Preconception Paternal Alcohol Exposure Decreases IVF Embryo Survival and Pregnancy Success Rates in a Mouse Model.

Running Title: **Paternal Alcohol Exposures and IVF Outcomes**

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Abstract

Increasingly, couples struggling with fertility turn to assisted reproductive techniques, including *in vitro* fertilization (IVF), to have children. Despite the demonstrated influence of periconception male health and lifestyle choices on offspring development, studies examining IVF success rates and child health outcomes remain exclusively focused on maternal factors. Using a physiologically relevant mouse model, we tested the hypothesis that chronic paternal preconception alcohol intake adversely affects IVF success and negatively impacts IVF offspring fetoplacental growth. Using a voluntary, binge-like mouse model, we exposed sexually mature C57BL/6J males to three preconception treatments (0% (Control), 6% EtOH or 10% EtOH) for six weeks, isolated and cryopreserved caudal sperm from treated males, and then used these samples to fertilise oocytes before assessing IVF embryo developmental outcomes. We found that preconception paternal alcohol use reduced IVF embryo survival and pregnancy success rates in a dose-dependent manner, with the pregnancy success rate of the 10% EtOH treatment falling to half those of the Controls. Mechanistically, we found that preconception paternal alcohol exposure disrupts embryonic gene expression, including *Fgf4* and *Egfr*, two critical regulators of trophectoderm stem cell growth and placental patterning, with lasting impacts on the histological organization of the late-term placenta. The changes in placental histoarchitecture were accompanied by altered regulation of pathways controlling mitochondrial function, oxidative phosphorylation and some imprinted genes. Our studies indicate that male alcohol use may significantly impede IVF success rates, increasing the couple's financial burden and

emotional stress, and highlights the need to expand prepregnancy messaging to emphasize the reproductive dangers of alcohol use by both parents.

Keywords: *in vitro* fertilization; epigenetics; paternal; alcohol; sperm; Fetal Alcohol Spectrum Disorder; mitochondrial dysfunction; placenta

Introduction

In vitro fertilization (IVF) is a medical technique where spermatozoa fertilize oocytes outside the body, generating embryos that are subsequently transferred into the female reproductive tract and carried to term (Serafini, 2001). Couples experiencing infertility who desire a biological family turn towards IVF when they have failed to conceive naturally (Bavister, 2002; Johnson, 2019). After almost half a century of research and advancement, over 9 million infants have been conceived via IVF, and today, assisted reproductive technologies (ARTs) account for 2% of all infants born in the United States (Carson and Kallen, 2021).

To date, most investigations into pregnancy loss and poor IVF success rates have focused on the influence of maternal stressors and periconceptional health (Homan *et al.*, 2007; Rossi *et al.*, 2011; Nicolau *et al.*, 2014; Wdowiak *et al.*, 2014; Hornstein, 2016). However, a recent wave of research demonstrates that a wide range of stressors and toxicants alters the sperm-inherited developmental program, negatively affecting offspring fetoplacental growth and long-term health (Fleming *et al.*, 2018). Unfortunately, despite the demonstrated importance of the sperm epigenome to offspring development, studies examining IVF outcomes seldom consider paternal exposures or explore dimensions of male fertility beyond sperm morphology and motility.

In the United States, alcohol use is widespread (Esser *et al.*, 2014; Mahnke *et al.*, 2019), with annual sales reaching 252 billion US dollars in 2019 alone (Castaldelli-Maia *et al.*, 2021). It is well established that men drink more than women, and significantly, 72% of men consume alcohol on

a weekly basis (Naimi *et al.*, 2003; White *et al.*, 2006; Kanny *et al.*, 2018). However, despite the emerging importance of paternal alcohol use to child health and development, very few studies consider the impact of male alcohol use on reproductive function, while the small number of studies that do are confounded and often contradictory. For example, some studies using animal models suggest chronic moderate-level (Anderson *et al.*, 1980; Salonen *et al.*, 1992) and supraphysiological alcohol exposures induce lower fecundity, decrease in gonad function and alter semen parameters (Akingbemi *et al.*, 1997; Emanuele *et al.*, 2001; Lee *et al.*, 2010; Sánchez *et al.*, 2018). In contrast, studies examining limited binge-like exposures suggest no impacts (Bedi *et al.*, 2019). Clinical studies also provide conflicting data on the impacts of alcohol intake on hormone production, sperm count and morphology, and overall fertility (Gümüş *et al.*, 1998; Muthusami and Chinnaswamy, 2005; Jensen *et al.*, 2014; Condorelli *et al.*, 2015; Van Heertum and Rossi, 2017). Therefore, the effects of alcohol use on male reproductive physiology remain unclear. Nevertheless, alcohol is associated with intranuclear changes in mature spermatozoa, resulting in irregular chromatin condensation (Sánchez *et al.*, 2018) as well as modifications to the sperm epigenome (Rompala *et al.*, 2018; Bedi *et al.*, 2019, 2022).

Unfortunately, most human studies examining interactions between alcohol use and IVF either neglect the male's drinking habits and exclusively focus on female drinking (Rossi *et al.*, 2013; Wdowiak *et al.*, 2014; Dodge *et al.*, 2017; Lyngsø *et al.*, 2019) or are significantly confounded by multiple lifestyle factors (Nicolau *et al.*, 2014; Hornstein, 2016). Only two studies have examined the effects of both maternal and paternal alcohol use on IVF outcomes. A five-year IVF and gamete intrafallopian transfer (GIFT) study in California found decreased chances of achieving a

live birth and increased risks of miscarriage when the father consumed alcohol. Notably, alcohol intake did not significantly affect sperm concentration, motility or morphology (Klonoff-Cohen *et al.*, 2003). In contrast, a ten-year IVF analysis in Boston revealed no statistically significant association between live births and male drinking (Rossi *et al.*, 2011). Unlike the California report, the Boston study identified significantly lower sperm counts and irregular morphologies in semen samples from men who drank but only those who drank wine (Rossi *et al.*, 2011). However, these limited studies were too underpowered to reliably detect an impact of paternal alcohol use and are significantly confounded by differing genetics, environmental exposures and often unknown causes of infertility. Therefore, the influence of paternal alcohol use on IVF outcomes remains poorly described (Nicolau *et al.*, 2014; Hornstein, 2016).

In clinical studies, IVF babies are predisposed to preterm birth, lower birth weights and increased rates of congenital disabilities (Chang *et al.*, 2020). In support of these clinical observations, several preclinical studies have revealed that ovarian stimulation, IVF and embryo culture lead to placental defects, including placentomegaly, reduced placental efficiency, modified histoarchitecture and altered metabolic function (Collier *et al.*, 2009; Delle Piane *et al.*, 2010; Bloise *et al.*, 2012; de Waal *et al.*, 2015; Tan *et al.*, 2016; Dong *et al.*, 2021; Bai *et al.*, 2022). Notably, the IVF-associated changes in placental growth correlate with disruptions in imprinted gene expression, suggesting that epigenetic mechanisms potentially contribute to these pathological outcomes (Mann *et al.*, 2004; de Waal *et al.*, 2015).

Our previous studies using a C57BL/6J mouse model reveal that preconception paternal alcohol exposures transmit an intergenerational stressor that also negatively affects placental growth, efficiency, histology and metabolism (Bedi *et al.*, 2019; Chang *et al.*, 2019b, 2019a; Thomas *et al.*, 2021, 2022). Specifically, paternal alcohol use correlates with sex-specific, dose-dependent changes in placental histoarchitecture and alterations in the control of imprinted gene expression (Thomas *et al.*, 2021, 2022). Based on these shared phenotypic outcomes, we hypothesized that alcohol-induced changes in the paternal epigenetic program would exacerbate the placental growth phenotypes induced by IVF, resulting in increased rates of congenital disabilities and pregnancy loss. Using a physiologically relevant mouse model of chronic alcohol exposure, we found that male alcohol use negatively impacts IVF embryo development and pregnancy success rates. Critically, our data reveal that evaluating male exposure history and lifestyle choices is essential to optimizing the chances of achieving a healthy pregnancy and that male alcohol use may be a crucial, unrecognized factor affecting IVF outcomes.

Materials and Methods

Ethics Statement

We conducted all experiments under AUP 2017–0308, approved by the Texas A&M University IACUC. All experiments were performed following IACUC guidelines and regulations. Here, we report our data per ARRIVE guidelines.

Animal husbandry and preconception paternal alcohol exposures

In our studies, we used C57BL/6J strain (RRID:IMSR_JAX:000664) mice, which we obtained and housed in the Texas A&M Institute for Genomic Medicine, fed a standard diet (catalog# 2019, Teklad Diets, Madison, WI, USA), and maintained on a reverse 12-h light/dark cycle. To reduce stress, a known confounder modulating paternal epigenetic inheritance (Chan *et al.*, 2018), we subjected males to minimal handling and provided them with additional cage enrichments, including shelter tubes (catalog# K3322, Bio-Serv, Flemington, NJ, USA).

Before initiating the Ethanol (EtOH) and Control preconception treatments, we acclimated male mice to individual housing conditions for one week. We then randomly assigned sexually mature, postnatal day 90 male mice to one of three treatment groups (n=8). Using a prolonged version of the Drinking in the Dark model of voluntary alcohol consumption (Rhodes *et al.*, 2005), we exposed males to the Control or EtOH treatments for four hours per day, beginning three hours

after the initiation of the dark cycle. Using methods described previously (Thomas *et al.*, 2022), we replaced the home cage water bottle with a bottle containing either: 0% (Control), 6% (6% EtOH) or 10% (10% EtOH) w/v ethanol (catalog# E7023; Millipore-Sigma, St. Louis, MO, USA). We simultaneously exchanged the water bottles of Control and EtOH-exposed males to ensure identical handling and stressors. We recorded the weekly weight of each male (g) and the amount of fluid consumed (g) and then calculated weekly fluid consumption and average daily EtOH dose as described previously (Thomas *et al.*, 2022).

During the mating phase, we bred exposed males to naive postnatal day 90 C57BL/6J females. We synchronized the female reproductive cycles using the Whitten method (Whitten *et al.*, 1968), then placed the female in the male's home cage immediately after the male's daily exposure window. After eighteen hours, we recorded the presence of a vaginal plug and returned females to their original cages.

In vitro fertilization and embryo culture

The process used for IVF and embryo culture is described in Figure 1A. After ten weeks of exposure, we sacrificed exposed males using CO_2 asphyxiation followed by cervical dislocation, then extracted mature caudal sperm, which we cryopreserved using CARD MEDIUM (catalog # KYD-003-EX, Cosmo Bio USA, Carlsbad, CA, USA) and methods described by (Nakagata, 2011). We visually confirmed sperm motility, then transferred frozen sperm to liquid nitrogen for long-term storage. We then superovulated three to five-week-old C57BL/6J female mice using timed

intraperitoneal injections of 6.25 IU PMSG (catalog # HOR-272, Prospec Bio, East Brunswick, NJ, USA), followed 48 hours later by 6.25 IU HCG (catalog # C1063, Millipore-Sigma, St. Louis, MO, USA). We then sacrificed the superovulated females and isolated cumulus-oocyte complexes, which we placed in prewarmed CARD MEDIUM. Next, we thawed sperm straws in a 37˚C water bath, then added 10µl of sperm to 90µl of FERTIUP preincubation medium (catalog # KYD-002-05-EX, Cosmo Bio USA, Carlsbad, CA, USA) in a 37˚C incubator (5% CO_2 in air). We placed cumulus-oocyte complexes in 90µl of CARD MEDIUM. After 35 minutes of capacitation in FERTIUP, we added 10µl of sperm for a total culture volume of 100ul, then incubated sperm and cumulus-oocyte complexes for four hours.

We counted 1-cell stage embryos after 3-5 hours of culture, then collected 2-cell embryos after 1.5 days of culture, which we transferred (maximum of 8-14 two-cell embryos per dam) into 10–12-week-old pseudopregnant recipient C57BL/6J females (see below). Finally, we cultured the remaining embryos for a total of 60 hours, collected morula-stage embryos, then snap-froze pools of 10-15 morulae in 350µl of RLT lysis buffer (catalog # 74136, Qiagen, Germantown, MD, USA) containing 1% 2-Mercaptoethanol (catalog # M6250, Millipore-Sigma, St. Louis, MO, USA). We then sent samples to Quick Biology (Pasadena, California, USA) for mRNA isolation and deep sequencing.

We bred 10–12-week-old recipient C57BL/6J females to vasectomized C57BL/6J males and selected pseudopregnant dams based on the presence of a copulation plug. After anesthetizing the recipient dams, we opened the abdominal cavity under a dissection stereomicroscope,

created an incision in the oviduct, then inserted a glass capillary containing the 2-cell embryos and expelled the embryos towards the ampulla. Finally, we pushed the ovary, oviduct and uterine horn back into the abdomen, closed the wound using wound clips, then kept the mice warm on a 37°C warming pad until the animal recovered from the effects of anesthesia.

Fetal dissection and tissue collection

We co-housed recipient dams and provided them with additional nesting materials and cage enrichment, including igloo huts (catalog# K3570, Bio-Serv). The 2-cell embryos were transferred after 1.5 days of culture. We then sacrificed recipient dams 15 days later, corresponding to embryonic day 16.5, using CO_2 asphyxiation followed by cervical dislocation. Subsequently, we dissected the female reproductive tract and recorded fetoplacental measures. We either fixed tissue samples in 10% neutral buffered formalin (catalog# 16004-128, VWR, Radnor, PA, USA) or snap-froze the tissues on dry ice and stored them at −80°C.

Fetal sex determination

We isolated genomic DNA from the fetal tail using the HotSHOT method (Truett *et al.*, 2000) and then determined fetal sex using a PCR-based assay described previously (Thomas *et al.*, 2021).

Placental RNA isolation and RT-qPCR gene expression

We isolated RNA from gestational day 16.5 placentae using the Qiagen RNeasy Plus Mini Kit (cat# 74136, Qiagen, Germantown, MD, USA). We then seeded approximately one µg of RNA into a reverse transcription reaction using the High-Capacity cDNA Reverse Transcription Kit (catalog# 4368814, Thermo-Fisher, Waltham, MA, USA). Using published methods and primer sequences (Thomas *et al.*, 2021, 2022), we determined the relative levels of candidate gene transcripts using the AzuraView GreenFast qPCR Blue Mix LR kit (Cat No. AZ-2320: Azura Genomics, Raynham, Massachusetts, USA). We describe the data normalization and handling procedures below.

Analysis of placental histology

To increase tissue contrast, we treated tissue samples with phosphotungstic acid (Lesciotto *et al.*, 2020) and then processed samples for imaging using MicroComputed tomography (microCT), using methods described previously (Thomas *et al.*, 2021, 2022). Briefly, we incubated half placentae in 5% phosphotungstic acid (w/v) dissolved in 90% methanol for four hours and then held samples in 90% methanol overnight. We then incubated samples in progressive reductions of methanol (80%, 70%, 50%) each for one day, then moved placentae into PBS with 0.01% sodium azide for long-term storage. Finally, we embedded placentae in a 50/50 mixture of polyester and paraffin wax to prevent tissue desiccation, then imaged samples on a SCANCO vivaCT 40 (SCANCO Medical AG Brüttisellen, Switzerland) using a 55 kVp voltage x-ray tube and 29uA exposure. The resulting microCT image voxel size was 0.0105mm^3, with a resolution of 95.2381 pixels/mm. After scanning, we used the open-source medical image analysis software

Horos (Version 3.3.6; Nibble Co LLC, Annapolis, Maryland, USA; https://horosproject.org/) to measure placental features.

To contrast placental glycogen levels between treatments, we processed and sectioned paraffin-embedded placental samples as described previously (Thomas *et al.*, 2022), then stained the slides using a Periodic Acid Schiff (PAS) Stain Kit (Mucin Stain) (catalog #ab150680, Abcam, Boston, MA, United States) following the manufacturer's provided protocol. We imaged samples using the VS120 Virtual Slide Microscope (Olympus, Waltham, MA, United States) and analyzed the images with the included desktop software, OlyVIA (Version 2.8, Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Using Photoshop (Version 21.0.1, Adobe, San Jose, CA, United States) and ImageJ (Version 1.53f51, Wayne Rasband and contributors, National Institutes of Health, United States), we calculated the PAS-stained area of the decidua and junctional zones, then normalized this value to the area of the decidua and junctional zone.

Informatic analysis

We used the open-source, web-based Galaxy server (Afgan *et al.*, 2018) (usegalaxy.org) to process and analyze our data files. First, we used MultiQC (Ewels *et al.*, 2016) to perform quality control on the raw paired-end, total RNA sequence files and then trimmed the sequences of Illumina adapters using Trimmomatic (Bolger *et al.*, 2014). Next, we used RNA STAR (Dobin *et al.*, 2013) to map the reads to the Mus musculus reference genome (UCSC version GRCm39/mm39). We then used featureCounts (Liao *et al.*, 2014) with a minimum mapping quality per read of 10

to determine read abundance for all genes, followed by annotation versus M27 GTF (GENCODE, 2020). Finally, we analyzed the generated featureCounts files using the DESeq2 (Love *et al.*, 2014) function with default parameters to generate the PCA plots and the Volcano Plot function to produce graphical representations of the (log2FC, q-value <0.05) gene expression levels for the top 25 significant genes. Next, we exported the differentially expressed genes (log2FC, unadjusted p-value <0.05) into the Ingenuity Pathway Analysis software package and conducted gene enrichment analysis (Jiménez-Marín *et al.*, 2009; Krämer *et al.*, 2014).

We generated heatmaps for differentially expressed genes using the 'pheatmap' (Kolde, Raivo. "Pheatmap: pretty heatmaps." R package version 1.2 (2012): 726.) package on R version 4.2.1 (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) We transformed the raw counts using variance stabilized transformation (VST) in DESeq2 (Love *et al.*, 2014) and used the VST counts to plot the heatmap. We calculated z-scores using the scale = 'row' function. We used Venny 2.1 (Oliveros, J.C. (2007-2015) Venny, an interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html) to generate Venn diagrams comparing differentially expressed genes between treatments.

Data Management

The data generated in this study were managed using a detailed data management plan that prioritizes safe and efficient data handling. For long-term storage, retrieval and preservation, we

have stored all data on Google Drive. The sequence files generated from this project can be obtained in the GEO database under accession number GSE214726.

Statistical analysis

We initially collected physiological measures for each exposed male, embryo, or fetus by hand and then transcribed these data into Google Sheets or Microsoft Excel, where we collated the data. We then transferred the physiological and molecular data into the statistical analysis program GraphPad Prism 9 (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, United States), set the statistical significance at alpha = 0.05, used the ROUT test (Q = 1%) to identify outliers, and then verified the normality of the datasets using the Shapiro-Wilk test. If data passed normality (alpha = 0.05), we employed either a One-way or Two-way ANOVA or an unpaired, parametric (two-tailed) t-test. If the data failed the test for normality or we observed unequal variance (Brown Forsythe test), we ran a Kruskal-Wallis test followed by Dunn's multiple comparisons test or a non-parametric Mann–Whitney test.

For measures of fetoplacental growth, we determined the male and female average for each litter and used this value as the individual statistical unit. As we observed differences in litter size (Figure 2B), which can exert stage-specific impacts on fetal weight (Ishikawa *et al.*, 2006), we also compared the collective measures of individual fetuses and reported the analysis of both datasets. To calculate placental efficiency (Hayward *et al.*, 2016), we divided fetal weight by placental weight. For offspring organ weights and the analysis of placental histology, we

randomly selected male and female offspring from each litter and used measures of these samples as the statistical unit. For RT-qPCR analysis of gene expression, we imported the replicate cycle threshold (Ct) values for each transcript into Excel, then normalized expression to the geometric mean of two validated reference genes, including transcripts encoding Phosphoglycerate kinase 1 (*Pgk1*) and 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*Ywhaz*). We then used the -ΔΔCT method (Schmittgen and Livak, 2008) to calculate the relative fold change for each biological replicate. Data presented are mean +/− standard error of the mean. For each figure presented, we provide detailed descriptions of each statistical test in Supplementary Table SI.

Results

A physiologically relevant mouse model to examine the impacts of chronic preconception paternal alcohol use on IVF outcomes.

To understand the impact of preconception paternal alcohol use on IVF outcomes, we employed an established mouse model of voluntary alcohol exposure that mimics chronic binge drinking (Boehm *et al.*, 2008), isolated sperm from exposed males, then used these samples in an established IVF protocol (Nakagata, 2011) (Figure 1A). Using a physiologically relevant, prolonged version of the limited access Drinking in the Dark model, we exposed postnatal day 90, C57BL/6J male mice to 6% and 10% (w/v) ethanol (EtOH) treatments. We have previously found that these treatments exert dose-dependent effects on offspring placental growth and histology, with the 6% treatment increasing litter average placental weights while the 10% treatment (2.1-4.86 g/kg) was associated with placental growth restriction (Thomas *et al.*, 2022). We exposed Control males to water alone and ensured identical handling by concurrently switching between two identical water bottles. We maintained treatments for six weeks, then bred exposed males to naïve C57BL/6J dams during a three-week mating phase while maintaining the preconception treatments. We did not observe any differences in fluid consumption between the Control and EtOH treatment groups (Figure 1B). During the three-week breeding phase, a subset of the exposed males produced litters (Supplementary Table SII). After resting for one week, we sacrificed exposed males at ten weeks of total exposure, then collected and cryopreserved the sperm (Nakagata, 2011). Males in the 6% EtOH treatment group received an average daily dose

of 1.7g/kg, while males in the 10% EtOH treatment group received an average daily dose of 2.2g/kg (Figure 1C). Consistent with our previous studies using this model, plasma alcohol concentrations averaged ~100 and ~115 mg/dL for the 6% EtOH and 10% EtOH treatment groups, respectively (data not shown). We did not observe any difference in weekly weight gain between treatment groups (Figure 1D).

Preconception paternal alcohol use reduces IVF embryo survival and pregnancy success rates.

Using sperm isolated from treated males, we employed an established IVF protocol (Nakagata, 2011) to generate preimplantation embryos. Despite the potential for stressors associated with superovulation and *in vitro* embryo culture to obscure paternal epigenetic effects on offspring development, we found that, compared with Control IVF offspring, embryos generated using alcohol-exposed sperm exhibited decreased development rates and survival. For example, compared to sperm isolated from Control males, we observed a dose-dependent ($p > 0.0134$) decrease in fertilization rates, as measured by the generation of 1-cell zygotes after 36 hours of culture (Table I). However, we did not observe any significant differences in the number of 1-cell transitioning to 2-cell stage embryos (Table II). We transferred 2-cell stage embryos into naïve recipient dams and examined pregnancy rates at gestational day 16.5, a developmental phase where we have previously characterized alcohol-induced alterations to fetoplacental growth and patterning (Bedi *et al.*, 2019; Thomas *et al.*, 2021, 2022). Preconception paternal EtOH exposure significantly decreased both the number of surviving offspring from each embryo transfer (Figure 2A) and the total number of two-cell embryos surviving to gestational day (GD)16.5 (Table III),

with the 6% EtOH treatment group exhibiting a 24% decline and the 10% EtOH treatment group displaying a 32% reduction in total embryo survival, compared to sperm derived from males in the Control treatment. In contrast to our previous studies, which did not identify any impacts on litter size (Chang *et al.*, 2017, 2019b; Bedi *et al.*, 2019; Thomas *et al.*, 2021, 2022), both the 6% EtOH and 10% EtOH IVF treatment groups exhibited reductions in the number of live offspring (Figure 2B). Finally, we observed a significant (Chi-square analysis, p=0.0448) reduction in our pregnancy success rates, with the 6% EtOH treatment displaying a 20% decline, while pregnancy rates from the 10% EtOH treatment were half those of the Control treatment (Figure 2C). For the full dataset, please see Supplementary Table SIII. These results demonstrate that chronic preconception male alcohol use significantly decreases IVF embryo survival and pregnancy success rates.

Paternal alcohol exposures disrupt embryonic gene expression patterns.

To better understand the molecular basis of the reduced IVF success rates, we used deep sequencing to contrast the embryonic transcriptome between the three treatment groups. Previous studies demonstrate that IVF procedures alter the allocation of cells between the embryonic and extraembryonic lineages, disrupting the earliest phases of placental development initiated at the blastocyst stage (Bai *et al.*, 2022). We suspected that alcohol might exacerbate these molecular changes and wanted to determine their developmental origins. Therefore, we focused our analyses on the morula stage, which precedes differentiation into the founding lineages. To this end, we pooled 10-15 morulae per male, isolated RNA, sequenced the generated

cDNA libraries (n=3 per treatment), and compared gene expression patterns using DESeq2. After adjusting for false discovery (log2-fold change, q-value < 0.05), our analysis identified a small number of differentially expressed genes (DEGs) between treatment groups (16 Control vs. 6% EtOH, 35 Control vs. 10% EtOH, and 30 6% EtOH vs. 10% EtOH). Of note, when comparing the 10% EtOH treatment to Controls, we identified increased enrichment of transcripts encoding genes functioning as critical regulators of trophectoderm stem cell growth and placental patterning, including Fibroblast growth factor 4 (*Fgf4*), epidermal growth factor receptor (*Egfr*) and Marvel domain containing 1 (*Marveld1*) (Tanaka *et al.*, 1998; Chen *et al.*, 2016, 2018) (Supplementary Table SIV). These observations suggest that preconception paternal alcohol exposures may disrupt critical pathways regulating placental development.

To further understand the impacts of paternal drinking on embryonic development, we relaxed the stringency of our informatic analysis to include genes with an unadjusted p-value of less than 0.05 and used this list to conduct Ingenuity Pathway Analysis (Jiménez-Marín *et al.*, 2009). Using these criteria, we found a wide variation in embryonic gene expression patterns, with largely distinct sets of genes and genetic pathways emerging across comparisons between the Control-6% EtOH, Control-10% EtOH, and 6% EtOH-10% EtOH treatments (Figure 3A-H). Importantly, in our comparisons of morulae derived from the 6% EtOH and 10% EtOH treatment groups, we identified alterations in genetic pathways associated with mitochondrial dysfunction, oxidative phosphorylation and Sirtuin signaling (Figure 3I). We have previously identified disruptions in these same pathways in the gestational day 16.5 placentae of naturally conceived offspring sired by alcohol-exposed males (Thomas *et al.*, 2021, 2022).

Preconception paternal alcohol exposure alters the growth and development of IVF offspring.

We next examined the impact of paternal alcohol exposure on IVF fetal offspring growth and development at gestational day 16.5. Despite observing smaller litter sizes (6% EtOH p-value=0.0265, 10% EtOH p-value=0.0039), we did not observe any significant impacts of paternal EtOH exposure on gestational sac weights or fetal weights (Figure 4A-B). However, we did observe reductions in fetal crown-rump lengths for the male offspring of alcohol-exposed sires (Figure 4C). However, we only observed these differences when comparing individual offspring, not litter averages. Next, we compared offspring normalized organ weights to determine whether paternal EtOH could induce programmed changes in organogenesis. We did not observe any differences in normalized brain weights (Figure 4D). However, male IVF offspring from the 10% EtOH group displayed reduced heart weights (Figure 4E). Interestingly, male and female offspring generated using sperm from alcohol-exposed sires displayed reduced thymic weights (Figure 4F). The 6% EtOH and 10% EtOH treatment groups both displayed reduced thymic weights in males, while in the female offspring, only the 10% EtOH treatment group was significantly different. These observations indicate that paternal alcohol use programs dose and sex-specific changes in IVF offspring growth and organogenesis.

Paternal ethanol exposure modifies the histological organization of IVF-offspring placentae.

Given the identified up-regulation of genes controlling trophoblast differentiation and placental patterning, we next examined the gestational day 16.5 placenta. In contrast to our previous studies examining naturally conceived offspring (Bedi *et al.*, 2019; Thomas *et al.*, 2021, 2022), we did not observe any influence of paternal alcohol exposure on placental weights and only modest effects on placental diameter and placental efficiency (Figure 5A-C). Notably, we did not observe any differences when comparing litter averages, only when comparing individual offspring. Previous studies demonstrate that IVF procedures induce changes in placental histoarchitecture (Collier *et al.*, 2009; Delle Piane *et al.*, 2010; Bloise *et al.*, 2012; de Waal *et al.*, 2015; Tan *et al.*, 2016; Dong *et al.*, 2021; Bai *et al.*, 2022). Similarly, we have found that chronic paternal EtOH exposures program male-specific changes in the histological organization of the placenta (Thomas *et al.*, 2021, 2022). Therefore, we stained male and female placentae from each treatment group with phosphotungstic acid to enhance tissue contrast and then used microCT imaging to quantify the volumes of the different placental layers (Lesciotto *et al.*, 2020).

In contrast to our previous studies examining placentae derived from EtOH-exposed sires, we did not observe any differences in the proportional volumes of the chorion or the labyrinth layers (Figure 5D & G). We did, however, observe increases in the maternal decidua and decreases in the fetal junctional zone (Figure 5E & G). Unexpectedly, compared to our previous studies, which identified male-specific changes in placental histology, we observed histological changes in the decidua and junctional zone, as well as proportional changes in the labyrinth junctional zone, in

both sexes (Figure 5H & I). Finally, reductions in the junctional zone may be due to decreased phosphotungstic acid staining arising from increased glycogen content. Further, IVF procedures are known to increase the glycogen content of the placenta (Dong *et al.*, 2021). Therefore, we utilized Periodic acid Schiff (PAS)-stained tissue sections to quantify placental glycogen levels in male placentae. These analyses identified a significant increase ($p<0.05$) in glycogen levels in placentae isolated from male offspring in the 10% EtOH treatment group but not in the 6% EtOH treatment (Figure 5J). We did not observe any differences in the number of glycogen islands in the labyrinth layer between treatment groups (data not shown). Our morphometric and histological data reveal that the IVF offspring of EtOH-exposed sires display histological changes in placental patterning and increases in placental glycogen beyond those induced by IVF procedures (Dong *et al.*, 2021) and that, unlike natural matings, female IVF offspring also display EtOH-induced changes.

Chronic male alcohol exposure disrupts IVF-embryo placental gene expression.

Our comparisons of embryonic gene expression patterns between morulae derived from the 6% EtOH and 10% EtOH treatment groups identified alterations in genetic pathways associated with mitochondrial dysfunction, oxidative phosphorylation and sirtuin signaling. Given our previous identification of these gene sets and altered imprinted gene expression in placentae derived from alcohol-exposed males using natural matings (Thomas *et al.*, 2021, 2022), we assayed Control and 10% EtOH placentae to determine if this same transcriptional signature presents in IVF embryos. RT-qPCR analysis confirmed the disruption of multiple imprinted genes and genes

involved in the identified pathways in placentae derived from IVF offspring (Figure 6). These data reveal the persistence of an alcohol-induced transcriptional signature in placentae derived from IVF offspring generated using alcohol-exposed sperm.

Discussion

Emerging biomedical and clinical evidence convincingly demonstrates that epigenetic factors carried in sperm significantly influence the health of future generations (Lane *et al.*, 2014; Fleming *et al.*, 2018). However, across the spectrum of male reproductive biology, there is a foundational lack of knowledge concerning the impacts of epigenetic processes on sperm production and their heritable influences on embryonic development. This knowledge gap impedes our ability to recognize the importance of paternal health in the development of birth defects and disease, as well as larger aspects of male infertility. Consequently, through their maternal-centric focus, the medical community perpetuates the stigma that birth defects are exclusively the woman's fault and create an imbalance in clinical practice that forces females to bare the burden of male infertility (Barratt *et al.*, 2018). This inequity is especially evident in fertility clinics where the perception that IVF success rates are the exclusive consequence of maternal health and lifestyle while, in contrast, men are given a 'free pass', and paternal periconceptional health and lifestyle choices are neither scrutinized nor recorded.

Using a physiologically relevant mouse model of chronic ethanol exposure, our study demonstrates that paternal alcohol use significantly reduces IVF embryo survival and pregnancy

success rates. Furthermore, these adverse outcomes are associated with alterations in embryonic gene expression and downstream placental dysfunction, suggesting an epigenetic memory of preconception alcohol use transmits through sperm, disrupting the formation and function of the placenta. Given the higher prevalence of preterm birth, lower birth weights and congenital disabilities in IVF children (Chang *et al.*, 2020) and that chronic male alcohol use is widespread (Naimi *et al.*, 2003), we must investigate the impacts alcohol-induced alterations in sperm epigenetic programming have on IVF embryo growth and development. Further, given the established influences of poor placentation on infant health and adult onset of disease (Burton *et al.*, 2016), a better understanding of the impacts paternal alcohol use has on placental biology may help explain why the life expectancy of people with fetal alcohol syndrome is 34 years, less than half of the broader population (Thanh and Jonsson, 2016).

Our studies utilized C57Bl6/J mice, which, although an established model for studying the teratogenic effects of alcohol (Petrelli *et al.*, 2018), are an inbred strain exhibiting comparatively poor fecundity (Rennie *et al.*, 2012). Using this genetic background, preconception paternal alcohol exposures reduced embryo development rates to the point that obtaining the requisite number of litters required for our analysis necessitated conducting 1.5x to 2x the number of embryo transfers (Figure 2C). Although mouse models do not strictly translate to humans (mice metabolize alcohol 5.5 times faster than men and, therefore, require higher doses to feel the effects (Jeanblanc *et al.*, 2019)), the average daily dose observed in the 6% treatment group (1.7 g EtOH/kg of body weight) is roughly equivalent to a 75 kg man drinking two and a quarter beers per hour for four hours (total of nine), while the 10% treatment (2.2 EtOH/kg body weight) is

equivalent to a 75 kg man consuming twelve beers in four hours, both of which are exposure levels commonly observed among US males (Naimi *et al.*, 2003; White *et al.*, 2006; Kanny *et al.*, 2018). Therefore, if these data translate to humans, our research suggests that preconception paternal alcohol use may be an unappreciated yet easily modified factor that significantly impedes IVF success rates, increasing patient financial burden and emotional stress.

The junctional zone, located between the maternal decidua and the fetal labyrinth layer, functions as the endocrine component of the murine placenta, releasing a diverse suite of hormones, cytokines and growth factors, but also serves as the primary energy reserve via the storage of glycogen (Woods *et al.*, 2018). Previous studies have confirmed that the proportional size of the junctional zone significantly affects fetal growth. For example, gene loss-of-function and overexpression mouse models reveal that reductions, expansion or mislocalization of junctional zone glycogen cells correlate with fetal growth restriction (Li and Behringer, 1998; Rampon *et al.*, 2008; Esquiliano *et al.*, 2009; Tunster *et al.*, 2016a, 2016b). Additionally, experiments comparing offspring derived using *in vitro* embryo culture to naturally conceived offspring or maternal hypoxia reveal a late-term enlargement of the junctional zone and reductions in fetal weights (de Waal *et al.*, 2015; Higgins *et al.*, 2016; Vrooman *et al.*, 2020, 2022). In contrast, reductions in the junctional zone arise in mouse models of maternal nutrient restriction (Coan *et al.*, 2010; Sferruzzi-Perri *et al.*, 2011).

Here, we provide new data describing the impact of preconception paternal EtOH exposures on IVF placental morphology and fetal growth. IVF offspring of alcohol-exposed sires exhibited a

decrease in the junctional zone and the proportional relationship to the decidua (Figure 5F, 5H). Therefore, the histological changes we observe in the offspring of alcohol-exposed sires contrast with the changes normally induced by IVF and more closely resemble the relative changes induced by starvation or hypoxia. However, we did not observe any changes in fetal weights between treatments, possibly due to the reductions in litter size. These results suggest that the volume of the junctional zone and glycogen cell quantities are modified when the sire regularly consumes alcohol before an IVF procedure. However, whether this altered phenotype results from placental dysfunction or as a compensatory modification is unknown and remains to be elucidated.

Although compelling, there are several limitations to our study. First, we and others have observed alcohol-induced alterations in sperm non-coding RNAs and post-translational histone modifications but not DNA methylation (Chang *et al.*, 2017; Rompala *et al.*, 2018; Bedi *et al.*, 2019, 2022). However, the current study does not reveal which epigenetic factors carried in sperm are responsible for transmitting the observed phenotypes. Further, we do not know whether factors transported in the seminal plasma of alcohol-exposed males may also influence pregnancy outcomes (Bromfield *et al.*, 2014). Consequently, we do not know which phases of sperm production or maturation are impacted by alcohol and, therefore, cannot make informed recommendations as to the duration of time that may be required for the epigenetic impacts of alcohol to dissipate. Second, the processes of superovulation and *in vitro* embryo culture are known to disrupt maternal epigenetic factors regulating development and alter placental histoarchitecture (Collier *et al.*, 2009; Delle Piane *et al.*, 2010; Bloise *et al.*, 2012; de Waal *et al.*,

549 2015; Tan *et al.*, 2016; Dong *et al.*, 2021; Bai *et al.*, 2022). Therefore, we do not know how much
550 of the phenotypic changes or alterations in embryonic gene expression are directly attributable
551 to paternal alcohol exposures versus a combinatorial interaction with assisted reproductive
552 technologies. Third, because our IVF protocol prioritized the transfer of 2-cell embryos, we only
553 examined embryonic development to this stage and harvested the remaining morulae for
554 transcriptomic analysis. Therefore, our embryological analysis does not address the observed
555 pregnancy loss after the two-cell stage, nor do our experiments faithfully mimic human IVF
556 studies, which focus on blastocyst stage outcomes. We require additional studies to determine
557 how paternal alcohol use impacts blastocyst cell lineage specification, development rates and
558 quality, and to determine how this may impact post-implantation survival. Fourth, we selected
559 the 6% EtOH and 10% EtOH treatments as the average daily dose for these treatments (1.7g/kg
560 for 6% EtOH and 2.2 g/kg for 10% EtOH) spanned the previously described moderate and heavy
561 drinker threshold; moderate daily doses (1.14-2.0 g/kg/day) were associated with increases in
562 litter average placental weights while heavy drinking (2.1-4.86 g/kg/day) associated with
563 placental growth restriction (Thomas *et al.*, 2022). In our experiments, both treatments inhibited
564 IVF success rates and altered placental histology, while neither treatment impacted litter average
565 placental weights. Further, although we observed some overlap in altered embryonic gene
566 expression between the 6% EtOH and 10% EtOH treatments (Figure 3G-H), it was minimal,
567 suggesting different doses exert distinct impacts on the embryonic transcriptional program. A
568 further limitation of this study was that we pooled morulae and could not separate male and
569 female embryos. Thus, the transcriptional differences we describe may be confounded by an
570 unequal representation of male and female embryos across treatments, although we did not

observe any changes at GD16.5. Future studies will examine the time required for alcohol-induced changes in the sperm-inherited developmental program to dissipate and which epigenetic signals may drive the inheritance of these phenotypes and will employ single-cell sequencing approaches to more rigorously examine embryonic gene expression patterns.

The planning status of a pregnancy has a substantial impact on maternal behaviour, which in turn, has a positive influence on infant health at birth (Pregnancy and Medicine, 1995; Kost *et al.*, 1998; Mohllajee *et al.*, 2007). Accordingly, maternal education on topics like the benefits of folic acid intake has helped significantly increase children's health (Prevention and Health, 2000). However, in the United States, 70% of men drink, and 40% engage in repetitive patterns of binge drinking (Naimi *et al.*, 2003; White *et al.*, 2006; Kanny *et al.*, 2018). Although half of pregnancies are unplanned (Henshaw, 1998), many male partners are heavily engaged in family planning, particularly with couples struggling with infertility (Kost *et al.*, 1998; Mohllajee *et al.*, 2007). Our work, combined with other published studies (Klonoff-Cohen *et al.*, 2003; Rompala and Homanics, 2019), indicates that we need to expand health messaging around prepregnancy planning to include the father and change IVF clinical practice to emphasize the dangers of periconceptional alcohol use by both parents.

591 **Data Availability statement**

592 We deposited the sequencing data generated during this study into the GEO database under
593 accession number GSE214726.

594

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599

600 **Authors' roles**

601 Conceptualization, ANR, KNT, and MG. Methodology, ANR, KNZ, KNT, and MCG. Investigation,
602 ANR, KNZ, KNT, AB, and MG. Formal analysis and Visualization, ANR, KNT, AB, SSB, and MG.
603 Funding acquisition and Supervision, MCG. Writing, ANR and MG.

604

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608

609 **Conflicts of Interest**

610 The authors declare no conflicts of interest.

611

612 **Figure Legends**

613

614 **Figure 1. A mouse model to determine the impact of chronic paternal alcohol use on *in vitro***
615 **fertilization-embryo development and pregnancy success rates**. **A)** Experimental paradigm used
616 to investigate the impact of chronic paternal EtOH exposure on IVF offspring growth and survival.
617 Comparison of sire **B)** average weekly fluid consumption and **C)** average daily dose of ethanol
618 between treatment groups (n=8). **D)** Comparison of average weekly weight gain between
619 treatment groups (n=8). We compared treatments using either a one-way or two-way ANOVA.
620 Error bars represent the standard error of the mean, * $P < 0.05$, **** $P < 0.0001$.

621

622 **Figure 2. Chronic paternal alcohol exposure impedes IVF embryo survival and decreases**
623 **pregnancy success rates.** Chronic male alcohol exposure reduces **A)** the percentage of two-cell
624 stage embryos per transfer surviving to gestational day 16.5 and **B)** the number of live offspring
625 or litter size at gestational day 16.5. We arcsine transformed the percentage of surviving 2-cell
626 embryos and used a one-way ANOVA to compare treatments on each litter. **C)** Paternal alcohol
627 use reduces IVF pregnancy success rates. We used a chi-squared test to identify differences in
628 pregnancy success rates between treatments ($p=0.0448$, n=18 Control, n=27 6% EtOH, n=38 10%
629 EtOH litters). Error bars represent the standard error of the mean, * $P < 0.05$, ** $P < 0.01$, *** P
630 < 0.001.

631

632 **Figure 3. Chronic paternal alcohol exposures induce changes in morula-stage embryonic gene**
633 **expression patterns**. Analysis of differential patterns of embryonic gene expression between

Control and 6% EtOH morulae: **A**) Heatmap comparing gene expression patterns, **B**) volcano plot contrasting down- and up-regulated differentially expressed genes, and **C**) Ingenuity Pathway analysis of differentially expressed genes. Comparison of embryonic gene expression patterns between Control and 10% EtOH morulae: **D**) Heatmap comparing gene expression patterns, **E**) volcano plot contrasting down- and up-regulated differentially expressed genes, and **F**) Ingenuity Pathway analysis of differentially expressed genes. Venn diagrams comparing the number of overlapping **G**) up-regulated and **H**) down-regulated differentially expressed genes between treatment groups. Baseline comparisons were made to the Control treatment. **I**) Ingenuity Pathway analysis comparing differentially expressed genes identified between the 6% EtOH and 10% EtOH treatment groups. C1, C2 and C3 reference Control samples 1 through 3, while E1, E2 and E3 reference EtOH samples 1 through 3. (For the analysis presented above, we selected differentially expressed genes exhibiting a log2-fold change and unadjusted p-value of <0.05, n=3 pools of 10-15 morulae per treatment).

Figure 4. Chronic paternal ethanol exposure alters IVF-embryo growth and organ development. Comparison of gestational day 16.5 **A)** gestational sac weights, **B)** fetal weights, and **C)** crown-rump lengths between treatment groups (n=45 Control, 31 6% EtOH, 26 10% EtOH male offspring; n=44 Control, 23 6% EtOH, 16 10% EtOH female offspring). Comparison of body weight-normalized **D)** brain weights, **E)** heart weights and **F)** thymus weights between IVF offspring generated using sperm derived from Control and EtOH-exposed males. We randomly selected ~ two male and two female offspring from each litter to examine organ weights (n=23 Control, 17 6% EtOH, 13 10% EtOH male offspring; 20 Control, 13 6% EtOH, 7-11 10% EtOH female offspring).

We used a two-way ANOVA to contrast differences between sex and treatment or Kruskal-Wallis test, depending on the normality of the data. Sex differences are indicated above the figures, while treatment effects are demarcated directly above the bar graphs. Error bars represent the standard error of the mean, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 5. Preconception paternal alcohol exposure alters IVF-embryo placental development. Comparison of **A)** placental weight, **B)** placental diameter, and **C)** placental efficiency between IVF offspring generated using sperm derived from Control and EtOH-exposed males (n=45 Control, 31 6% EtOH, 26 10% EtOH male offspring; n=44 Control 23, 6% EtOH 16, 10% EtOH female offspring). Using microCT, we measured the proportional volume of each placental layer and used a two-way ANOVA to compare measures between male and female offspring across treatment groups. Volumes for the **D)** chorion, **E)** decidua, **F)** junctional zone and **G)** labyrinth are expressed as a ratio of the total placental volume. We randomly selected placentae from each litter to examine histological changes (n=32 Control, 23 6% EtOH, 23 10% EtOH males; 23 Control, 16 6% EtOH, 13 10% EtOH females). Ratios comparing the proportional volumes of the **H)** junctional zone to decidua and **I)** labyrinth to junctional zone between male and female offspring across treatment groups. **J)** Quantification of placental glycogen content in male offspring. PAS-stained area normalized to the decidua and junctional zone area, compared between treatment groups (n=11 Control, 8 6% EtOH, and 8 10% EtOH). Sex differences are indicated above the figures, while treatment effects are demarcated directly above the bar graphs. Error bars represent the standard error of the mean, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

678 **Figure 6. IVF placentae derived from alcohol-exposed males exhibit transcriptional changes in**
679 **select imprinted and mitochondrial genes. A**) Analysis of imprinted gene expression in the
680 placentae of the male offspring of Control and 10% EtOH-exposed sires. Comparison of
681 mitochondrial-encoded transcripts in placentae of **B**) male and **C**) female IVF offspring derived
682 from Control and 10% EtOH treated males. We analyzed gene expression using RT-qPCR. Gene
683 expression was normalized to transcripts encoding *Pgk1* and *Ywhaz*; (n = 8). For analysis, we used
684 an unpaired, parametric (two-tailed) t-test or a Mann-Whitney test (unpaired, non-parametric t-
685 test) if the data failed the test for normality. Error bars represent the standard error of the mean,
686 *p < 0.05, **p < 0.01.

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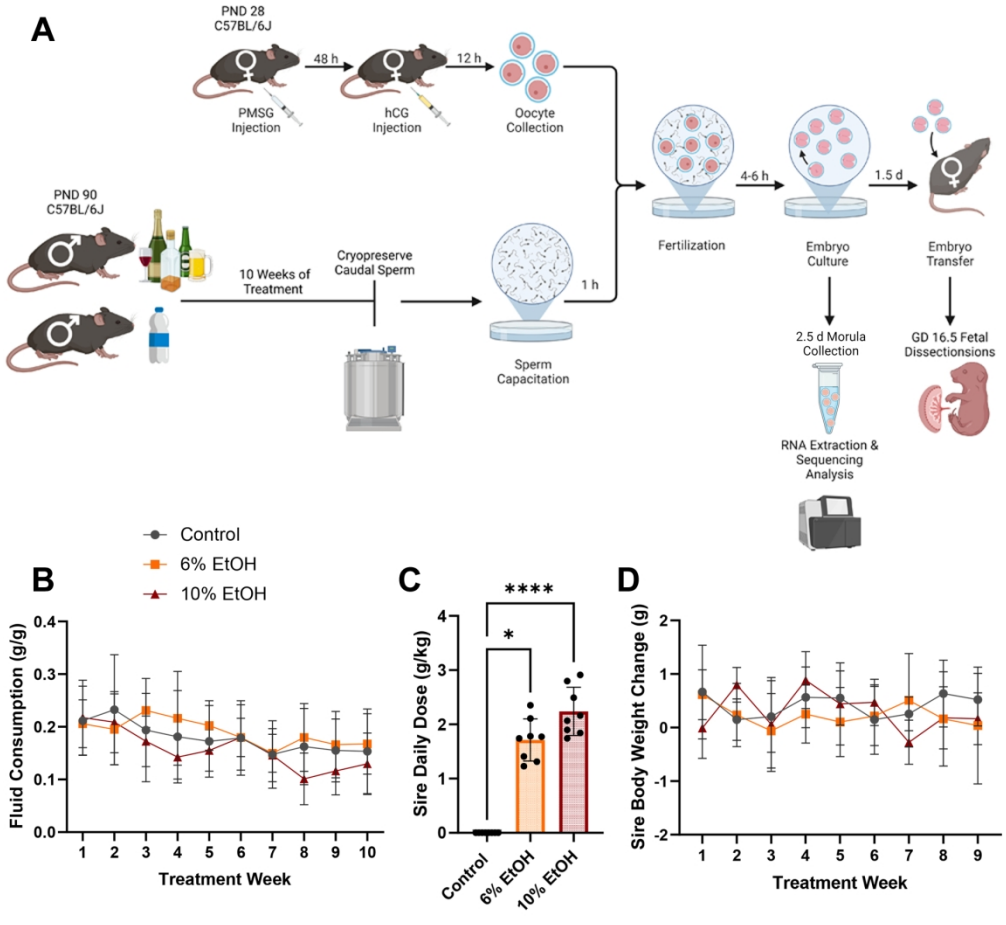

Figure 1. A mouse model to determine the impact of chronic paternal alcohol use on in vitro fertilization-embryo development and pregnancy success rates. A) Experimental paradigm used to investigate the impact of chronic paternal EtOH exposure on IVF offspring growth and survival. Comparison of sire B) average weekly fluid consumption and C) average daily dose of ethanol between treatment groups (n=8). D) Comparison of average weekly weight gain between treatment groups (n=8). We compared treatments using either a one-way or two-way ANOVA. Error bars represent the standard error of the mean, * $P < 0.05$, **** $P < 0.0001$.

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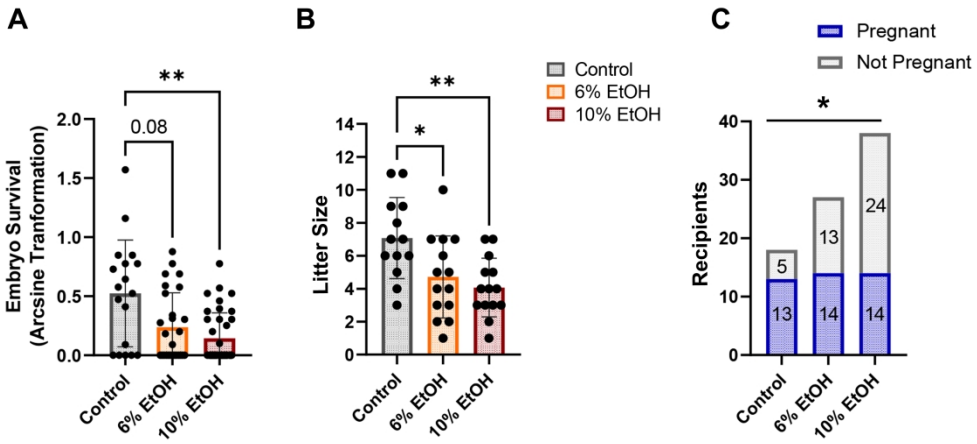

Figure 2. Chronic paternal alcohol exposure impedes IVF embryo survival and decreases pregnancy success rates. Chronic male alcohol exposure reduces A) the percentage of two-cell stage embryos per transfer surviving to gestational day 16.5 and B) the number of live offspring or litter size at gestational day 16.5. We arcsine transformed the percentage of surviving 2-cell embryos and used a one-way ANOVA to compare treatments on each litter. C) Paternal alcohol use reduces IVF pregnancy success rates. We used a chi-squared test to identify differences in pregnancy success rates between treatments ($p=0.0448$, n=18 Control, n=27 6% EtOH, n=38 10% EtOH litters). Error bars represent the standard error of the mean, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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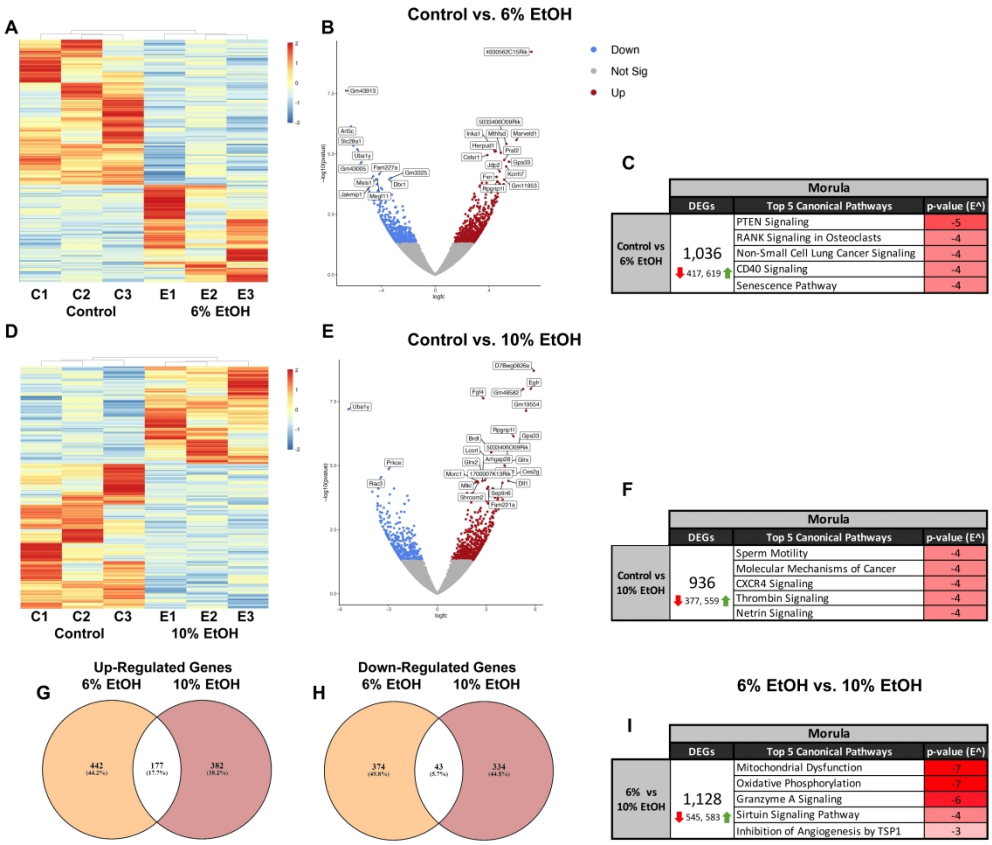

Figure 3. Chronic paternal alcohol exposures induce changes in morula-stage embryonic gene expression patterns. Analysis of differential patterns of embryonic gene expression between Control and 6% EtOH morulae: A) Heatmap comparing gene expression patterns, B) volcano plot contrasting down- and up-regulated differentially expressed genes, and C) Ingenuity Pathway analysis of differentially expressed genes. Comparison of embryonic gene expression patterns between Control and 10% EtOH morulae: D) Heatmap comparing gene expression patterns, E) volcano plot contrasting down- and up-regulated differentially expressed genes, and F) Ingenuity Pathway analysis of differentially expressed genes. Venn diagrams comparing the number of overlapping G) Up-regulated and H) Down-Regulated differentially expressed genes between treatment groups. Baseline comparisons were made to the Control treatment. I) Ingenuity Pathway analysis comparing differentially expressed genes identified between the 6% EtOH and 10% EtOH treatment groups. C1, C2, and C3 reference Control samples 1 through 3, while E1, E2, and E3 reference EtOH samples 1 through 3. (For the analysis presented above, we selected differentially expressed genes exhibiting a log2-fold change and unadjusted p-value of <0.05, n=3 pools of 10-15 morulae per treatment).

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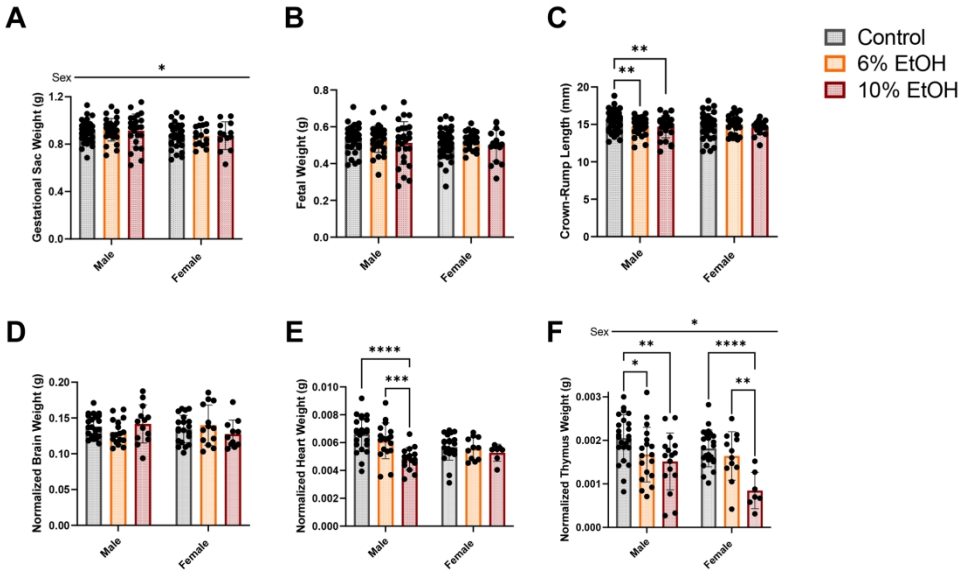

Figure 4. Chronic paternal ethanol exposure alters IVF-embryo growth and organ development. Comparison of gestational day 16.5 A) gestational sac weights, B) fetal weights, and C) crown-rump lengths between treatment groups (n=45 Control, 31 6% EtOH, 26 10% EtOH male offspring; n=44 Control 23 6% EtOH 16 10% EtOH female offspring). Comparison of body weight-normalized D) brain weights, E) heart weights, and F) thymus weights between IVF offspring generated using sperm derived from Control and EtOH-exposed males. We randomly selected ~ two male and two female offspring from each litter to examine organ weights (n=23 Control, 17 6% EtOH, and 13 10% EtOH male offspring; 20 Control, 13 6% EtOH, and 7-11 10% EtOH female offspring). We used a two-way ANOVA to contrast differences between sex and treatment or Kruskal-Wallis test, depending on the normality of the data. Sex differences are indicated above the figures, while treatment effects are demarcated directly above the bar graphs. Error bars represent the standard error of the mean, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

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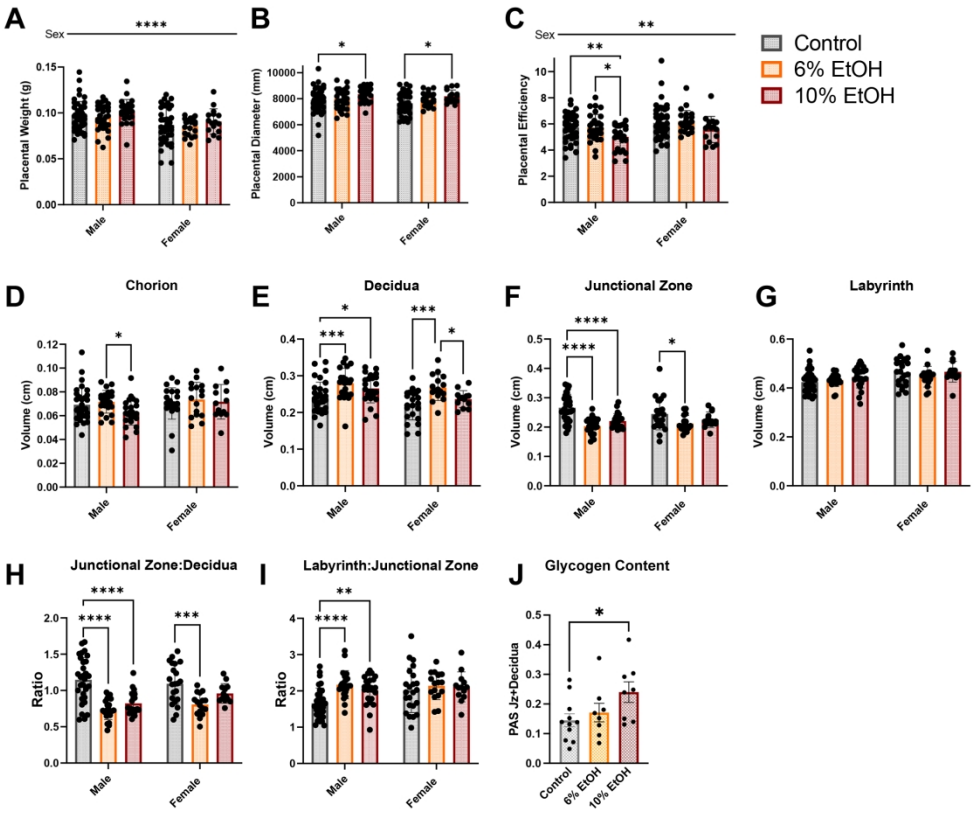

Figure 5. Preconception paternal alcohol exposure alters IVF-embryo placental development. Comparison of A) placental weight, B) placental diameter, and C) placental efficiency between IVF offspring generated using sperm derived from Control and EtOH-exposed males (n=45 Control, 31 6% EtOH 26 10% EtOH male offspring; n=44 Control 23 6% EtOH 16 10% EtOH female offspring). Using microCT, we measured the proportional volume of each placental layer and used a two-way ANOVA to compare measures between male and female offspring across treatment groups. Volumes for the D) chorion, E) decidua, F) junctional zone, and G) labyrinth are expressed as a ratio of the total placental volume. We randomly selected placentae from each litter to examine histological changes (n=32 Control, 23 6% EtOH, and 23 10% EtOH males; 23 Control, 16 6% EtOH, and 13 10% EtOH females). Ratios comparing the proportional volumes of the H) junctional zone to decidua and I) labyrinth to junctional zone between male and female offspring across treatment groups. J) Quantification of placental glycogen content in male offspring. PAS-stained area normalized to the decidua and junctional zone area, compared between treatment groups (n=11 Control, 8 6% EtOH, and 8 10% EtOH). Sex differences are indicated above the figures, while treatment effects are demarcated directly above the bar graphs. Error bars represent the standard error of the mean, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

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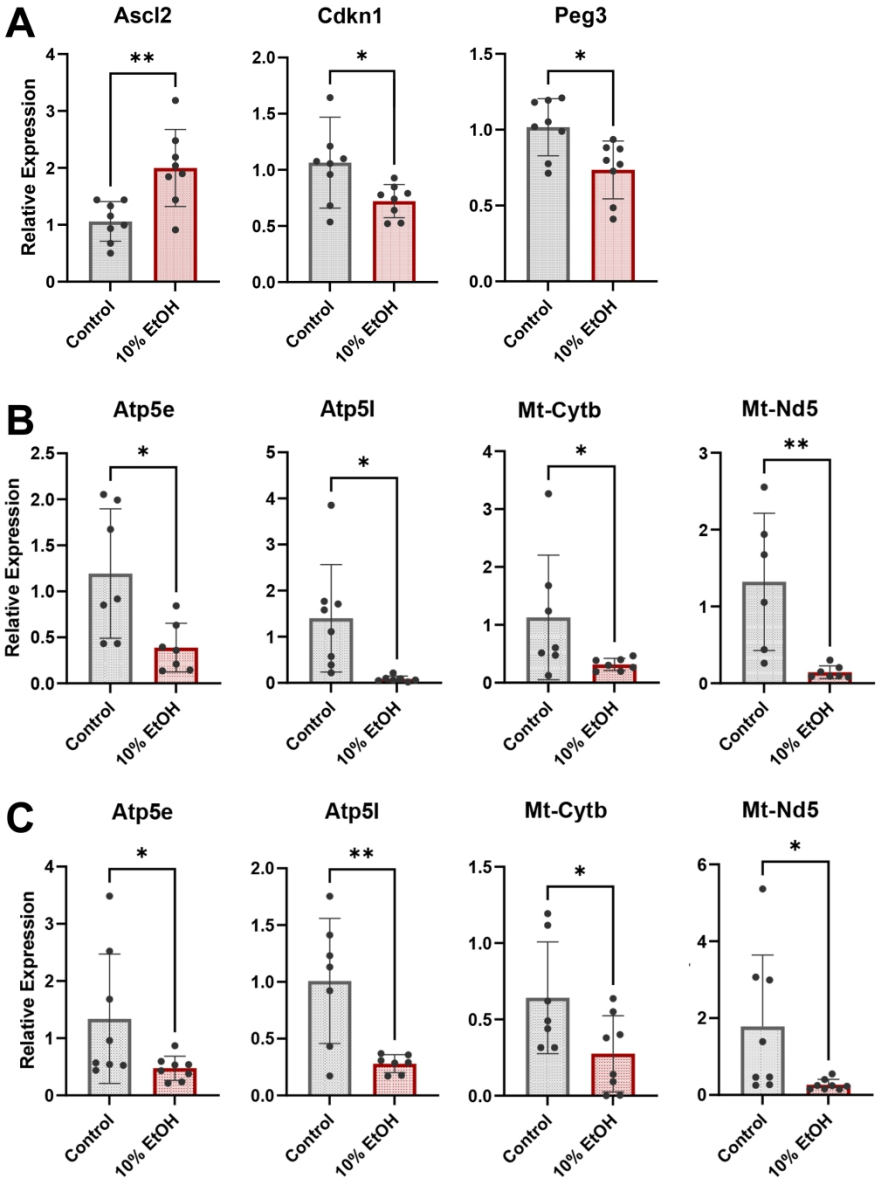

Figure 6. IVF placentae derived from alcohol-exposed males exhibit transcriptional changes in select imprinted and mitochondrial genes. A) Analysis of imprinted gene expression in the placentae of the male offspring of Control and 10% EtOH-exposed sires. Comparison of mitochondrial-encoded transcripts in placentae of B) male and C) female IVF offspring derived from Control and 10% EtOH treated males. We analyzed gene expression using RT-qPCR. Gene expression was normalized to transcripts encoding Pgk1 and Ywhaz; (n = 8). For analysis, we used an unpaired, parametric (two-tailed) t-test or a Mann-Whitney test (unpaired, non-parametric t-test) if the data failed the test for normality. Error bars represent the standard error of the mean, *p < 0.05, **p < 0.01.

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Table I. Fertilization success rates.

Sire Treatment	# Oocytes	# Oocytes to 1-cells	% Success
Control	967	515	53.25%
6% EtOH	1296	589	45.44% *
10% EtOH	1837	707	38.48% ****, #

Number of 1-Cell embryos after 4 hours culture. Comparisons to Control * $P < 0.05$, **** $P < 0.0001$; comparing 6% EtOH to 10% EtOH # $P < 0.05$.

Table II. Developmental progression of one-cell embryos to the two-cell stage.

Sire Treatment	# 1-cells	# 1-cells to 2-cells	% Success
Control	515	441	85.63%
6% EtOH	589	506	85.90%
10% EtOH	707	625	88.40%

Table III. Embryo Success Rate.

Sire Treatment	# 2-cells Transferred	# GD 16.5 Live Offspring	% Success
Control	197	90	45.69%
6% EtOH	301	66	21.93% ****
10% EtOH	414	57	13.77% ****, #

Total number of two-cell embryos transferred compared to the number of live offspring at gestational day 16.5. Comparisons to Control **** $P < 0.0001$; comparing 6% EtOH to 10% EtOH # $P < 0.05$.