MOLECULAR ANALYSIS OF A PATERNALLY TRANSMITTED PLACENTAL PHENOTYPE IN A MOUSE MODEL OF PATERNAL ALCOHOL EXPOSURE

An Undergraduate Research Scholars Thesis

by

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Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Michael Golding

May 2020

Major: Biomedical Sciences

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ABSTRACT

Molecular Analysis of a Paternally Transmitted Placental Phenotype in a Mouse Model of Paternal Alcohol Exposure

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This project investigates the reason why the placentas of mice sired by males exposed to alcohol prior to conception are larger and less efficient than placentas of control mice. This research question is important because it will help explain how preconception paternal alcohol exposure (PAE) influences the health and development of offspring, including symptoms such as low birth weight and metabolic irregularities. Research in the Golding lab has demonstrated that preconception paternal alcohol exposure is associated with a reduction in placental efficiency, however, the mechanism behind the enlargement of the placenta and reduction in placental efficiency is still unknown. To address this question, we assayed the impact of paternal drinking on an outbred cross and followed these experiments with an analysis of placental gene expression. Here, we find that differences in placental efficiency after preconception PAE are sex-specific as well as maternal-strain specific. Interestingly, the male offspring of alcohol-exposed males mated to C57BL6/J dams exhibited decreased placental efficiency, while in contrast, male offspring of alcohol-exposed males mated to ICR dams displayed increased placental efficiency. This suggests that the maternal uterine environment or fetal genetics play a

role in how the placenta develops in response to preconception PAE. Additionally, RT-qPCR was performed on placental samples in order to identify differentially expressed genes hypothesized to be involved with the observed placental phenotype. The genes encoding retinoid X receptor α (RXRα) and farnesoid X-activated receptor (FXR) were significantly upregulated in the placentas of female offspring sired by alcohol-exposed males mated to C57BL6/J dams. Since these genes are involved in placental development and lipid metabolism, it is possible that the upregulation of these genes allowed these alcohol-sired females to avoid decreased placental efficiency as seen in their male counterparts.

DEDICATION

I would like to dedicate this paper to the Golding Lab "Dream Team." I am so honored to be able to be a part of the research that is taking place in the Golding Lab.

I also want to dedicate this paper to the Lord. I am so excited to learn more about genetics, His incredibly complex language of life!

ACKNOWLEDGMENTS

Thank you to everyone in the Golding Lab who has invested time and energy to help me learn. I am so grateful for the opportunities that Dr. Golding has given me to learn in this lab and for his guidance and encouragement. A special thank you to Kara Thomas and Richard Chang, two extremely intelligent people who have spent a lot of time teaching me and walking through experiments with me.

Thank you to Texas A&M for the providing the opportunity to be exposed to some of the best research going on in the nation as an undergraduate! I am so thankful for the bright minds and resources that make Texas A&M such a stand-out institution for research.

Thank you to my family, friends, and coaches for encouraging and supporting me in everything I do.

NOMENCLATURE

ANOVA	Analysis of variance test
Apoa4	Apolipoprotein A4
Ct	Replicate cycle threshold
ddH_2O	Double distilled water
dNTP	Deoxynucleoside triphosphate
FASD	Fetal alcohol spectrum disorder
FXR	Farnesoid X receptor
GD	Gestation day
HRE	Hormone response element
LXRα	Liver X receptor alpha
LXRβ	Liver X receptor beta
PAE	Paternal alcohol exposure
PLTP	Phospholipid transfer protein
qPCR	Quantitative Polymerase Chain Reaction
RT-qPCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA- Seq	RNA- Sequencing

CHAPTER I

INTRODUCTION

A vast amount of research has been conducted regarding prenatal alcohol exposure and fetal alcohol spectrum disorders (FASDs). Research has demonstrated that maternal alcohol exposure during pregnancy can have a wide range of teratogenic effects, including growth deficiencies, problems with cognitive development, behavioral differences, and characteristic facial deformities[2,15]. In fact, studies have shown that gestational exposure to alcohol can impact almost every fetal organ system[13]. A myriad of previous studies indicate that prenatal alcohol exposure can impact the growth, health, and wellness of the organism from early life through adulthood[14].

Studies have indicated that between around 2%-5% of young children in the USA have FASD[15]. The same study that produced this statistic found that the variables that are most predictive of a child having FASD include the mother having late recognition of the pregnancy, the number of alcoholic drinks consumed by the mother three months prior to pregnancy, and the amount of alcohol that the father drank[15]. This last point is interesting to note considering that most of the research on FASDs focus on the impact of maternal drinking, with very little of FASDs research focusing on the impacts of paternal alcohol exposure on offspring.

Studies indicate that preconception paternal alcohol exposure (PAE) can play a role in FASDs via epigenetics inherited through the father's sperm[3]. Epigenetics refers to mechanisms that alter how the genes in a given genome are regulated and expressed. While epigenetics does not change a given genome, a multitude of epigenetic factors can impact what parts of the genome are expressed and to what level expression occurs. These programmed increases or

suppression of gene expression in turn impacts protein production, which in turn impact an organism's physiology. Some studies have shown that long-term preconception PAE can lead to decreased activity of DNA methyltransferases, causing hypomethylation, which in turn allows the activation of genes that would normally be silenced[4]. This increased expression of genes in the sperm is suspected to lead to the development of symptoms of FASDs because the normal levels of expression needed for regular development would be altered[4]. However, studies from the Golding lab challenge the claim that DNA methylation is the main contributor to preconception PAE-related FASDs, identifying seemingly minute changes in the methylation profile of sperm in contrast to the widespread changes observed in the resulting offspring that correlate with changes in growth and development[1]. While it is agreed that fathers can impact the health and development of offspring via epigenetics, the biochemical methods by which the memory of paternal alcohol exposure is transmitted has yet to be established.

The Golding lab has observed that preconception PAE is associated with a reduction in placental efficiency; however, the mechanism behind the enlargement of the placenta and reduction in placental efficiency is still unknown[1]. Additionally, the Golding lab has observed growth deficits in mice with preconception PAE is similar in severity to mice that had mothers drink alcohol during pregnancy[1]. Since low birth weight is one of the most prominent indicators of risk of chronic diseases in adulthood, it can be inferred that decreased placental efficiency can have an impact that goes beyond early development[14]. Understanding the mechanisms behind these observations will help explain how preconception paternal alcohol exposure influences the health and development of offspring, including symptoms such as low birth weight and metabolic irregularities. To add a layer of complexity, the Golding lab has observed maternal strain specific effects and fetal sex-specific effects of preconception PAE on offspring[1].

Based on previous findings in the Golding lab as well as questions that have been left unanswered, this project explores the effects of preconception PAE on offspring in relation to both the maternal genetic background (strain) and sex of the fetus. Additionally, this project explores and analyzes differentially expressed genes that are hypothesized to influence the placental phenotypes observed. The knowledge gained from answering theses research questions can bring us one step closer to understanding the implications of preconception alcohol exposure in humans and ideally will lead to changes that will benefit the health and development of the next generation.

CHAPTER II

METHODS

Animal work

All animal work was performed under AUP 2017-0308 in compliance with Texas A&M University IACUC. C57BL6/J male mice of reproductive age were divided into a control group and an alcohol group. The control group was given water, and the alcohol group was given 10% EtOH. The "Drinking in the Dark" model of access to ethanol was used, in which the mice were given ad libitum access to ethanol for four hours during the dark cycle for 70 days. This treatment lasted 70 days to ensure that the sperm was exposed to EtOH, because 70 days is about the length of 2 spermatogenic cycles[5]. The level of alcohol exposure in this model can be compared to a person's blood alcohol content being 2.5 times the legal limit.[1] The males were then mated with either ICR females or C57BL6/J females. Resulting pregnancies were terminated at gestational day (GD) 16.5 in attempt to access the earliest effects of PAE on offspring placentation. A range of fetal growth parameters were measured at GD 16.5, including gestational sac weight, fetal weight, placental weight, placental diameter, crown-rump length, snout-occipital distance, and biparietal distance.

Sex determination

Fetal DNA was isolated using the HotSHOT method. PCR amplification of the genes *Xist*, which is encoded on the X chromosome, and *Zfy*, which is encoded on the Y chromosome was performed. Gel electrophoresis of the amplified DNA was used to determine the presence of the target genes and determine the sex of the fetuses.

RNA isolation and analysis

The RNeasy Mini Kit (Qiagen. Catalog # 74104) was used to isolate GD 16.5 placental RNA according to the manufacturer's instructions. RNASeq libraries were prepared using 10ng of RNA with the TruSeq RNA Sample Preparation kit (Illumina) and pooled for sequencing on an Illumina HiSeq 2500 at Whitehead Institute of Genomic Research (Cambridge, MA). RNASeq data was analyzed and collected by Yudi Bedi.

cDNA synthesis protocol

The concentration of the GD 16.5 placental RNA was measured using a Thermo Scientific spectrophotometer nanodrop machine. The reaction mixture was prepared using a High Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific. Catalog # 4368813) according to the manufacturer's instructions. The reaction mixtures were placed in the Bio-Rad T100 Thermal Cycler using the "ABCDNASynthesis" program.

qPCR protocol

The cDNA produced from the GD 16.5 placental RNA outlined in the above procedure was retrieved. The DyNAmo Flash SYBR Green qPCR Kit (Thermo Fischer Scientific. Catalog # F-415L) was used according to the manufacturer's instructions. The reaction mixtures were placed in the Bio-Rad C1000 Touch Thermo Cycler (CFX384 Real-Time System) using the "Golding_Lab" program.

Statistical analysis

In this project, the significance level was set at 0.05. Two-way analysis of variance test (ANOVA) with Tukey's multiple comparison test were used to evaluate the independent variables. When analyzing gene qPCR results, the replicate cycle threshold (Ct) values for each gene were compared to that of two housekeeping genes, *Hprt* and *Ppia*. Relative expression

levels were calculated using the $\Delta\Delta$Ct method[9]. This data was then transferred to GraphPad (GraphPad Software, Inc., La Jolla, CA) to be analyzed with two-way ANOVA and Tukey's multiple comparison test. Statistical significance is signified with asterisks.

CHAPTER III

RESULTS AND DISCUSSION

Preconception PAE effects on placental efficiency are sex and maternal strain specific

Previous studies in the Golding lab have indicate that preconception paternal alcohol exposure correlates to decreases in placental efficiency[1]. Placental efficiency refers to the amount of fetus that a placenta of a given size can support, as seen in Equation 1. Higher placental efficiency means that a placenta can support the growth of a larger fetus.

$$\frac{Fetus\ (g)}{Placenta\ (g)} = Placental\ Efficiency \tag{1}$$

As seen in Figure 1, there were no significant differences in fetal weight noted between the groups. There was a significant decrease in placental weight from ICR control males to ICR ethanol males, but there were no other significant differences in placental weight between groups. Significant differences in placental efficiency between control and ethanol groups was seen in the male offspring, but not in females. The C57BL6/J ethanol males had a decrease in placental efficiency as compared to the control group, while in contrast, the ICR ethanol males displayed an increase in placental efficiency in comparison to the control ICR males. It was surprising to find that preconception paternal alcohol exposure was associated with changes in placental efficiency for males, but not for the female offspring. This indicates that preconception PAE has sex-specific effects on placental efficiency. It was also surprising to observe the increase in placental efficiency for ICR males in the ethanol group as compared to the control group, because this was contrary to our hypothesis. These findings show that preconception PAE

has maternal strain specific effects, implying that the differing genetics of the mother determines how preconception PAE will impact the offspring.

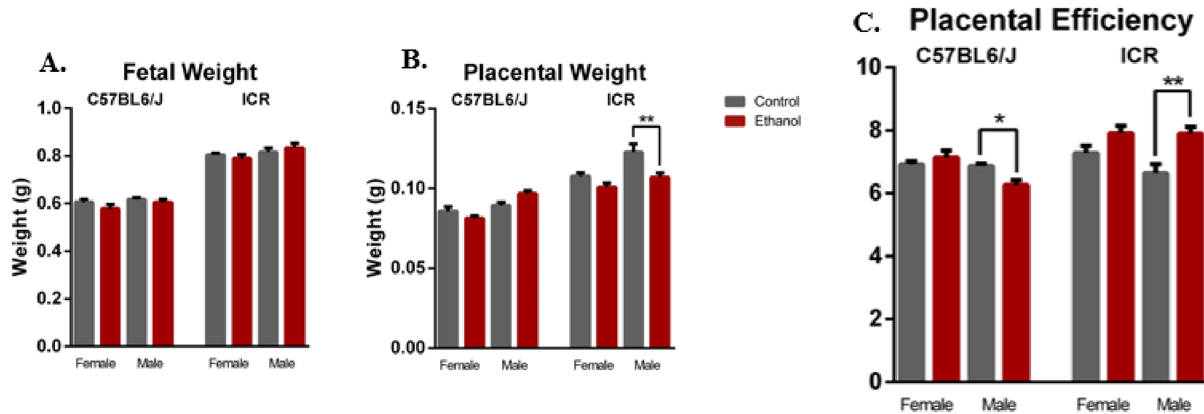

Figure 1. A) Average fetal weight across all litters. B) Average placental weight. C) Average placental efficiency. Data represent mean +/- standard deviation. *$*p<0.05$, ** $p<0.01$*. Statistics were calculated using a two-way ANOVA, with Tukey's multiple comparison test. N Value: C57BL6/J= Males: 15, Females: 24, ICR= Males: 27, Females: 25. Number of litters: C57BL6/J= Control: 4, Ethanol: 3, ICR= Control: 3, Ethanol: 3

It should be noted that the differences observed in placental efficiency among the groups in this project were not consistent with the previous findings in the Golding lab. In a previous project, the Golding lab observed a decrease in placental efficiency in ethanol females in comparison to control females, yet no significant change in placental efficiency for males[1]. Contrary to the findings in this project, the lab's previous project found that alcohol-sired females had a significant decrease in fetal weight in comparison to control-sired females, accounting for the increased placental efficiency[1]. However, considering that previous Golding projects involved the C57BL/6 (CAST7) strain, a cross between C57BL/6J and B6(CAST7) strains[1], these differences are likely explained by either maternal genetics or differences in the

uterine environment. Therefore, the discrepancies between previous findings in the Golding lab and this project further affirm the observation that preconception PAE has sex-specific and maternal strain specific effects on placental efficiency. Additionally, the Golding lab terminated pregnancies at GD 14.5 rather than GD 16.5, which could account for some variance in results[1].

Differential expression of genes related to placental development

The differential expression of six genes was analyzed in comparison to the expression of housekeeping genes using qPCR. Housekeeping genes are genes which have a relatively constant level of expression regardless of the treatment used in the experiment. The cDNA used for qPCR was synthesized from RNA that was isolated from the fetal placentas.

The two housekeeping genes used were *Hprt* and *Ppia,* which encode hypoxanthine-phosphoribosyl transferase and peptidylprolyl isomerase A respectively. Experiments preformed on other placental samples showed that these genes were expressed at a consistent level across treatment groups[1].

Four of the six candidate genes analyzed code for nuclear receptors: Nr1h3 (LXRα), Nr1h2 (LXRβ), Nr2b1 (RXRα), and Nr1h4 (FXR). Nuclear receptors are receptors located within the cell that typically act as transcription factors[6]. When activated, nuclear receptors bind to segments of DNA called a Hormone Response Element (HRE) to activate transcription of a target gene[6]. Nuclear receptors are normally activated by the presence of a lipid-soluble ligand that can cross the plasma membrane, enter the cell, and bind to the nuclear receptor, changing its shape and thus its function[6].

Differential expression of Nr2b1 gene is sex and maternal strain specific

The Nr2b1 gene encodes retinoid X receptor α (RXRα). RXRα is involved in placental development, and is especially important during the later stages of development[7]. RXRα also

plays a role in embryogenesis and eye development[7]. Mutations in this gene can decrease placental efficiency by thickening the labyrinthine trabeculae, which is where nutrients and gas are exchanged between the mother and fetus[7]. The results of the qPCR for RXRα, as seen in Figure 2, showed a significant upregulation of the Nr2b1 gene in the female C57BL6/J alcohol group in comparison to the corresponding control group. Since RXRα is involved in the development of the placenta and is crucial in the development of the labyrinthine trabeculae, it is possible that the upregulation of this gene was a method of compensating for preconception PAE and thus accounted for why the female C57BL6/J alcohol and control groups did not have a significant difference in placental efficiencies.

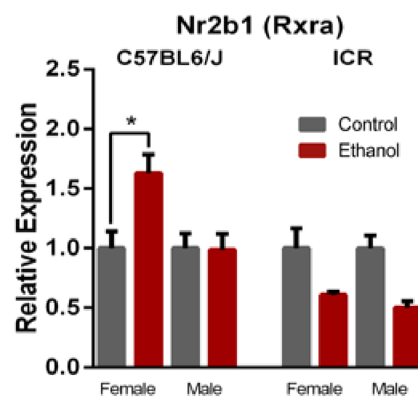

Figure 2. Average gene expression of Nr2b1 gene, encoding retinoid X receptor α (RXRα). RXRα was upregulated in female C57BL6/J offspring whose fathers consumed ethanol. Data represent mean +/- standard deviation. * $p<0.05$. Statistics were calculated using a two-way ANOVA, with Tukey's multiple comparison test. N Value: C57BL6/J= Control Males: 8, Control Females: 8, Ethanol Males: 6, Ethanol Females: 6, ICR= Control Males: 6, Control Females: 6, Ethanol Males: 6, Ethanol Females: 6. Number of litters: C57BL6/J= Control: 4, Ethanol: 3, ICR= Control: 3, Ethanol: 3

Differential expression of Nr1h4 gene is sex and maternal strain specific

The Nr1h4 gene encodes farnesoid X-activated receptor (FXR). FXR forms a heterodimer with RXR to enhance gene expression[8]. Previous research has suggested that FXR targets the gene for phospholipid transfer protein (PLTP), which changes low density lipoprotein into high density lipoprotein[8]. It is interesting to note that PLTP is expressed in very high levels in the placenta in comparison to other body tissues[8]. The results of qPCR for the Nr1h4 gene, as seen in Figure 3, showed a significant upregulation of this gene in female alcohol-sired C57BL6/J mice in comparison to the female control-sired C57BL6/J mice. There was no significant difference in the expression of the FXR gene between any of the other groups. Considering the fact that PLTP is abundant and likely important in the metabolism of lipids in the placenta, it is possible that the upregulation of this gene was also a method of compensation for preconception PAE much like what we theorized to be the case for the Nr2b1 gene.

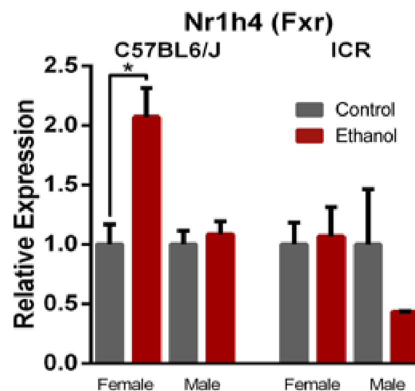

Figure 3. Average gene expression of Nr1h4 gene, encoding farnesoid X-activated receptor (FXR). FXR was upregulated in female C57BL6/J offspring whose fathers consumed ethanol. Data represent mean +/- standard deviation. * $p<0.05$. Statistics were calculated using a two-way ANOVA, with Tukey's multiple comparison test. N Value: C57BL6/J= Control Males: 8, Control Females: 8, Ethanol Males: 6, Ethanol Females: 6, ICR= Control Males: 6, Control

Females: 6, Ethanol Males: 6, Ethanol Females: 6. Number of litters: C57BL6/J= Control: 4, Ethanol: 3, ICR= Control: 3, Ethanol: 3

Expression of Nr1h3 and Nr1h2 genes

The Nr1h3 gene encodes liver X receptor α (LXRα) and the Nr1h2 gene encodes liver X receptor β (LXRβ). Like FXR, LXR nuclear receptors also form heterodimers with RXR[10]. In general, LXR plays a role in lipid absorption, storage, and use, as well as in the regulation of the concentration of sterols within a cell[10]. Studies have shown that LXRs are present early on in the process of placental development and are concentrated at the fetomaternal interface[10]. Studies suggest that these receptors play a role in lipid metabolism, hormone production, and fetomaternal immune tolerance[10]. No significant difference in expression was seen between groups, as seen in Figure 4.

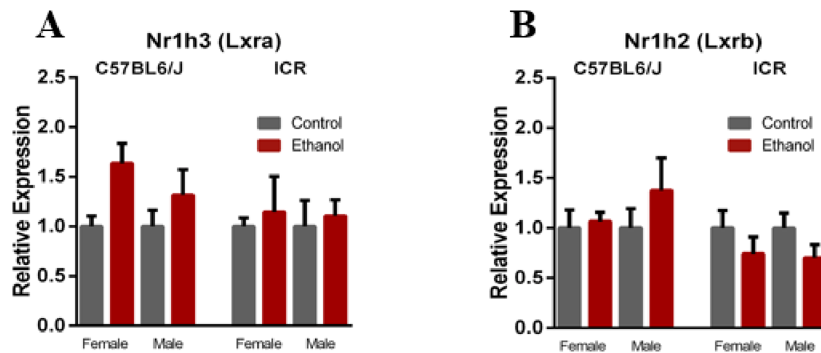

Figure 4. Average gene expression of (A) Nr1h3 gene, encoding liver X receptor α (LXRα) and (B) Nr1h2 gene, encoding liver X receptor β (LXRβ). There are no significant differences in expression levels between groups. Data represent mean +/- standard deviation. * $p<0.05$. Statistics were calculated using a two-way ANOVA, with Tukey's multiple comparison test. N Value: C57BL6/J= Control Males: 8, Control Females: 8, Ethanol Males: 6, Ethanol Females: 6,

ICR= Control Males: 6, Control Females: 6, Ethanol Males: 6, Ethanol Females: 6. Number of litters: C57BL6/J= Control: 4, Ethanol: 3, ICR= Control: 3, Ethanol: 3

Expression of Col4a1 gene

The Col4a1 gene codes for a protein that is part of type IV collagen[11]. This gene helps create networks that become the main component of basement membranes, which support and separate cells in tissues[11]. For this reason, the Col4a1 gene is important in cell maturation, differentiation, proliferation, migration, and survival[11]. Results from qPCR for the Col4a1 gene showed no significant differences between groups, as seen in Figure 5.

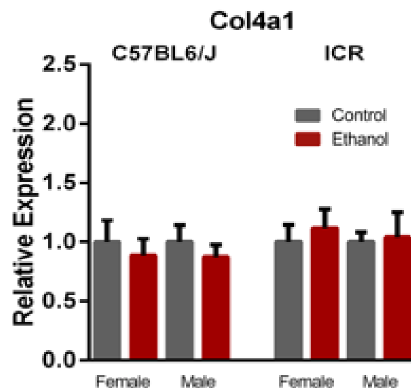

Figure 5. Average gene expression of Col4a1 gene. There are no significant differences in expression levels between groups. Data represent mean +/- standard deviation. * *p<0.05.* Statistics were calculated using a two-way ANOVA, with Tukey's multiple comparison test. N Value: C57BL6/J= Control Males: 8, Control Females: 8, Ethanol Males: 6, Ethanol Females: 6, ICR= Control Males: 6, Control Females: 6, Ethanol Males: 6, Ethanol Females: 6. Number of litters: C57BL6/J= Control: 4, Ethanol: 3, ICR= Control: 3, Ethanol: 3

Expression of Apoa4 gene

The Apoa4 gene codes for apolipoprotein A4. This protein is involved in the plasma lipid transport system[12]. This apolipoprotein will interact with lipids and acts as a carrier for these lipids in both intravascular and extravascular spaces[12]. The results of the qPCR for this gene showed no significant differences between groups, as seen in Figure 6.

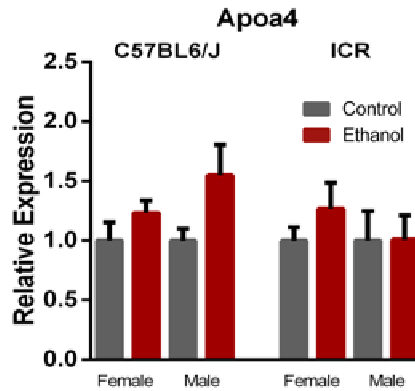

Figure 6. Average gene expression of Apoa4 gene. There are no significant differences in expression levels between groups. Data represent mean +/- standard deviation. * *p<0.05.* Statistics were calculated using a two-way ANOVA, with Tukey's multiple comparison test. N Value: C57BL6/J= Control Males: 8, Control Females: 8, Ethanol Males: 6, Ethanol Females: 6, ICR= Control Males: 6, Control Females: 6, Ethanol Males: 6, Ethanol Females: 6. Number of litters: C57BL6/J= Control: 4, Ethanol: 3, ICR= Control: 3, Ethanol: 3

CHAPTER IV

CONCLUSION

The relationship between preconception paternal alcohol exposure and placental efficiency varied depending on maternal genetic background and the sex of the fetus. Males showed a greater difference in placental efficiency between control and alcohol groups than females. Among males, preconception paternal alcohol exposure had maternal strain specific effects, with decreases in placental efficiency for those with C57BL6/J mothers yet increases for those with ICR mothers. These surprising results imply interaction between the father's sperm and the uterine environment to impact placental efficiency. Both RXRα and FXR genes showed significantly increased expression in C57BL6/J females with alcohol-exposed fathers in comparison to those with control fathers. These genes code for nuclear receptors associated with placental development/efficiency and lipid metabolism respectively. The upregulation of these genes paired with the insignificant difference in placental efficiency between control and alcohol female C57BL6/J groups suggest that the C57BL6/J females express these genes as a way to compensate for the stress caused by PAE.

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