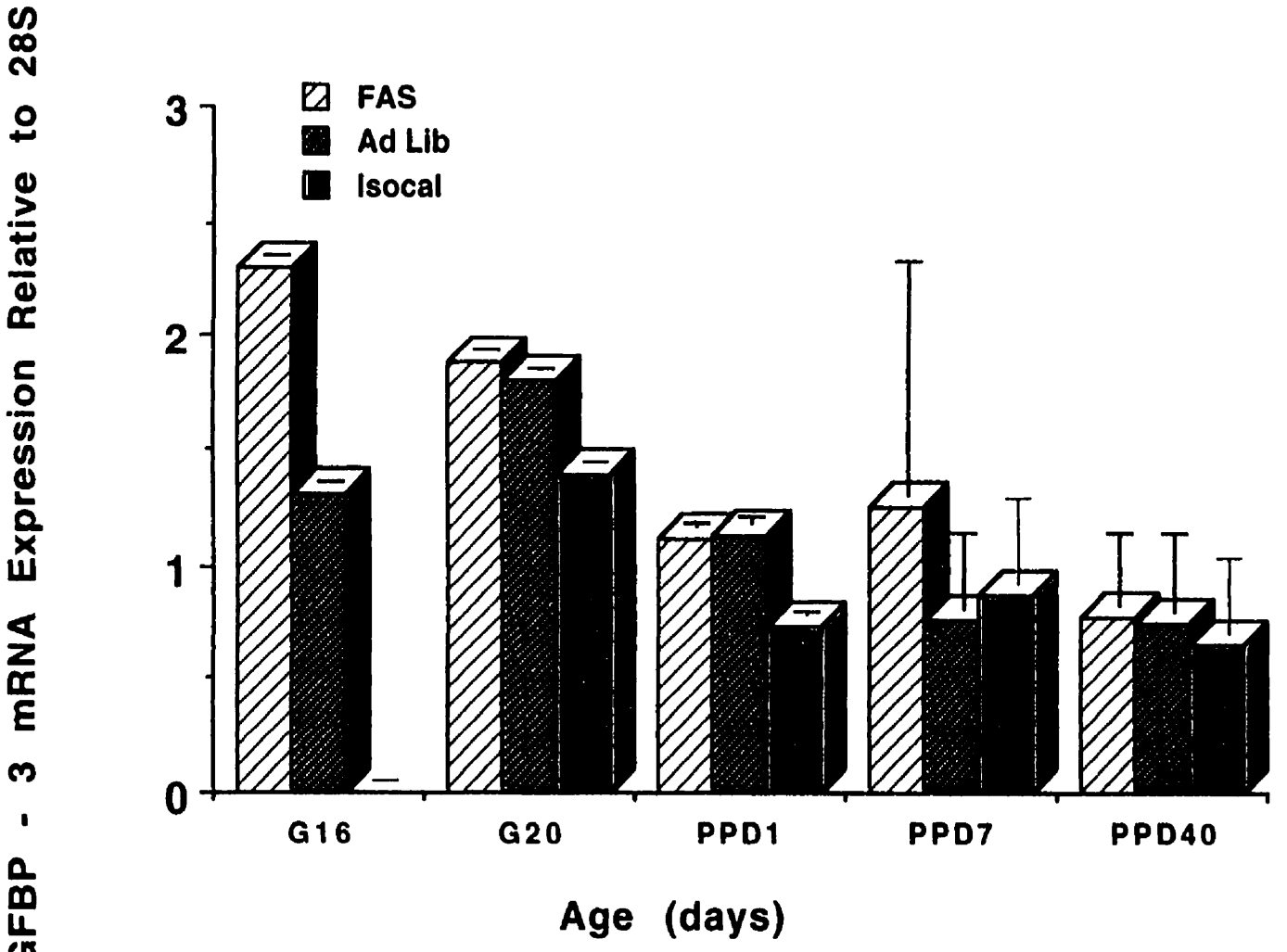
IGFBP - 3 mRNA Expression Relative to 28S
FAS
Ad Lib
Isocal
3
2
1
0
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 10: Northern blot analyses of Insulin – like growth factor binding protein - 3 mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, *Ad lib*, and Isocal rats.

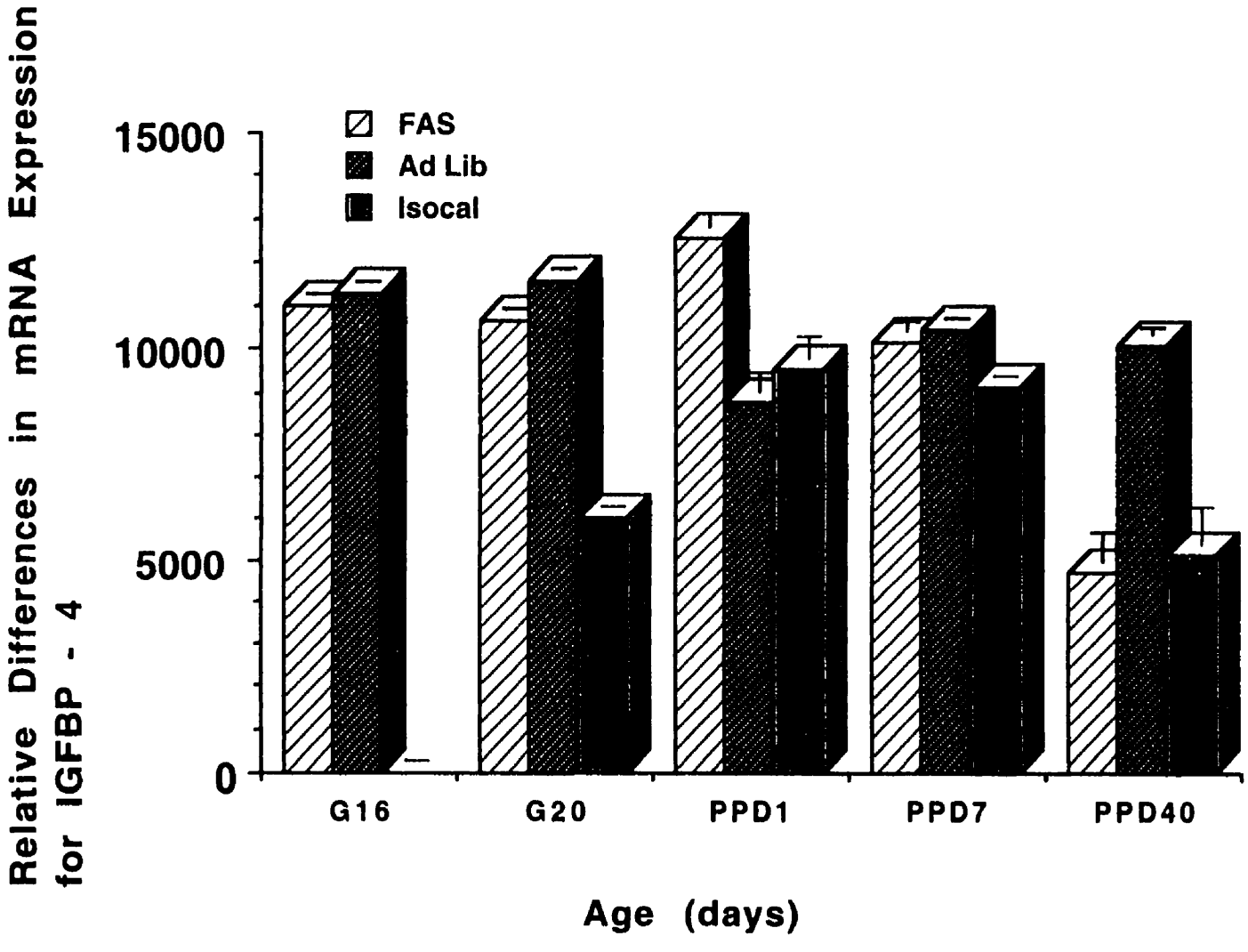
Relative Differences in mRNA Expression for IGFBP - 4
15000
10000
5000
0
FAS
Ad Lib
Isocal
G16
G20
PPD1
PPD7
PPD40
Age (days)

Figure 11: Northern blot analyses of Insulin – like growth factor binding protein - 4 mRNA expression at G16, G20, PPD1, PPD7, and PPD40 in FAS, *Ad lib*, and Isocal rats.

To determine whether the observed changes in the growth hormone / IGF / IGFBP axis altered glucose homeostasis in FAS pups, serum glucose, insulin, and glucagon levels, and insulin resistance assays were also performed. Table III provides the results of serum glucose analyses in FAS and isocaloric control rats at 40 days of age. Serum glucose levels in the FAS animals were approximately twice the levels seen in controls ($p<0.05$).

Serum insulin levels are provided in Table IV. As with serum glucose levels, in FAS pups, the serum insulin levels were approximately twice those seen in controls. (12.12 ± 0.68 μU/ml for FAS vs. in isocaloric controls, 45.18 ± 3.17 μU/ml, $p<0.001$).

Serum glucagon levels are provided in Table V. At 40 days of age, serum glucagon levels in the FAS group were 43.9 ± 2.79 pg/ml and 31.9 ± 2.76 pg/ml, $p<0.05$.

To document the sensitivity to insulin in these animals, the rapid insulin sensitivity test (RIST) was applied. The results of this analysis showed that FAS animals displayed significant insulin resistance when compared with control animals. FAS pups disposed of only 104 ± 11 mg/kg glucose during the test, while the control pups disposed of 320 ± 29 mg/kg glucose despite equal amounts of insulin administration (50 mU/kg).

STUDY GROUP	N	AGE (days)	AVERAGE GLUCOSE CONCENTRATION (Mmol/L)
FAS	5	40	6.88 ± 0.89*
ISOCAL	5	40	3.47 ± 1.09

Table III: Serum glucose concentrations in FAS pups and isocaloric fed Controls (see materials and methods for complete description of Group identification).

* $p < 0.05$

STUDY GROUP	N	AGE(days)	%MAX. BOUND	LEVEL (μU/ml)
FAS	4	40	59.75	12.12 ± 0.68*
ISOCAL	4	40	30.42	45.18 ± 3.17

Table IV: Serum insulin concentrations in FAS pups and Isocaloric fed controls at post partum day 40

* $p < 0.001$

STUDY GROUP	N	AGE (days)	% MAX. BOUND	LEVEL (pg/ml)
FAS	4	40	66.1	43.89 ± 2.79*
ISOCAL	4	40	74	31.86 ± 2.76

Table V: Serum glucagon concentrations for FAS and Isocaloric fed controls animals at post partum 40 as determined by radioimmunoassay.

* $p < 0.05$

5. DISCUSSION

Exposure to ethanol during gestation is known to produce serious alterations in development. These changes include modified skeletal development, reductions in body length and weight, altered levels of adipose tissue, craniofacial abnormalities, neurological disturbances and cardiac anomalies (Jones *et al.*, 1973; Jones *et al.*, 1974; Green, 1974; Abel and Dintcheff, 1978; Clarren and Smith, 1978; Detering *et al.*, 1979; Streissguth *et al.*, 1980; Abel, 1981; Persaud, 1988). The mechanism(s) responsible for this constellation of alcohol - related birth defects remains to be identified. Suggestions have been posed regarding the importance of not only ethanol *per se*, but also the contribution of the metabolites of ethanol. It has also been reported that the toxic effects of ethanol occur in the absence of malnutrition (Lieber, 1988). Induction of perturbations in physiology, endocrinology and gene expression are all plausible explanations for the maldevelopment seen in the offspring of mothers who consume ethanol during pregnancy.

Understandably, an in depth exploration into the pathogenesis of FAS is not possible to perform clinically and therefore, appropriate animal models of FAS are essential to our understanding of the syndrome. Over the years a number of proposed models have been designed and analyzed (Chernoff, 1977; Schwetz *et al.*, 1978; Henderson *et al.*, 1979; Testar *et al.*, 1988). Many factors must be established for a model to be considered appropriate, and

Persaud (1988) outlined a number of criteria that must be present for an accurate investigation of the effects of *in utero* exposure to ethanol on embryogenesis and development. In addition, a number of alcohol "diets" have been developed, and Lieber and De Carli were fundamental in establishing a proper balance for these diets in their application to the animals (1982).

The establishment of appropriate animal models of FAS allowed for investigations into the mechanisms involved. In the last two decades many studies have been conducted utilizing these models in the hope of further characterizing the condition and in search of the causes(s) for the malformations that result. Although much progress has been made in establishing the presence of neurological and musculo-skeletal abnormalities, additional information is still needed, particularly in identifying other organ systems involved and the reasons behind the maldevelopment.

A number of investigators have identified the effects of exposure to ethanol *in utero* on liver weights (Buts *et al.*, 1992; Pullen *et al.*, 1988; Henderson *et al.*, 1979; Través and López - Tejero, 1994), hepatic protein synthesis (Rawat, 1976), liver microsomal enzyme activity (Través and López - Tejero, 1994; Buts *et al.*, 1992), and hepatocytes in culture. Generally, regardless of exposure to ethanol acutely or chronically, liver weights were reduced in the offspring of pregnant rats who were administered ethanol during gestation. Similarly, fetal exposure to ethanol results in a decreased ability of

the fetal liver to synthesize proteins. Regarding the microsomal enzyme activity in the liver following *in utero* exposure to ethanol, the two studies cited above are in agreement in declaring that there is no difference in the activity and distribution of the enzymes. However, there are fundamental differences in the methods used in these studies that preclude comparisons being made with our data in FAS - for example, the method of ethanol administration varied from gastric intubation to provision in the drinking water or in the diet, the length of exposure to the ethanol varied in some studies from acute exposure to chronic consumption, the concentration of ethanol provided was 6% in two of the studies, 25% in two studies, and 30% in two studies. The age of the animals when the parameters were examined varied from study to study as well - many examinations were performed solely at gestational day 20. In addition, with the exception of the paper examining the effect of ethanol on fetal rat hepatocytes (Devi *et al.*, 1993), functionality of the liver was not described.

As mentioned earlier, there have been previous studies documenting the effects of ethanol exposure on the regenerating liver (Sessa and Perin, 1997; Minuk *et al.*, 1995; Diehl *et al.*, 1988; Luk, 1986; Duguay *et al.*, 1982). These investigations reported earlier termination of liver growth (perhaps premature maturation) as a result of ethanol exposure. It was largely on the basis of these results and the fact that "catch up" growth of liver weights does not occur in FAS pups that we proposed liver development ceases prematurely in FAS pups. That is, a developing liver exposed to ethanol in utero may

terminate further development earlier than would be the case in the absence of ethanol. This theory would be supported if there were reports of ethanol causing premature maturation of other tissues or if parameters of liver development were found to appear, peak, and fall to adult levels earlier in FAS pups than in controls. At this time, the former data (premature maturation of other tissues) is not in place, but neither has it been looked for. The small stature of FAS children would be in keeping with this possibility.

In rat pups, the inability to obtain liver tissues prior to gestational day 16 hindered attempts to address this possibility in its entirety i.e. documenting the first appearance of the selected liver developmental genes was not possible. None - the - less, one could interpret the data obtained from gestational day 16 and beyond in terms of presumed peaks in mRNA expression and falls to adult levels. Specifically, in FAS pups, albumin mRNA expression appeared to peak earlier in gestation than in control pups. However, an alternative explanation of the same data is that ethanol merely suppressed albumin mRNA expression throughout gestation. With respect to AFP mRNA findings, the data would suggest that the differences in FAS pups relate more to nutritional differences than ethanol exposure *per se*. Although not originally analysed as a marker of maturation, IGF - II mRNA expression can also be employed as a parameter of maturation. Here, the earlier and increased levels of IGF - II mRNA expression seen in FAS pups in the gestational and early post - partum period support the hypothesis of premature maturation. However, it could also be argued that the

changes observed in IGF - II mRNA expression relate more to the documented changes in glucose homeostasis than to the developmental process. Finally, even if the IGF - II findings reflected changes in maturation rather than glucose homeostasis, one would expect all the parameters of maturation - albumin, AFP, and IGF - II - to be consistent with early maturation.

An alternative explanation for the small livers seen in FAS involves disturbances of the GH / IGF / IGFBP axis. Because it is likely that GH and / or the IGFs are involved in development of the liver, the possibility exists that ethanol can adversely affect liver development by interfering with the GH / IGF / IGFBP axis. Numerous previous studies have established that ethanol inhibits the release of GH (Conway *et al.*, 1997; Tentler *et al.*, 1997; Ekman *et al.*, 1996; Fernstrom *et al.*, 1995; Xu *et al.*, 1995; Badger *et al.*, 1993; Soszynski and Frohman, 1992; Emanuele *et al.*, 1992). Other studies have documented that ethanol decreases plasma levels and hepatic mRNA expression for IGF - I, and IGFBP - 3 during pregnancy (Breese and Sonntag, 1995). The data presented here, however, was not supportive. With the significant decrease in Ghr mRNA expression not occurring in FAS rats until early adulthood, it is unlikely that this aspect of the GH / IGF / IGFBP axis has been impacted upon. Regarding other aspects of the axis, the finding that the levels of mRNA expression for IGF - I (a growth hormone receptor - mediated event) were increased rather than decreased would also not support suppression of the GH / IGF / IGFBP axis as an explanation for the small livers in FAS. Finally, the lack of consistent

changes in the expression of the IGFBPs that bind IGF - I (IGFBP-1, IGFBP-3) further suggest that significant, or the appropriate directional changes in the GH / IGF / IGFBP axis do not occur in FAS.

It must be noted, however, that because growth hormone was not measured directly, it's involvement (or lack thereof) in FAS cannot be excluded with complete certainty. The pulsatile, cyclical changes in growth hormone release from the pituitary gland that occur throughout the day render such determinations impractical (Hochberg *et al.,* 1993).

The possibility that ethanol exposure during pregnancy alters IGF - I in a growth hormone independent fashion must also be considered. The results of a previous study describing decreased levels of IGF - I following chronic exposure to ethanol support the need to address this possibility (Sonntag and Boyd 1989). However, unlike our own study where tissue IGF - I mRNA levels were directly measured, in the Sonntag and Boyd study, the focus was on plasma IGF - I levels which can be influenced by peripheral clearance mechanisms. Because IGF - I is more likely to act in an autocrine or paracrine fashion for liver development, our failure to identify decreased levels of IGF - I mRNA expression further argues against suppression of the growth hormone / IGF / IGFBP axis as being operative in FAS.

In agreement with the results of the present study is a report describing

the effects of maternal ethanol consumption on rat fetal plasma IGF and IGFBP levels (Mauceri et al., 1993). This investigation describes results which show no significant difference in IGF - I levels between ethanol - exposed and control pups.

Yet another possible explanation for why the liver remains small in FAS would be as a result of the presence of fibrotic or diseased tissue. As described in the results section of this paper, our histological findings did not support this possibility.

An additional interesting discovery resulting from this study was the effect of maternal ethanol consumption on glucose homeostasis in the pup. Together with the appearance of decreased GHR mRNA expression, hyperglycemia, hypoinsulinemia, hyperglucagonemia and significant resistance to insulin, these findings appear to reflect a diabetic profile. Interestingly, a recent clinical study compared plasma IGF - I and growth hormone levels in children with FAS and those born to mothers who abstained from alcohol consumption during pregnancy (Halmesmaki et al., 1989). This study described increased levels of umbilical growth hormone and lower levels of IGF -I in infants with FAS which the investigators ascribed to the presence of liver dysfunction in FAS infants. However, evidence of liver dysfunction was not provided and our results offer an alternative explanation for this finding. Obviously, these observations require further investigation for a more complete

understanding of the effects of ethanol exposure during gestation on glucose homeostasis.

In conclusion, it cannot be stated unequivocally that the data presented here supports either of the two explanations put forward in the hypothesis as an attempt to explain the mechanisms involved in the existence of sustained small livers seen in the fetal alcohol syndrome. Further studies are required to better define the events involved and to study the effects of maternal ethanol consumption on liver development. Additional parameters that may be involved include other growth promoters such as hepatic stimulatory substance (HSS) or hepatocyte growth factor (HGF) and / or growth inhibitors such as transforming growth factor - beta (TGF - ß) and gamma aminobutyric acid (GABA). Additional studies are also required to determine whether maternal consumption of alcohol during pregnancy predisposes their offspring to juvenile and / or insulin - resistant diabetes later in life.

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IMAGE EVALUATION
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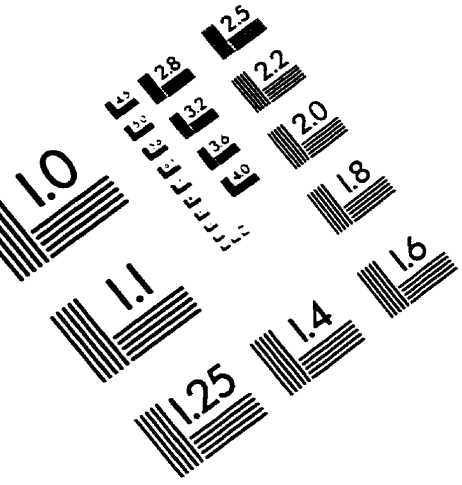

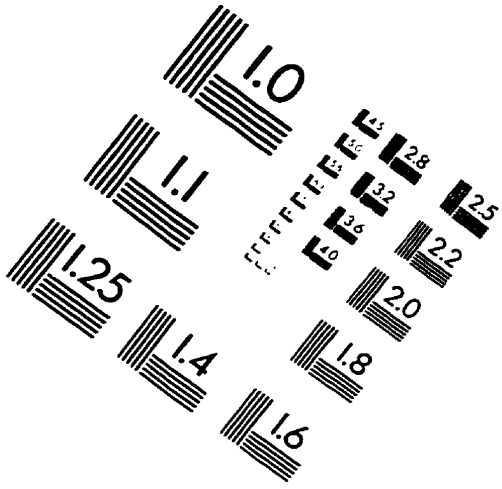

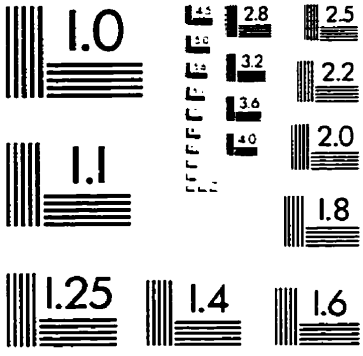

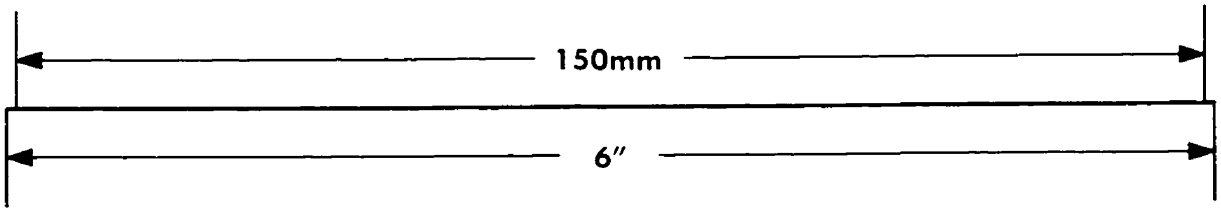

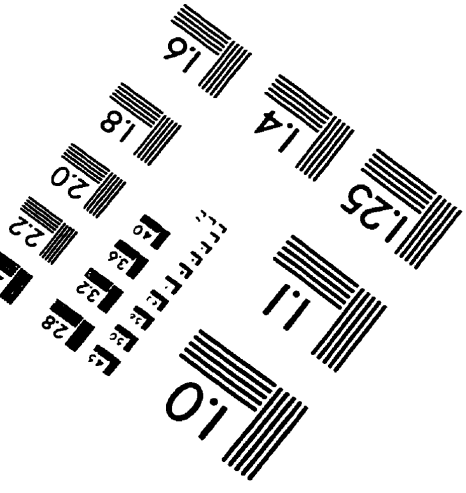

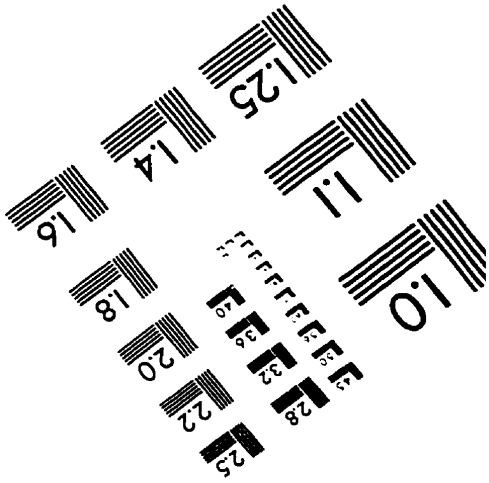

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