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Transgenerational effects of binge drinking in a primate model: implications for human health

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Abstract

Objective—To determine if binge ethanol prior to ovulation affects oocyte quality and gene expression and subsequent embryo development

Design—Binge ethanol given twice weekly for 6 months followed by standard IVF cycle and subsequent natural mating.

Setting—Research University – National Primate Research Center

Animals—Adult female rhesus monkeys

Interventions—Binge ethanol twice weekly for 6 months prior to standard in vitro fertilization cycle with or without embryo culture. With in vivo development, ethanol treatment continued until pregnancy was identified.

Main Outcome Measures—Oocyte and cumulus/granulosa cell gene expression, embryo development to blastocyst and pregnancy rate

Results—Reduced embryo development in vitro, changes in oocyte and cumulus cell gene expression, and an increase in spontaneous abortion during very early gestation

Conclusions—This study provides evidence that binge drinking can affect the developmental potential of oocytes even after alcohol consumption had ceased.

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Keywords

Cumulus cells; granulosa cells; reproduction; fetal alcohol syndrome; transcriptome; cDNA array

Introduction

The incidence of binge drinking in the United States continues to increase and in 2010 occurred in 28% of people aged 18 to 34 years, with an average drink consumption per episode of 9 drinks for men and 5.9 drinks for women (1). On college campuses, 44% of students report binge drinking and binge drinking accounts for 91% of alcohol consumed on campuses (2). Overall, 90% of alcohol consumed by underage youth and 75% of alcohol consumed overall is consumed during binge drinking (http://www.cdc.gov/alcohol/fact-sheets/binge-drinking.htm). Heavy alcohol use has been associated with alcohol related neurodevelopment disorders and fetal alcohol syndrome (FAS) (3). Avoidance of alcohol during pregnancy will prevent fetal alcohol syndrome, but by the time pregnancies are confirmed, major embryonic events may have already occurred (4). Case reports documenting FAS pregnancies in women that drink ethanol only during the first trimester or only until a positive pregnancy test (5) support the hypothesis that the early stages of pregnancy are significantly affected by alcohol consumption.

The timing of the origins of FAS has been difficult to determine, in part because of the challenge associated with in vivo studies of the peri-implantation stage of embryonic development. Recent studies add further weight to the consideration that effects can arise prior to fetal development. There is growing evidence in rodent and domestic animal models that maternal nutritional state and other environmental factors influence follicle selection, oocyte quality, and embryo growth, with exposures during the peri-conceptional period being especially concerning (6, 7, 8).. These observations highlight the importance of understanding the effects of maternal exposures pre- and peri-conception.

Only a few studies evaluating the effects of ethanol on oocyte/early embryo function have been performed, all of which have utilized the mouse model. In vitro exposure of murine embryos to ethanol and acetaldehyde impair embryo development (9). Embryos derived from ovulated oocytes exposed to ethanol in vitro exhibit an elevated rate of non-disjunction of chromosomes during the first mitotic division (10). Follow-up studies of ethanol exposure in vivo at a time that would insure exposure of recently ovulated oocytes resulted in similar levels of non-disjunction that was exclusively confined to the oocyte-derived chromosome set (11). It has long been recognized that drinking as little as one ounce of absolute ethanol only twice per week can increase the risk of spontaneous abortion in women (12). These murine studies provide a glimpse into one mechanism by which that effect might be accomplished, but other mechanisms may also apply.

Oocyte growth and maturation is a process that occurs over many months in primates, including humans and monkeys. Therefore, binge ethanol consumption has the potential to affect oocytes well before the time of ovulation and the menstrual cycle in which pregnancy may be initiated. The potential for ethanol to affect meiosis and early mitotic divisions of the embryo has significant impact on the approach that must be taken to prevent adverse effects

of alcohol consumption. Therefore, it is important to determine if ethanol affects oocytes and associated follicle cells even before fertilization. This study reports the effects of ethanol on gene expression patterns in cumulus and mural granulosa cells that have been recovered from rhesus monkey females after at least six months of binge ethanol dosing. Using oocytes from these animals, effects on subsequent in vitro embryo growth and survival are demonstrated. After the cycle of controlled ovarian stimulation, the animals were naturally mated and early pregnancy loss is also observed. These data reveal a previously unappreciated severe effect of binge ethanol drinking on oocyte quality and early embryogenesis.

Methods

Adult female rhesus macaques (Macaca mulatta), housed at the California National Primate Research Center, were housed as described (13) except that animals were not socially paired on days when dosing occurred. All procedures for maintenance and handling of the animals were reviewed and approved in advance by the Institutional Animal Use and Care Administrative Advisory Committee at the University of California at Davis. The criteria for selection included age range from 6 to 12 years, history of successful pregnancy, and normal menstrual cycles. The control group consisted of seven females 8.5 ± 3.1(mean ± SD) years of age and weight 8.01 ± 1.78kg. The treatment group consisted of nine females 8.2 ± 1.9 years of age and weight 8.08 ± 1.57kg. Menstrual bleeding was monitored daily and body weights were recorded weekly for the duration of study.

Binge ethanol treatment: For the ethanol treatment group, two days per week animals were hand caught and administered ethanol in water via nasogastric tube. A gradual increasing dosing regime was used to initiate treatment in which animals were administered a low dose the first week, a mid-range dose the second week and a high dose for the remainder of the study. The low, middle and high doses of ethanol were 0.75, 1.125, and 1.50g/kg respectively. The high dose level was continued for the remaining treatment time. The high dose was selected to be equivalent, on an alcohol/body weight basis, to the 4 to 5-drink consumption that usually defines a binge-drinking episode in women. Animals received ethanol treatment for at least 6 months prior to controlled ovarian stimulation to obtain oocytes and prior to natural mating, to assure that all phases of follicular growth and development were exposed to ethanol. Dosing continued through the cycle of controlled ovarian stimulation, but did not occur on the day of, or the day prior to oocyte retrieval.

Controlled ovarian stimulation, embryo culture and natural mating: Oocytes were obtained from ethanol treated (n=9) or untreated (n=6) female rhesus monkeys by controlled ovarian stimulation with twice daily injections of human rFSH (37.5 IU) for 7 days and 1000 IU of hCG on Day 8. On Day 9, oocytes were obtained by ultrasound-guided needle aspiration as described in detail (14). Oocyte-cumulus complexes were recovered from the follicular aspirate, dissociated, and then the oocytes and cumulus cells processed according to established procedures (15,16). Granulosa cells were also recovered from the follicular aspirate and processed as described (17). Oocytes were observed for maturation status and a randomly selected subset (usually n=8) of mature oocytes (MII) were placed in Picopure buffer and stored frozen for gene expression analysis. The remaining oocytes were used for

in vitro fertilization and subsequent embryo culture as described (14). Blastocyst stage embryos were fixed, stained and analyzed for differential cell counting as described (14). The percentage of blastocysts was calculated for each animal based on the number of MII oocytes per animal. After resting one menstrual cycle following oocyte retrieval, females were naturally mated (n=7 control and n= 9 ethanol) with proven males each month at mid-cycle until the female became pregnant or up to 6 months. Ethanol dosing was discontinued once pregnancy was confirmed by ultrasound examination (18), at approximately 19 – 20 days gestation. This time of pregnancy confirmation would be similar to a woman detecting pregnancy less than a week after missing a menstrual period.

Blood ethanol measurement: Prior to oocyte collection or natural mating, blood samples were collected to determine ethanol serum concentrations. This time frame ranged from 32–35 weeks following the beginning of ethanol dosing. Two mL of blood was obtained by venipuncture for a baseline sample prior to dosing and at 1, 2, and 3 hrs post administration. Samples were placed in a serum collection tube, centrifuged and placed in −20C freezer until analysis. Serum ethanol concentrations were performed by the Department of Pathology at the University of California Medical Center and determined by an enzymatic rate method using the Beckman UniCel DxC800 (Beckman-Coulter, Brea, California). The rate of change of absorbance of NADH, which is directly proportional to the concentration of ethyl alcohol in the sample, was measured at 340 nanometers. The calculation of ethanol concentration is based on a two-point calibration curve. The limit of quantification (LOQ) is 4 mg/dL. Within-run and total precision of the assay are 3.0% and 4.5% respectively.

Gene expression analyses

Affymetrix rhesus genome arrays were used to obtain whole transcriptome mRNA expression profiles for cumulus cells and mural granulosa cells obtained from ethanol treated and control females described above. To minimize the impact of inter-follicle or inter-female variability on array results, a pooling strategy was adopted. Samples from three females were combined to yield each array sample. The granulosa and cumulus cells from an individual female were pooled according to cell type, and then samples from three females were pooled to create the final samples for array analysis, with the exception of two of the normal IVF cumulus samples and one of the normal IVF granulosa samples (two females each). We processed 3–4 samples for array analysis for each cell type (cumulus, mural granulose, oocyte) and condition (control, ethanol treatment) combination.

Total RNA was isolated from cells using the PicoPure RNA isolation kit (In Vitrogen). Up to 50 ng of total RNA from each array sample were subjected to two rounds of cDNA synthesis using the Arcturus RiboAmp HS Plus kit (In Vitrogen. Labeled cRNA was produced using either the Affymetrix GeneChip Expression 3’ Amplification for IVT Labeling Kit (cumulus and granulosa samples) or the ENZO Bioarray High-Yield RNA Transcript Labeling Kit (T7) (ENZO Life Sciences, Farmingdale, NY). The biotin-labeled cRNA samples were fragmented and 10 μg were hybridized onto arrays. Post-hybridization washing, staining and scanning were performed as described in the Affymetrix GeneChip Expression Analysis Technical Manual.

Array data analysis

We obtained high quality data for 3–4 arrays for each cell type/condition combination. Array data were analyzed with scripts written in R (http://www.R-project.org), utilizing routines from Bioconductor (19) and SAM (20) packages. The quality of data from individual arrays was assessed by examining standard indicators: minimum, maximum and average background, percentage of present calls by MAS5 algorithm, scaling factor, ratios of expression between 3' and 5' probes for spike-in probesets. The array quality control parameters for those samples accepted for further analysis were all within the acceptable ranges (Table S1). Probeset expression values were summarized and normalized using Robust Multi-array Analysis (RMA) (21). Groups of samples from treated and control females within each cell type were compared to identify differentially expressed genes using Significance Analysis of Microarrays (SAM) algorithm (20). Probesets with all expression values below 100 were excluded, as well as the probesets with at least one absent call in both treatment groups. The parameters for SAM analysis were: false discovery rate threshold for q-value 0.01, number of permutations 1000. To eliminate the effects of random sampling, SAM analysis was performed five times and a probeset was deemed significantly differentially expressed only if it passed FDR control all five times. Full datasets were deposited in NCBI's Gene Expression Omnibus. Data are also available in a searchable format at the Primate Embryo Gene Expression Resource (www.preger.org).

To reduce the potential impact of cross-hybridization to off-target mRNAs, and to maximize the confidence and number of gene annotations applied to probesets, a custom set of probeset definitions and annotations was used instead of those provided by Affymetrix. Probeset definitions for the Affymetrix Rhesus gene expression assays were validated by aligning probe sequences to rhesus macaque mRNA Reference Sequences (RefSeq). Probes that were not aligned to any mRNA sequences, or were aligned to two or more unrelated mRNA sequences, were removed from the respective probesets. Updated probeset annotations were compiled from gene and mRNA records downloaded through NCBI Entrez eUtils system and FTP server (http://www.ncbi.nlm.nih.gov/) on July 17, 2013. The gene symbol assignments were combined with assignments provided by Dr. Robert Norgren, University of Nebraska Medical Center http://www.unmc.edu/rhesusgenechip/RhesusGeneChipAnnot3.xlsx] – which was based on alignment of rhesus assay probes to human mRNA sequences – to assign the most relevant gene symbols. This was especially important for those probesets that were not assigned any rhesus genes (possibly due to incomplete or wrong RefSeq mRNA sequences), or for which the initially assigned rhesus genes had generic symbols (e.g., LOCnumber). Human-to-rhesus synteny alignment data [ref ftp://hgdownload.cse.ucsc.edu/goldenPath/rheMac2/vsHg19/] was used to resolve ambiguities or inconsistencies between symbols for homologous human and rhesus genes. An annotation tool to enable the acquisition of gene annotations for Affymetrix rhesus genome array probeset IDs is available at the Primate Embryo Gene Expression Resource (www.preger.org).

In order to evaluate possible cooperative effects amongst genes showing significant differences in expression between control and ethanol treated groups, lists of these genes were analyzed – separately for each cell type – using the Ingenuity Pathway Analysis (IPA)

program (Ingenuity Systems, Rockwood, CA). IPA provides the opportunity to identify biofunctions, canonical pathways and potential upstream regulators with statistically significant overlap between their lists of involved genes and the submitted target gene list. Statistical significance is evaluated using Fisher's exact test (p-value threshold 0.05). Another statistic used to evaluate and characterize significance of biofunctions is the z-score, which measures the congruence between the observed change (increased or decreased) in genes' expression and the previously reported genes' activity related to the particular biofunction, and is used to predict the effect of treatment on biofunctions' activation state (decreased or increased). Similarly, z-score can be used to predict the activation state (activated or inhibited) of predicted upstream regulators, by measuring the congruence between the change in genes' expression and the reported effects (upregulation or downregulation) of their upstream regulators. IPA also constructs networks that incorporate genes from the target lists with previously reported relationships that can provide insight into possible pathways leading to observed changes in gene expression.

Real-time quantitative RT-PCR analysis

Total RNA was isolated using the PicoPure RNA isolation kit. TaqMan quantitative RT-PCR gene expression assays were performed using primers from Applied Biosystems/Life Technologies and the ABI StepOne Plus instrument according to the manufacturer's recommendations. Purified RNA was subjected to reverse transcription and then whole transcriptome amplification using the QuantiTect whole transcriptome kit (Qiagen, Valencia, CA), and 100 ng of cDNA used for each assay. The mRNA abundance of each target gene was normalized to the endogenous mitochondrial ribosomal protein S18C gene (MRPS18C) for sample-to-sample comparisons and the relative expression was calculated by the comparative CT method (16). Samples for qRT-PCR analysis included portions of the original pooled cumulus cell RNA samples used for array analysis. The pooled and individual samples for ethanol treatment groups were each compared to the four pooled, normal IVF control samples individually or collectively. Primers for the genes assayed were obtained from Applied Biosystems/Life Technologies were: Assay IDs Rh02911705_m1 (GRP), Rh02795191_m1 (RGS4), Rh02793827_m1 (SERPINA3), Rh01063214_m1 (STC2), Rh02836908_m1 (SPOCK3), and custom primers for RNASE2 (forward, CCAGCTGGATCAGTTCTCACA; reverse, CCCAACAGAAGAAGCAGACAAATTG; reporter, CCATGTTTTCCCAGTCTCC).

Statistical Analysis

All IVF outcome data were expressed as the mean ± SEM. Percentage of metaphase II oocytes and percentage of blastocyst-stage embryos were compared using a two-tailed paired Student's t test. Total cell count, ICM cell count, TE cell count, and percentage of ICM to TE cells in blastocyst-stage embryos were assessed with Student's t tests. Data were analyzed with Prism software (GraphPad Software). Differences were considered statistically significant at $p < 0.05$. Pharmacokinetic (PK) analysis of the serum ethanol concentrations was performed using WinNonlin v5.3 software (Pharsight Corp., Mountain View, CA, USA) to estimate time of peak (Tmax) ethanol concentrations using a one compartmental model and nonlinear regression analysis based on the Gauss-Newton (Levenberg and Hartly) method.

Results

Binge drinking prior to ovulation negatively affects pregnancy

Blood alcohol concentrations (mean ± SD) for the binge ethanol treatment group are presented in Figure 1. The serum ethanol concentrations had a mean (±SD) maximum concentration (Cmax) of 156.7±24.4 mg/dL and ranged from 134.0 to 200.0 mg/dL, similar to those of women engaged in binge drinking. The mean (±SD) for Tmax was 1.4±0.5 hrs and ranged from 1.0 to 2.0 hrs. The ethanol concentration at 3 hrs post administration had a mean (±SD) of 140.3±31.3 mg/dL and ranged from 99.0 to 187.0 mg/dL. The estimated Tmax from PK analysis had mean of 1.4±0.6 hrs and ranged from 0.8 to 2.3 hrs.

The outcome of the cycle of controlled ovarian hyperstimulation and IVF for binge ethanol treatment or controls is detailed in Table 1. The number of oocytes retrieved, the percentage maturing to MII and fertilization rate was similar in both groups. However, the percent of embryos resulting from IVF of MII oocytes that developed to the blastocyst stage was significantly lower in the ethanol treatment group. The total number of blastocysts produced was 90 for control and 85 for ethanol; a subset of blastocysts was examined for cell number (32 for ethanol, 43 for controls). There were no differences in the total cell number of inner cell mass/trophectoderm ratio of blastocysts from the two treatment groups.

Six of the seven control animals became pregnant within six positive natural matings with proven males and all pregnancies progressed normally to term. Time to pregnancy was not different between groups. In the ethanol treatment group, seven of nine animals became pregnant (not different than controls), but 2 ended in spontaneous abortion at approximately gestation day 30 (term = GD 165); thus, only 5 of the 9 ethanol treated animals had offspring. The treatment dosing ended on GD 17 + 3 for binge ethanol animals and GD 19 + 2 for controls. For the two pregnancies ending in spontaneous abortion, ethanol treatment ended on GD 8 and 15. Thus, treatment had ceased 2 to 3 weeks prior to spontaneous loss of pregnancy.

Effects of binge ethanol drinking on gene expression in follicle cells

The results described above were striking because they revealed a significant diminishment in developmental capacity of progeny developing from oocytes that had been exposed to ethanol during folliculogenesis, even though that treatment was terminated well before ovulation. This suggested that some aspect of oocyte quality was likely compromised, possibly through disruptions in the oocyte-follicle cell dialogue during folliculogenesis, and that this reduced oocyte quality impaired subsequent development. Our earlier studies revealed that gene expression in cumulus and granulosa cells can provide a more insightful evaluation of oocyte quality than gene expression in the oocyte itself (15,16). We therefore examined cumulus and granulosa cells for differences in gene expression. A small number of probesets was significantly affected in either cell type; however, this is not a reliable indicator for the magnitude of the effect of ethanol treatment on gene expression, as many more differences in expression may have gone undetected due to noise and/or the relatively small number of assays. Mural granulosa cells displayed 30 affected probesets (Table S2), all of which showed increased intensities in the ethanol treated group. Cumulus cells

displayed 51 affected probesets (Table S3), with 36 decreased and 15 increased in intensity in the ethanol treated group. Quantitative RT-PCR analysis was applied to six genes that appeared to be affected by ethanol treatment in cumulus cells (Fig. 2). The qRT-PCR data yielded fold changes in the same direction as seen for arrays for all six genes, with consistent differences for *SPOCK3, STC2* and *SERPINA3*, and more variable results for *RGS4, RNASE2* and *GRP*. The IPA biofunction analysis for cumulus cells (Table S4) revealed significant effects on cell death and survival, cell cycle, apoptosis, cell growth and proliferation, cell movement, carbohydrate metabolism, and cell signaling, along with ovarian cancer. Thirteen canonical pathways, six of which are related to signaling, involved a significant number of affected genes (Table S5); most of these pathways contain downregulated IL1R1, IL1RAP, and some of them contain downregulated SERPINA3 and upregulated TCF4. Three virtually disjoint large networks were constructed around the affected genes (Table S6). Cell morphology and embryonic development are two of the three top functions for genes in Network 1 (i.e., the network with the highest score). IPA identified four molecules (TNF, NFkB complex, IL1A, IL1B) that, based on their previously reported effects on genes affected in cumulus cells and the observed changes in those genes' expression, were predicted to be upstream regulators inhibited by maternal ethanol treatment (Table S7 and Figure 3). Three more regulators (chorionic gonadotropin complex, CD3 complex, OSM) were assigned z-scores that were not deemed significant. It is important to note that all statistics reported by IPA are dependent on the current knowledge database and are subject to change as the knowledge database is augmented with new findings. For many regulators the knowledge database does not contain sufficient findings that would characterize their effect on downstream molecules (i.e. upregulates or downregulates), which may lead to their z-scores being unrealistically small, or even impossible to calculate. In summary, the upstream regulator analysis for cumulus cells revealed a number of exogenous ligands in the affected networks. These included chorionic gonadotropin, luteinization hormone, interleukin 1, tumor necrosis factor, oncostatin M, and inhibin a. Interestingly, interleukin 1 receptor (IL1) and its accessory protein IL1RAP were both downregulated in cumulus cells of treated females, and were constituents of the top affected network and 12 of 13 affected canonical pathways.

Effects of binge ethanol drinking on gene expression in oocytes

To determine effects of maternal ethanol treatment on oocyte gene expression, we analyzed oocyte (MII stage) mRNA composition using microarrays. Significance analysis yielded 37 probesets indicating increased mRNA expression (1.54–7.36 fold change) and 82 probesets indicating decreased mRNA expression (1.42–5.05 fold change) (Table S8). Analysis of affected biofunctions (Table S9) with two or more member genes revealed a potential endocrine system effect related to pregnenolone synthesis. Among the affected biofunctions with the largest numbers of member genes and significant z-scores were cell death, lipid metabolism and synthesis, and necrosis. Canonical pathways analysis (Table S10) yielded predominantly pathways with three or fewer member genes, but these included genes related to PPARα/RXRα and PXR/RXR activation (PRKACB, MED23, PLCB1, CYP2C9, PRKCA, UGT1A9), a range of lipid metabolism pathways (PLCB1, PLCH1, MTMR2, AGPAT9), melatonin signaling, inositol signaling, and other forms of cellular signaling. IPA constructed six networks around the submitted list of affected genes (Table S11), and these

were related to cell-cell interactions, cell movement, or cellular assembly/morphology. We performed the IPA upstream regulator analysis, focusing on cellular regulators and excluding some molecule types, e.g., exogenous chemicals (Table S12). This revealed several potential effects acting on a subset of affected target molecules: PLCB4, PLCD1, CD24 acting on PLCB1; Ccl6, PDE4D, GUSB, NKX3-1, and hCg complex acting on CTSL2 (Note: IPA uses CTSV as identifier for CTSL2); NR1I3, HNF4A, PXR ligand-PXR-Retinoic acid-RXRα complex and NR3C1 acting on CYP2C9; BDNF, HOPX, AVPR1A and PLN acting on MYH3; and NR3C1 acting on IER2. Upstream regulators affecting the largest number of affected target molecules were nearly all nuclear receptors or transcription factors, and included HNF4A (17 targets), NR3C1 (7 targets), NR1I3, KDM5B, EPAS1 (3 targets each), and NKX3-1 (2 targets).

Discussion

Our results are the first to evaluate in a primate the effects of ethanol binge drinking on oocyte quality, and on subsequent in vitro embryo development occurring after exposure has ceased. We demonstrate that binge ethanol consumption for 6 months prior to controlled ovarian stimulation and oocyte retrieval altered follicle cell gene expression and that this was associated with an effect on oocyte quality as reflected in reduced preimplantation embryo development. Continued binge ethanol consumption until pregnancy was detected after natural mating (about GD 19–20) was associated with an elevated rate of spontaneous abortion at GD 30. These observations have major implications for the management of the reproductive health of women, as it reveals potential significant long-term effects on offspring phenotype even if alcohol consumption is terminated before pregnancy is established.

The amount of alcohol administered in these studies was the equivalent of four to five drinks in young women, based on observed blood alcohol level, validating our rhesus monkey model for binge drinking in young women. In a study conducted by Donovan (23), estimates of BAC in children and young adults were determined from public use data and BACs obtained in emergency departments. This study estimated the maximum BAC following the consumption of 4 or 5 drinks in 17 and 18 year old females to be between 137.6 mg/dL and 191.1 mg/dL. Additionally, a study looking at the ethanol pharmacokinetics of women following consumption of four drinks on two test days demonstrated a mean Tmax of 1.75 hrs (23). These data correspond remarkably well to the data obtained in the present study with BAC ranging from 134.0 to 200.0 and Tmax occurring an average of 1.4 hrs post ethanol administration. Khaole et al. conducted a study in women that allowed them to drink for 2.5 hrs under a semi-controlled setting to simulate a binge drinking event followed by breathalyzer determined BAC (BrAC) (24). The peak BrAC ranged from 51–139 mg% following consumption of 27.2–68.0 g ethanol, which is similar to the amount administered in the present study ranging from 41 to 54 g ethanol and the higher end of the BrAC range being similar to our results. Studies specifically investigating ethanol kinetics in young females are limited; however, published literature provides BAC in apprehended drivers, ages 15 to 20 years, with mean (±SD) BAC reported from 107±53 mg/dL to 145±63 mg/dL, respectively (25). Additionally, the same study estimated that BAC in the range of 100–249 mg/dL is consistent with consuming 3.8 to 6.7 drinks. The present study's findings are thus

based on outcomes following ethanol consumption to yield BACs approximating those expected in young adult women who engage in binge drinking.

The increased rate of spontaneous abortion in ethanol treated females after natural mating is striking, because spontaneous abortion is rare in rhesus monkeys, and further highlights the potentially high impact of binge drinking on reproductive function in women. A possible association of ethanol consumption around the time of conception with spontaneous abortion has been noted in women. In a study that included recall data on the amount of alcohol women drank around the time of conception, the number of days per week that alcohol was consumed correlated with rate of spontaneous abortion ranging from 0% for abstainers to 40% for those that drank more than 3 days per week (26). Interestingly, almost half of the women that consumed alcohol in that study were described as binge drinkers, consuming 4 or more drinks per episode. However, an association of spontaneous abortion with alcohol exposure during pregnancy in women was not always evident, some studies showed an association and others did not (27). It is also noteworthy that the Henderson et al. review identified only 14 studies that were relevant to the topic of fetal effects of binge drinking, despite the fact that binge drinking is now the most popular form of alcohol consumption among young women. One confounding factor with human studies is that increased public awareness of the dangers of alcohol to pregnancy may lead to some women denying or underreporting alcohol use (28). The issues of inaccurate reporting of alcohol use and relatively small numbers of clinical studies supports the importance of a primate model for effects of binge drinking. The lack of other studies on binge ethanol consumption and oocyte quality underscores the uniqueness of the data in this study.

The reduced developmental capacity of the oocytes retrieved after 6 months of binge alcohol treatment is the first evidence of the in vivo effects of alcohol on oocyte quality. One possible explanation for the fact that embryos resulting from in vitro fertilization of these oocytes have a substantially reduced competence to reach the blastocyst stage is that they could experience increased rates of aneuploidy. An alternate possibility is that there are changes in the epigenetic state and/or macromolecular content of the oocytes, and that such changes have long-lasting effects on development. The duration of alcohol dosing was designed to be at least 6 months because the growth and maturation of oocytes from primordial follicle to ovulation takes place over many months in humans and nonhuman primates. Therefore, we cannot be sure if the effect of binge alcohol has a specific window of activity or if the reduced developmental capacity would occur at any stage of growth. However, this study demonstrates that binge alcohol can affect oocytes in a way that perturbs subsequent embryo development. This outcome has serious implications for when to advise women that intend to become pregnant to reduce alcohol intake. Moreover, they indicate a potential for maternal binge ethanol drinking to affect the health of progeny even into adulthood, as seen with other factors that induce developmental origins of adult disease (8).

What is the mechanism by which long-term effects of maternal binge drinking might be imposed on developing progeny? The cumulus and granulosa cells were analyzed for gene expression because a previous study in the rhesus monkey demonstrated that when oocytes of known compromised quality are obtained, the changes in gene expression in oocytes are

not as great as the changes that occur in the somatic follicle cells (15,16). Thus, the gene expression patterns of cumulus and granulosa cells within the follicle may be better predictors of oocyte quality than that of the oocyte itself. Among the small number of changes seen in cumulus cells, the effects on three genes appear especially notable. First, SERPINA3 inhibits cathepsin activity, so that the reduction in SERPINA3 expression could compromise oocyte quality and later development, as reported in cattle (29). Second, SPOCK3 suppresses the MMP activity (30), which may play a crucial role during folliculogenesis, ovulation, and luteinization (31,32). SPOCK3 genetic polymorphism is associated with age of menarchy (33). Excess expression of SPOCK3 may disrupt the balance of MMP activity and affect the quality of the oocyte by altering ovarian function, or may be symptomatic rather than causal. Third, stanniocalcin 2 (STC2) mRNA expression is increased. STC2 opposes STC1 activity, (34,35), and negatively modulates store operated calcium entry (36). This could lead to decreased uptake of external calcium, possibly compromising ion content in the oocyte.

In addition to these effects in cumulus cells, the upstream regulator analysis indicates disruption in several significant pathways, including inhibition of signaling through IL1A, IL1B, TNF and NFkB. Those factors are not themselves altered at the mRNA level (and indeed some are ligands produced by other cell types), but the data reveal reduced mRNA expression of their downstream target genes.

One pathway that emerged in the upstream regulator analysis of both cumulus cells and oocytes was the pathway for response to chorionic gonadotropin. This pathway was inhibited in both oocytes and cumulus cells, and regulates the top networks observed for effects in oocytes and cumulus cells of treated females. This indicates that binge ethanol consumption is negatively affecting endocrine regulation of ovulation, most likely related to reduced signaling via LH, which emerged as an upstream regulator of the top network for cumulus cells. LH stimulation promotes meiotic maturation and cumulus expansion, and other processes such as luteinization and implantation.

Additional effects on other key regulators were also evident in oocytes (Table S13). Previous studies indicated that for oocytes of compromised quality, the relative number of genes affected in oocytes is smaller than those in cumulus cells (15,16). Thus we expected that the number of genes that displayed altered expression in the MII oocytes in response to maternal binge ethanol exposure was relatively small. However, these few changes are correlated with a dramatic reduction in the ability of oocytes to develop to blastocysts. These include effects on the expression of mRNAs encoding proteins related to steroid and lipid metabolism and calcium signaling; [cytochrome P450 subfamily IIC peptide 9 (CYP2C9), and phospholipase C beta 1 (PLCB1)] , which may play key roles in oocyte metabolism and activation, It is also interesting that the upstream regulators associated with each affected network in oocytes are predominantly transcription factors. However, the mRNAs for those regulators were not themselves affected by treatment. We note that the timely activation of genes in early embryos is regulated in part by temporal control of translation of maternal mRNAs encoding transcription factors (42,43,44). Although the abundances of the mRNAs encoding these upstream regulators was not significantly altered in the array expression profiles, the data raise the interesting possibility that translational control of such maternal

mRNAs could be defective after fertilization, contributing to the reduced embryo viability. Post-translational effects at the level of transcription factor activity could also be affected. Either process could be affect by e.g., PLCB1 acting through the phosphoinositide pathway, and the observed effects on inositol signaling.

While the association of alcohol exposure with spontaneous abortion in the mated rhesus monkeys and the reduced developmental capacity of the oocytes from an assisted reproductive cycle indicate a possible reduction in oocyte quality as being responsible for the early pregnancy loss, other mechanisms may also contribute. An alternative explanation for the spontaneous abortion rate in the binge alcohol group is that alcohol can affect various reproductive hormones. Chronic alcohol perturbs menstrual cycles and anterior pituitary and ovarian hormones in women and rhesus monkeys (45,46). Binge alcohol consumption also was associated with increased testosterone, prolactin, ACTH and cortisol in young women presenting at hospital emergency rooms (47). Therefore, it is conceivable that the binge alcohol consumption in this study would be sufficient to cause disruption in the hormonal milieu necessary to maintain early pregnancy.

The biological effects of binge ethanol consumption demonstrated here in a primate model coupled with the widespread incidence of binge ethanol drinking in the human population together point to an important public health concern that needs to be addressed. A combination of nonhuman primate modeling to study the reproductive biology, molecular and cellular mechanisms or these effects is needed, along with greater surveillance of binge drinking and its effects on women's fertility, and on the long-term health consequences on children born subsequent to maternal binge drinking. A better understanding of these interactions could improve women's reproductive health and reduce the incidences of costly health problems in their children.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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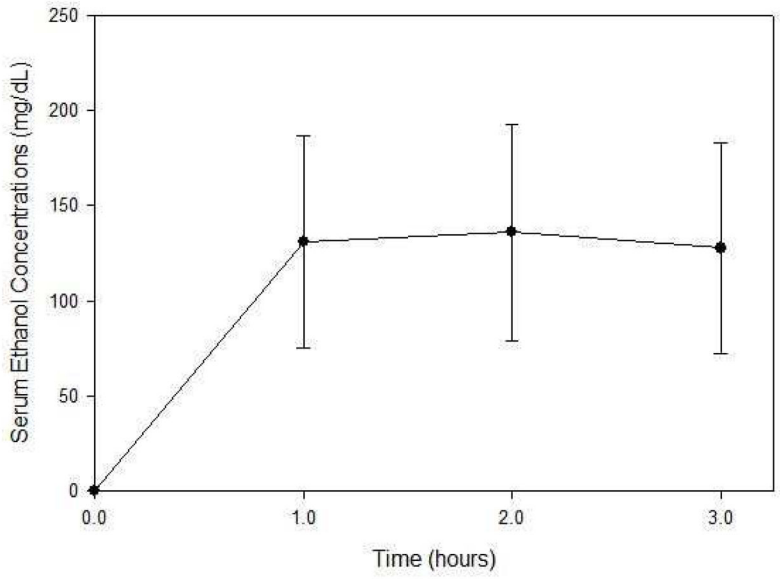

Figure 1.

Mean ± s.d. of serum ethanol concentrations at predetermined time points following nasogastric tube administration of ethanol 1.5 g/kg bwt. Time of ethanol administration was designated as time 0.

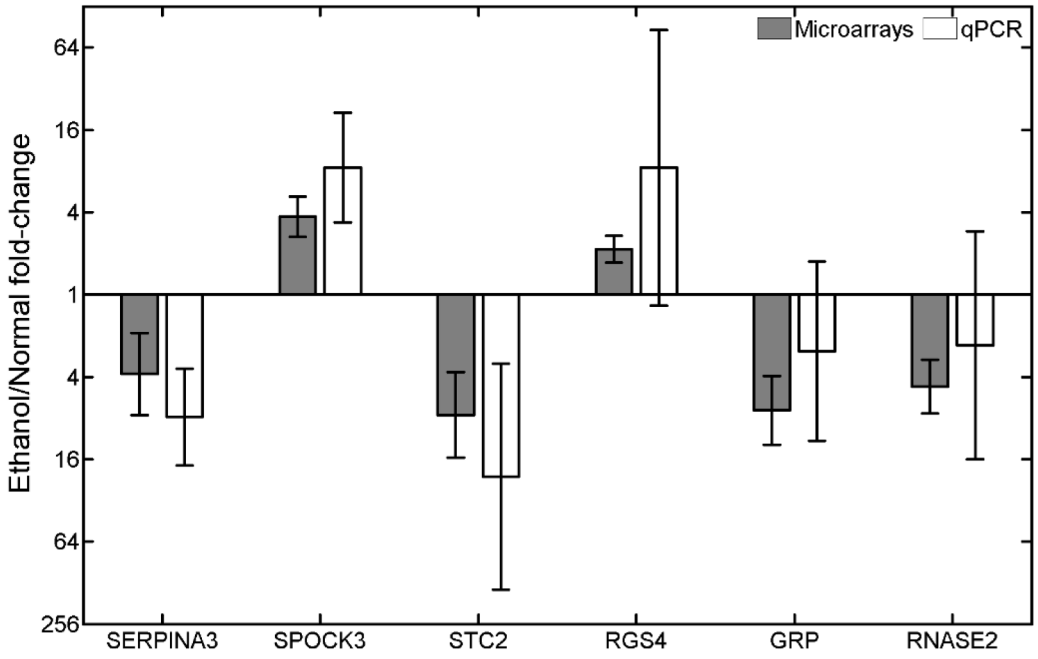

Figure 2.

Comparison of array and qRT-PCR expression data for selected genes between cumulus cells from treated and untreated females. Bars indicate the average fold change values (+ S.D.); black bars show results from the array data and white bars show the results from the qRT-PCR assay.

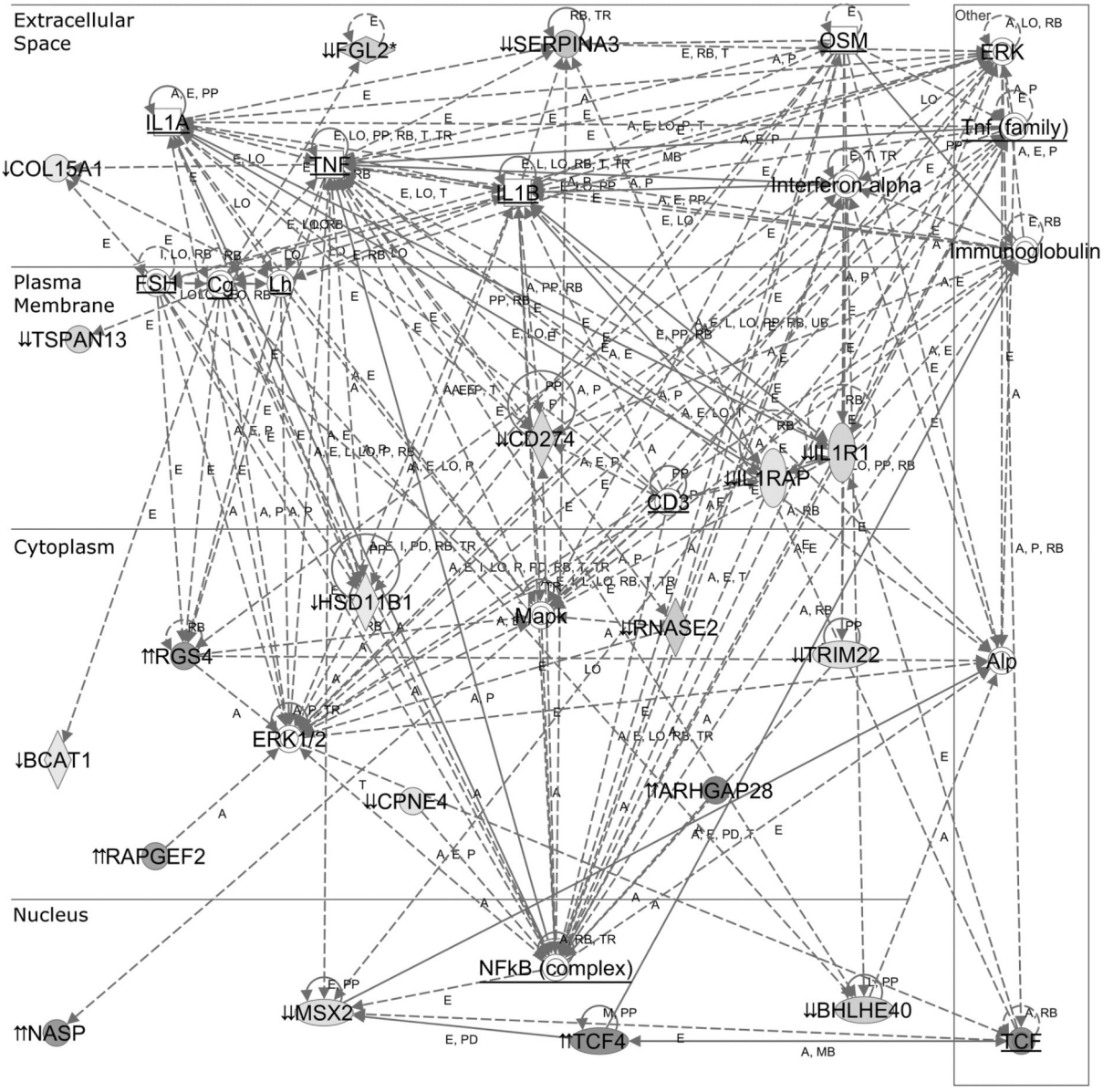

Figure 3.

Ingenuity Pathway Analysis network number 1 from cumulus cell analysis with selected upstream regulators included (see Table S6). The network illustrates important functional connectison between the reguators and affected downstream target gene. Names of molecules for which mRNA expression is affected by maternal binge ethanol treatment are preceded by ↑ (increased) and ↓ (decreased by treatment). Upstream regulators are marked with a thicker border. IPA employs the following symbols in its networks: A, activation; CP, chemical-protein interaction; E, expression; I, inhibition; LO, localization; M, biochemical modification; MB, group/complex membership; nTRR, non-targeting RNA-RNA interaction; P, phosphorylation/ dephosphorylation; PD, protein-DNA interaction; PP, protein-protein interaction; PR, Protein-RNA binding; RB, regulation of binding; RE, reaction; T , transcription; TR, translocation; UB, ubiquitination; line, binding only; line

with filled arrowhead, downstream effect; line with bar and arrowhead, inhibition and downstream effect; line with outlined arrowhead, translocation; solid line, direct interaction; broken line, indirect interaction; vertical diamond, enzyme; horizontal diamond, peptidase; horizontal oval, transcription regulator; vertical oval, transmembrane receptor; trapezoid, transporter; down-pointing triangle, kinase; square, cytokine; vertical rectangle, G-protein coupled receptor, horizontal rectangle, ligand-dependent nuclear receptor; dashed square, growth factor; dashed rectangle, ion channel; double circle, complex/group; circle, other.

Table 1

Outcome of IVF-embryo culture and subsequent natural mating conducted after at least 6 months of binge ethanol treatment.

Treatment	Total Oocytes / animal	MII Oocytes	% MII Oocytes	# MII Oocytes for IVF	% Blastocysts	Early loss during natural pregnancy
Control	28.8 ± 5.5	25.1 ± 5.2	86.0 ± 1.9		61.4 ± 5.1*	0/6
Ethanol	35 ± 7.3	27.3 ± 6.7	76.1 ± 3.7		36 ± 3.7*	2/7

*Ethanol treatment significantly different than control $p < 0.05$