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Alterations in sperm-inherited noncoding RNAs associate with late-term fetal growth restriction induced by preconception paternal alcohol use

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Abstract

Using a mouse model, our group recently described an association between chronic paternal alcohol use prior to conception and deficits in offspring growth. Here, we sought to determine the impact of alcohol exposure on male reproductive physiology and the association of sperm-inherited noncoding RNAs with the transmission of the observed growth defects. Alcohol exposure did not appreciably alter male reproductive physiology or fertility. However, chronic alcohol use reproducibly induced late-term fetal growth restriction in the offspring, which correlated with a shift in the proportional ratio of transfer RNA-derived small RNAs to Piwi-interacting RNAs, as well as altered enrichment of microRNAs miR21, miR30, and miR142 in alcohol-exposed sperm. Although our dataset share similarities to prior works examining the impact of paternal stress on offspring phenotype, we were unable to identify any changes in plasma corticosterone, indicating alcohol may alter sperm-inherited noncoding RNAs through distinct mechanisms.

Keywords

paternal alcohol use; preconception exposure; epigenetic programming; growth restriction; sperm; noncoding RNAs; microRNAs; epigenetic

1. Introduction

Developmental plasticity refers to the dynamic ability of one genotype to produce multiple phenotypes in response to different environmental stimuli[1]. This phenomena enables the best chances of reproductive success but can also associate with the development of functional deficits and disease. Indeed, there are multiple instances where the predisposition of offspring to develop diseases later in life can be traced to a fetal compensation to an early

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life stressor[2]. For example, small-for-gestational-age babies have an increased risk of developing metabolic diseases, such as type two diabetes, and both cardiovascular morbidity and mortality as adults[3,4]. These observations helped found the Developmental Origins of Health and Disease hypothesis, and established the current recognition of the importance intrauterine development has in lifelong health.

Less well defined are the abilities of parental life history to influence organism phenotype. Although not as well characterized as intrauterine encounters, parental exposures prior to conception also exert a significant impact on offspring health and development[5]. Thus, in addition to uterine programming, the processes of germline programming that occur during gametogenesis also have the ability to impact offspring phenotype and influence the developmental plasticity of the next generation. Recently, preconception male exposures to a range of environmental factors have been linked to alterations in the developmental program of sperm and correlated with increased rates of structural and metabolic defects in the next generation[6–25]. These studies challenge the singular importance of maternal in utero exposures and implicate paternal exposure history as an additional and important mediator of both developmental defects and environmentally-induced disease.

The molecular mechanisms by which preconception stressors heritably influence cellular phenotypes are still very poorly understood. Molecular processes that allow the stable propagation of either chromatin-states or epigenetic information from one generation of cells to the next are hypothesized to play a role in transmitting the cellular memories of past exposures through gametogenesis to the offspring[26]. Specifically, mature sperm carries epigenetic information in patterns of DNA methylation, the region-specific retention of histones and DNA binding factors (like CTCF), as well as populations of small noncoding RNAs (ncRNAs). However, studies examining paternally-inherited abnormalities in growth and metabolic function have provided evidence to both support and refute the involvement of sperm-inherited changes in DNA methylation in the transmission of these phenotypes[7–9,16,17,23,25]. Similarly, although select regions of the sperm genome retain histones, it is unclear if this epigenetic information persists through the remodeling of the paternal genome during syngamy, or have the ability to transmit through the cell cycle[27,28]. The strongest candidate to date has been the transmission of paternally inherited ncRNAs. In these studies, the injection of either total sperm RNAs or a subset of sperm RNAs (for example, microRNAs or tRNA-derived small RNAs) into normal zygotes can recapitulate some paternal phenotypes induced by mental or metabolic stressors[29]. However, the mechanisms through which the effects of sperm-inherited ncRNAs persist into later life remain poorly defined[30].

In the United States, 70% of men drink and 40% drink heavily, with 8.3% reporting the routine consumption of more than two drinks per day[31–33]. Despite the nearly ubiquitous and constant exposure during reproductive ages, we currently have a very poor understanding of the effects chronic preconception alcohol use has on male reproductive physiology and the sperm-inherited developmental program. Using a mouse model of voluntary consumption, our lab recently described an association between chronic preconception paternal alcohol use and deficits in both placental function and offspring growth[23,34]. Importantly, these phenotypes did not associate with any measurable

alterations in sperm-inherited patterns of DNA methylation[23]. Therefore, the question of how the memory of chronic alcohol use transmits to the offspring remains unresolved.

Mechanistic studies in rodents have revealed that alcohol impairs the endocrine-reproductive axis, indicating male alcohol use may impact foundational aspects of reproductive function[35,36]. In addition, multiple studies in humans and rodents have indicated that chronic alcohol use negatively impacts the integrity of the sperm nucleus[37–42]. Finally, using an inhalation model of exposure, Rompala and colleagues recently described alcohol-induced alterations in the profile of sperm derived ncRNAs[24]. However, whether facets of male reproductive function, sperm nuclear structure or the profile of sperm-inherited ncRNAs are altered in our model of voluntary alcohol consumption and associate with the development of the observed growth defects remains to be resolved.

2. Materials and Methods

2.1 Animal work

All experiments were conducted under AUP 2017–0308 and approved by the Texas A&M University IACUC. Individually caged C57BL/6J (RRID:IMSR_JAX:000664) postnatal day 90 adult males were 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 12-hour light/dark cycle. In this study, we employed a voluntary model of alcohol exposure known as Drinking in the Dark. This model of exposure avoids the stress associated with forced feeding and mitigates the known impact of stress on the sperm-inherited epigenetic program[13]. Using methods described previously[23], males were provided limited access to ethanol during a four-hour window, beginning one hour after the initiation of the dark cycle. During this four-hour window, experimental males were provided access to a solution of 10% (w⁄v) ethanol (catalog# E7023, Millipore-Sigma, St. Louis, MO, USA) and 0.066% (w⁄v) Sweet'N Low (Cumberland Packing Corp, Brooklyn NY, USA). Control males received a solution of 0.066% (w⁄v) Sweet'N Low alone. After each session, the amount of fluid consumed by each mouse was recorded.

Once consistent patterns of drinking were established, males were maintained on this protocol for a period of 70 days. Subsequently, two naturally cycling females were placed in a new cage along with each exposed male. During these matings, males were not provided access to the alcohol/control preconception treatments. The next morning, matings were confirmed by the presence of a vaginal plug and both the male and female mice were returned to their original cages. Males were allowed a 24-hour rest period, during which the preconception exposure was resumed and then used in a subsequent mating. This procedure was repeated until each male had produced a minimum of two pregnancies. Subsequently, males were euthanized by CO_2 asphyxiation and cervical dislocation, blood collected post-mortem and the male reproductive tract excised. Pregnant dams were maintained on a Breeder diet (catalog# 5058, LabDiet, St. Louis, MO, USA), subjected to minimal handling and euthanized by CO_2 asphyxiation and cervical dislocation on gestational day 16.5. The female reproductive tract was excised, the gestational sac removed and fetal tissues weighed.

2.2 Measurement of Physiological Parameters

Male plasma alcohol concentrations were measured using the Ethanol Assay Kit (catalog# ECET100, BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocol. Levels of serum testosterone were determined using an ELISA at the Ligand Assay and Analysis Core at the University of Virginia Center for Research in Reproduction. Serum levels of corticosterone were measured using the Corticosterone ELISA kit (catalog# EC3001–1, AssayPro, St. Charles, MO, USA) according to the recommended protocol.

2.3 Sperm and Tissue Collection & Histology

The male reproductive tract, liver, and spleen were excised, trimmed of fat and their weights recorded. To collect mature sperm, cauda epididymides and roughly 1 cm of vas deferens were placed in 500 μL of pre-warmed (37ºC) Human Tubal Fluid (HTF) medium (catalog# ZHTF-100, Zenith Biotech, Blue Bell, PA, USA) in 12 well plates. Four or five incisions were made to the cauda to allow sperm to swim out and the vas deferens were carefully squeezed using forceps to expel their contents. Plates were allowed to incubate at 37ºC for 10 minutes. Sperm counts were performed by diluting a 10μl aliquot 1:50 in diH_2O and counting on a Neubauer chamber slide. Post-incubation, HTF media containing mature sperm were carefully layered over a pre-warmed (37ºC) gradient consisting of 350 μL each of 40% and 80% PureSperm (catalog#s PS40 and PS80, Spectrum Technologies, Healdsburg, CA, USA) in 1.5 mL microcentrifuge tubes and spun at 650g for 25 min. The pellet was then washed in 1mL of 1% BSA in PBS, and either immediately fixed for microscopic analyses or spun down, snap frozen in liquid Nitrogen and stored at −80ºC. To determine cell counts, 10 μL of caudal sperm from the last wash was used. Using microscopy, the purity of all samples was judged to be greater than 99%. After removal of encapsulating tunica, one testis from each animal was snap frozen in liquid nitrogen. The other testis was punctured with a 21 G needle and then fixed overnight in Bouin's solution. Bouin's fixed testes were paraffin embedded and stained using standard procedures for Hematoxylin and Eosin (H&E). H&E stained sections were imaged under bright field using the Leica DMi8 microscope (Leica microsystems, Germany). Cross-sectional area of tubules were measured using the area line tool in the Leica Application Suite X (LAS X) analysis software package. All tubules exhibiting longitudinal sectioning were excluded, while all tubules cut transversely into cross-sections were included in the analysis. Two non-sequential stained sections per animal were analyzed and a total of three animals per treatment were used in the comparison of calculated areas.

2.4 CMA3 staining

An equal volume of 3:1 methanol:acetic acid was added to PBS suspended sperm and samples incubated at 4ºC for 5 min. Samples were spread on glass slides and allowed to air dry at room temperature. Slides were treated for 20 minutes with 100 μL CMA3 solution: 0.25 mg/mL CMA (catalog# C2659, Millipore-Sigma, St. Louis, MO, USA) and 10mM $MgCl_2$, McIlvain's buffer (17 mM citric acid, 164 mM Na_2HPO_4, pH 7.0). Slides were rinsed with PBS and mounted with Prolong Gold Antifade Mountant (catalog# P36930, Thermo-Fisher, Waltham, MA, USA) and then kept at 4ºC overnight. Evaluation of fluorescence was done for a minimum of 200 spermatozoa on each slide.

2.5 Sperm TUNEL assay

The APO-DIRECT kit (catalog# 556381, BD Pharmingen, San Jose, CA, USA) was used for terminal deoxynucleotidyl transferase dUTP nick-end-labeling (TUNEL) to assess DNA damage. Approximately 3 million sperm per sample were resuspended in 4% PFA at room temperature for 1 hour. Samples were then spun down at 300xg for 5 minutes and washed twice with PBS. Pellets were suspended in ice-cold 70% ethanol and held at −20ºC for at least 12–18h. Following this incubation, preparation and staining of samples and appropriate controls were carried out as per manufacturer's instructions. Stained samples were settled onto poly-L-lysine coated glass slides, mounted in Prolong Gold Antifade Mountant, and visualized on a Leica DMi8 microscope with SOLA SE 365 light source and Chroma filters for FITC detection. Total cell counts and sperm morphology of the PFA fixed sperm was visualized by differential Interference contrast microscopy at 63x. At least three fields of view and 200 sperm were scored for each sample to determine percent TUNEL positive.

2.6 Sperm RNA Isolation

Approximately 10 million mature sperm per sample were lysed in 1mL TRIzol Reagent (catalog# 15596018, Thermo-Fisher, Waltham, MA, USA) plus 10 μl β-ME (catalog# M3148, Millipore-Sigma, St. Louis, MO, USA) using homogenizing pestles (catalog# 6478820, Electron Microscopy Sciences, Hatfield, PA, USA). Subsequently, 200μl of 1-Bromo-3-chloropropane (catalog# B9673, Millipore-Sigma, St. Louis, MO, USA) was added to separate the aqueous phase, which was collected and precipitated using an equal volume of isopropanol. After two ice-cold, 70% ethanol washes, RNA samples were reconstituted in RNase free water and stored at −80ºC.

2.7 Sperm RNA Sequencing

Illumina single-end cDNA libraries were synthesized from size-selected RNAs (<50 bases) derived from 100 ng of sperm total RNA using the TruSeq Stranded mRNA kit. Four biological replicates per treatment group were multiplexed and sequenced on a single HiSeq2000 lane (Illumina) within the sequencing core of the Whitehead Institute. Using Bowtie2 (RRID:SCR_016368) and Tophat (RRID:SCR_013035), small RNA reads were aligned to the Mus musculus (UCSC version mm10) reference genome[43]. Small RNA reads with a single allowable mismatch were selected for further analysis. Small RNA annotation was performed by separately aligning reads to the microRNA database(http://www.mirbase.org/), tRNA database(http://gtrnadb.ucsc.edu/), and piRNA database(http://pirnabank.ibab.ac.in/index.shtml), as described previously[44]. Differentially expressed microRNAs were quantified using the miRDEEP2 ver2.0.0.7 pipeline[45]. The referenced tRNA database holds sequences for both tRNA anticodons and tRNA fragments (tRFs), which are selected as reads partially matching the tRNA anticodon but are less than 34 nucleotides in length[19]. To quantify differentially expressed tRFs, piRNAs, and tRNAs, total counts of mapped reads were calculated using the featureCounts pipeline[46] and then normalized to the total mapped reads of each class of small RNA species, as described previously[19]. Generated volcano plots contrast the differential enrichment of ncRNAs by raw p-value and not by FDR selection.

2.8 Data Handling and Statistical Analysis

For all experiments, measures were input into the statistical analysis program GraphPad (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, USA) and statistical significance was set at alpha = 0.05. For all datasets, normality was first verified using the Brown-Forsythe test. In this study, the effect of treatment was assessed using either an unpaired Student's t-test or two-way analysis of variance test (ANOVA), with differences among the means evaluated using Sidak's posthoc test of contrast. In all instances, we have marked statistically significant differences with an asterisk. For the comparisons of testicular, epididymal and seminal vesicle weights expressed as a percentage of total paternal body weight, as well as the percentage of TUNEL and CMA3 positive sperm, data were arcsine transformed and an unpaired t-test with Welch's correction applied. To calculate the tRFs:piRNA ratio, the percentage of mapped tRFs per sample was divided by the percentage of mapped piRNAs and differences compared between treatments using an unpaired student's t-test.

3. Results

3.1 Daily ethanol exposures induce pharmacologically meaningful blood alcohol concentrations but do not impact paternal body weight.

To define the long-term impact alcohol exposure has on both reproductive function and the male-inherited developmental program, we returned to our established mouse model of chronic alcohol exposure[23, 34]. Here, postnatal day 90 adult males were provided limited access to either the ethanol or control preconception treatments during a four-hour window, that began one hour into the night cycle. Once consistent patterns of alcohol consumption were established, males were maintained on this protocol for a period of 70 days, which corresponds to the length of two complete spermatogenic cycles and ensures that sperm formed prior to alcohol treatment are not able to confound the resulting phenotypes. For each male, the amount of fluid consumed per day was recorded. No differences in fluid consumption between the two preconception treatment groups were observed (Fig. 1A).

The rodent model we employed (Drinking in the Dark) promotes the daily, voluntary consumption of ethanol in sufficient quantities to achieve pharmacologically meaningful blood alcohol concentrations, typically around 125 mg/dL or 1.5x the legal limit (Fig. 1B). In the United States, 16% of men report engaging in high-risk drinking, which is defined as exceeding five or more standard drinks on any day within a given week and importantly, 8.3% of men routinely consume more than two drinks per day[33,47]. Therefore, the blood alcohol levels observed in our model of preconception male alcohol exposure are physiologically relevant. Further, chronic, daily alcohol use among males is both prevalent and a significant health concern. During the treatment course, no differences in body weight or changes in the rate of weight gain were observed between the preconception treatment groups (Fig 1C–D).

3.2 Chronic paternal alcohol exposure induces late-term fetal growth restriction and reductions in placental efficiency within the offspring.

In our previous studies examining the offspring of alcohol-exposed males, we identified fetal growth restriction in only the female offspring at gestational day 14.5[23], while at birth, both male and female offspring exhibited significant growth restriction[34]. Therefore, we examined fetal growth at day 16.5 of gestation, which corresponds to the period when the mouse fetus experiences a dramatic increase in growth rate[48]. After 70 days of exposure, males undergoing the described preconception treatments were mated to unexposed females, and at gestational day 16.5, dams were sacrificed and offspring evaluated for growth. No differences in litter size were observed between the preconception treatment groups (Fig. 2A). At this developmental stage, the male and female offspring of alcohol-exposed sires displayed a ~15% reduction in fetal weight ($p < 0.01$), a ~10% reduction in crown-rump length ($p < 0.01$) and a respective 9% and 14% reduction in the weight of the gestational sac ($p < 0.05$) (Fig. 2B–D). These reductions in fetal growth were accompanied by an 18% increase in the placental weight of the male offspring of alcohol-exposed sires, while placental weights of the female offspring were identical to the controls (Fig. 2D). Collectively, a respective 28% and 17% reduction in placental efficiency (grams of fetus produced per gram of placenta[49]) was observed for the male and female offspring of the alcohol-exposed sires (Fig. 2E). These observations indicate that the growth restriction associated with paternal alcohol use predominantly manifest during the later phases of pregnancy and correlate with reductions in placental efficiency.

3.3 Chronic paternal alcohol use does not overtly impact male reproductive physiology

To better understand how the memory of chronic paternal alcohol use transmits to the offspring, we began by examining large-scale measures of male reproductive function. No differences in the absolute or proportional weights of the testis, epididymis or seminal vesicles, as well as the spleen or liver could be identified (Fig. 3A–E). Mean values and variations in serum testosterone levels we similar between the treatment groups and were consistent with established values[50] (Fig. 3F). Histological examinations of testicular sections could not identify any overt morphological differences and no changes in the mean surface area of the seminiferous tubules could be identified between the preconception treatment groups (Fig 3G–I). From these results, we conclude that the dose and duration of chronic alcohol exposure employed here do not overtly impact male reproductive physiology.

3.4 Chronic paternal alcohol use does not impact sperm production, morphology, viability or large-scale measures of chromatin structure

We next assayed the impact of chronic male alcohol use on sperm production. No differences in total sperm counts or changes in sperm morphology could be identified between treatment groups (Fig. 4A–B). Using the TUNEL assay, we were unable to identify any increases in sperm DNA fragmentation (Fig. 4C). Chromomycin A3 (CMA3) is a fluorochrome, which has been shown to compete with protamines for binding to the minor groove of DNA and is, therefore, indicative of compromised nuclear packaging. Using this stain, we could not detect any increases in the sperm of males chronically exposed to alcohol

(Fig. 4D). Thus, we were unable to identify any large-scale changes in sperm production, DNA fragmentation (TUNEL assay) or nuclear packaging (CMA3 staining).

3.5 Chronic paternal alcohol alters the profile of sperm-inherited non-coding RNAs.

Using an inhalation model of exposure, Rompala and colleagues recently described alterations in sperm derived ncRNAs induced by a 5-week exposure to alcohol[24]. These studies achieved similar blood alcohol levels (125–175mg/dL) to those observed in our model. However, whether these separate models of exposure and different durations induce similar or distinct impacts on the profile of sperm-inherited ncRNAs is unknown. To examine this further, mature sperm were collected from the cauda epididymides and vas deferens of the control and ethanol-exposed males used to sire the offspring analyzed in Figure 2 (see materials and methods). The purity of sperm was judged to be greater than 99% as evaluated by microscopy. Similar to previous reports[51], isolated RNAs predominantly ranged from 20 to 40 nucleotides in length (Figure 5A). Small RNAs from control and ethanol-exposed males were subjected to deep sequencing analysis, with an average of 25 million mappable reads obtained per sample (n=4). Similar to previous studies describing the small RNA profiles of mouse sperm[19,24,51], we found that the majority of small RNA reads mapped to transfer RNA-derived small RNAs (~60% tRFs) and Piwi-interacting RNAs (~30% piRNAs) (Fig. 5B). The remaining small RNAs predominantly mapped to transfer RNAs (~5% tRNAs) and microRNAs (~5% miRNAs) (Fig 5B). A recent study by Sharma et al., described proportional changes in the ratio of tRFs and piRNAs as sperm undergo maturation in the epididymis[44]. Here, a progressive increase in tRFs and a loss of piRNAs were observed as sperm mature. Although sperm derived from alcohol-exposed males tended to have proportionally fewer mappable tRFs and a greater abundance of piRNAs, individually, these trends did not reach statistical significance ($p=0.0552$ and $p=0.1086$) (Fig. 5C–D). However, a ratio comparing tRFs:piRNAs revealed a significant shift ($p<0.05$) between the two preconception treatment groups (Fig. 5E). Further, while populations of tRNAs were similar between treatments, we observed a significant ($p=0.03$), ~30% increase in the abundance of miRNAs in sperm derived from alcohol-exposed males (Fig 5F–G). These observations reveal that chronic alcohol consumption shifts the profile of sperm-inherited non-coding RNAs, with miRNAs exhibiting the greatest change.

3.6 Alterations in the abundance of miR21, miR30, and miR142 in alcohol-exposed sperm.

Using the Bowtie2 and miRDEEP2 pipelines, we compared the abundance of individual candidate small non-coding RNAs between control and alcohol-exposed sperm. No differentially enriched tRFs, piRNAs or tRNAs could be identified between treatment groups (Fig. 6A–C). In contrast, three differentially enriched miRNAs could be identified between treatments (miR21, miR30, and miR142) (Fig. 6D). Of these, miR21 and miR142 were abundantly enriched in both treatment groups (miR21 500 (C) and 900 (A) fpkm, miR142 600 (C) and 300 (A) fpkm). However, differences in the abundance of these two candidates offset each other, and therefore, do not explain the 30% increase in miRNA enrichment observed in alcohol-exposed sperm (Fig. 5F). In contrast to these candidates, the remaining miRNAs identified displayed large variations both across and within treatment groups (Fig. 6E). Collectively, these observations indicate that the 30% increase in miRNA abundance represents a general increase and is not linked to any specific candidate. Recently, alterations

in the profile of sperm-inherited miRNAs induced by chronic stress have been directly linked to increased circulating levels of corticosterone[52]. We, therefore, assayed the levels of this hormone in our model. No differences in corticosterone could be identified between preconception treatment groups (Fig. 6F). Therefore, the 30% increase in miRNA enrichment and differences in miR21, miR30 and miR142 cannot be linked to alcohol-induced changes in the profile of circulating corticosterone.

4. Discussion

Using a mouse model of voluntary alcohol consumption, our group recently described an association between chronic preconception paternal alcohol use and deficits in both placental function and fetal growth within the offspring[23]. Subsequent studies revealed that these alcohol-induced growth phenotypes were accompanied by a prolonged period of fetal gestation and sex-specific patterns of postnatal growth restriction[34]. These deficits in growth are similar to phenotypes described in long-term clinical studies of children with fetal alcohol spectrum disorders[53,54] and join a growing body of literature indicating preconception paternal alcohol use is a significant, yet under-recognized contributor to alcohol-induced growth defects (reviewed here[55–57]). However, the question of how the memory of chronic alcohol use transmits from father to offspring remains unresolved.

The literature examining the impacts of chronic alcohol use on male reproductive physiology is varied and highly inconsistent[58,59]. To this point, of three published studies using similar rodent models of exposure, two identified systemic decreases in testosterone concentrations[60,61], while the third was unable to identify any reproducible changes[62]. Similarly, the human literature is equally varied, with studies describing both alcohol-associated decreases and increases in testosterone levels[63–65]. However, combined with the negative correlations observed between alcohol use and successful outcomes in human in vitro fertilization[66,67], the prevailing feeling is that this teratogen exerts a negative effect on male reproductive function[58,59].

In this study, we returned to our voluntary model of alcohol consumption to determine the impact chronic ethanol use has on male reproductive physiology and the association of sperm-inherited noncoding RNAs with the transmission of alcohol-induced growth defects. Here, we first confirmed that chronic preconception paternal alcohol exposure induced fetal growth restriction in the offspring and extended our previous findings by demonstrating that these deficits in growth primarily manifest during the later phase of pregnancy (Fig. 2). We then assayed large-scale measures of male reproductive health, including testicular, epididymal and seminal vesicle weights, as well as testicular morphology and testosterone levels. However, we were unable to identify changes in any of these criteria. In addition, no differences in total sperm counts, sperm DNA fragmentation or sperm nuclear packaging were observed between the preconception treatment groups. Combined with the observed similarities in litter size between preconception treatments, we conclude that the dose and duration of alcohol exposure employed in our model do not impact macro-measures of male reproductive physiology.

As no observable changes in either sperm DNA methylation[23] or macro-measures of nuclear structure (Fig. 4) could be observed, we examined alcohol-induced alterations in the profile of sperm-inherited noncoding RNAs. Using an inhalation model of exposure, Rompala and colleagues recently identified alcohol-induced changes in the profile of tRFs and miRNAs, as well as select mitochondrial small mRNAs in sperm[24]. In contrast to these observations, we were only able to identify differences in select miRNAs, none of which were consistent with this published study. Although both models result in sex-specific patterns of postnatal growth restriction[68], these two separate models of exposure may induce distinct epigenetic changes. However, we did observe some similarities between our dataset and previous works examining the impact of paternal stress on offspring phenotype. Specifically, miR-30 and miR-21 were both up-regulated in the present study as well as in that of three previous reports examining stress-induced changes in sperm noncoding RNAs[13,52,69]. Although the levels of plasma corticosterone observed in our mice were higher than those reported by Rodgers et al.[69], we presume this difference is due to the techniques used to measure corticosterone levels and note that our results are consistent with those of two other publications employing ELISA-based measurements[52,70]. Regardless, we could not identify any changes in the levels of plasma corticosterone between treatment groups, indicating alcohol may alter sperm-inherited noncoding RNAs through distinct mechanisms. Importantly, the three differentially enriched candidate miRNAs identified in this study are all known to be modulated by alcohol exposure[71,72].

Recent studies indicate that dynamic changes in the levels of tRFs, piRNAs, and miRNAs are a core feature of sperm maturation[44]. Specifically, progressive increases in tRFs are observed during epididymal transit, while conversely, piRNAs become reduced. These changes are hypothesized to be integral to sperm maturation and the reproductive success of the conceptus[44]. In this study, we observed a shift in the ratio of tRFs and piRNAs indicating that the sperm of alcohol-exposed males has proportionally fewer tRFs and more piRNAs. This may indicate that the complement of non-coding RNAs in alcohol-exposed sperm are less mature than the controls. Of note, miR21, which is comparatively rare in testicular sperm populations, is nearly absent in the in the epididymal soma but is highly abundant in epididymosomes of the caput region of the epididymis[73–75]. This suggests that the alcohol-induced shifts in noncoding RNAs may primarily be mediated by effects on epididymal trafficking. However, further studies are needed to confirm this hypothesis.

At this point, we do not know if the modest changes in noncoding RNA abundance identified in this study are directly linked to the alcohol-induced growth restriction phenotypes observed in the offspring. The comparative contribution of sperm-inherited RNAs to the vast repertoire found in the early conceptus is negligible[76] and further, no mechanisms have been identified by which this small contribution could stably alter the gene expression profile of the early embryo and the long-term health of the offspring. In light of this, it is challenging to see how modest changes (50% decrease in miR142, 1.5 fold increases in miR21 and miR30) in the identified candidates could induce fetal growth restriction with the consistency observed in our model, as well as mediate the long-term impacts on offspring metabolic health. One possibility is that the paternally-inherited RNAs are post-transcriptionally modified to confer dramatically enhanced stability, which may potentiate their impacts beyond preimplantation development and influence the processes of

lineage specification[29]. It may also be that unrelated alterations in sperm histone retention and chromatin looping mediate the observed effects or a combinatorial interaction between multiple epigenetic mediators. Finally, we have not ruled out the possibility that these phenotypes may be due to alcohol-induced changes in the seminal plasma. In previous studies, ablating the seminal vesicle gland induced placental hypertrophy in late gestation and sex-specific effects on the long-term growth and metabolic health of the offspring[77]. Although the effects on offspring growth were opposite to those observed in our model, the impact on placental growth is compelling. Future studies using IVF will be necessary to determine the impact of male seminal plasma in mediating the effects of paternal alcohol use on the offspring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ncRNAs	noncoding RNAs
tRFs	transfer RNA-derived small RNAs
piRNAs	Piwi-interacting RNAs
tRNAs	transfer RNAs
miRNAs	microRNAs
CMA3	Chromomycin A3
fpkm	fragments per kilobase of transcript per million mapped reads

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Highlights:

- Chronic preconception paternal alcohol exposure associates with late-term fetal growth restriction and a loss of placental efficiency in the offspring.
- The model of chronic alcohol consumption employed in this study did not impact macro-measures of male reproductive physiology or negatively affect fertility.
- Chronic alcohol use alters the ratio of transfer RNA-derived small RNAs to Piwi-interacting RNAs, which has recently been identified as a core facet of the epigenetic maturation of sperm.
- Chronic alcohol exposure induces a 30% increase in the abundance of sperminherited miRNAs, with miR21, miR30 and miR142 exhibiting the greatest changes.
- Although our data share some similarities to recent work examining stressinduced changes in paternally-inherited miRNAs, we did not observe any differences in the levels of plasma corticosterone, indicating a novel mechanism underlies the observed changes.

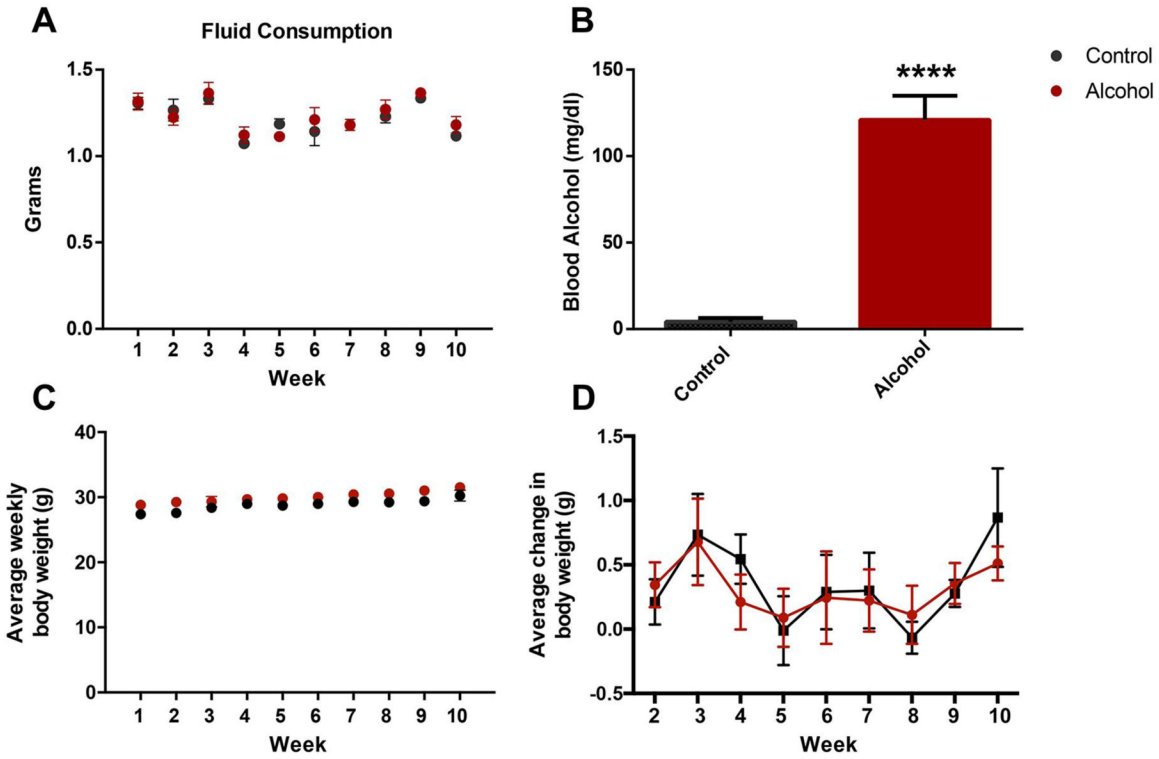

Figure 1.

Chronic alcohol exposure using a limited access model induces physiologically relevant plasma alcohol levels but does not alter paternal weight. A) Average daily fluid consumption compared between the preconception treatment groups (n=11). B) Average plasma alcohol levels between preconception treatment groups, as measured one month after the beginning of treatment (n=9). C) Average weight of males in each treatment group over the experimental course (n=11). Average weekly weight gain of males in each preconception treatment group (n=11). Data were analyzed using an unpaired t-test, error bars represent the standard error of the mean (**** $p < 0.0001$).

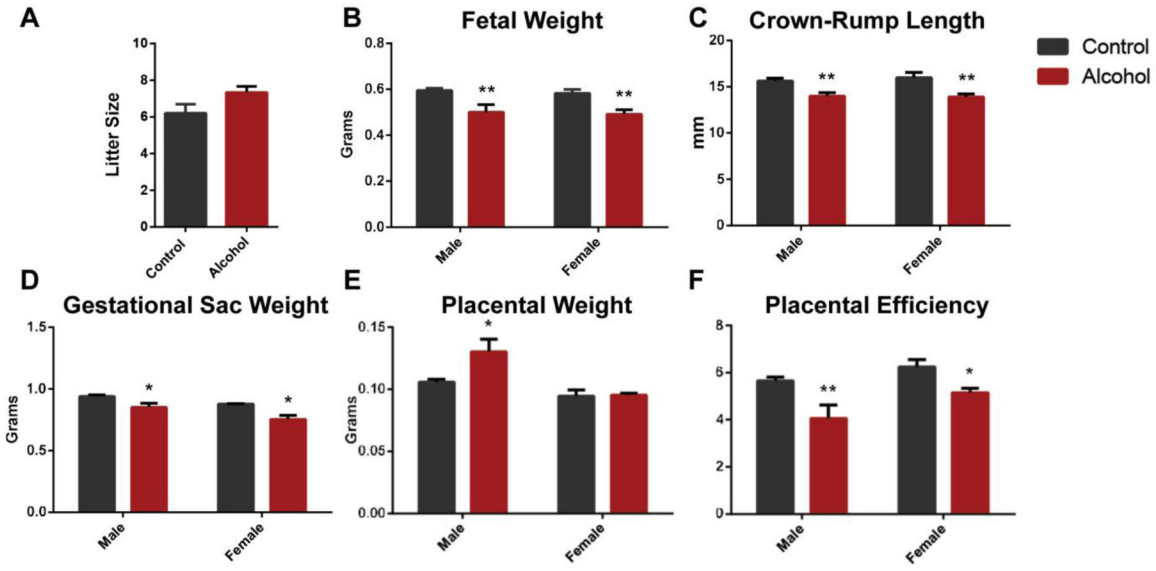

Figure 2.

Chronic preconception paternal alcohol exposure induces fetal growth restriction and decreased placental efficiency in the offspring at gestation day 16.5. A) Comparison of litter size between matings sired by control and ethanol-exposed males (n=5 control 6 alcohol). Comparisons of B) fetal weight, C) crown-rump length, D) gestational sac weight and E) placental weights between male and female offspring sired by control and ethanol-exposed males (n=10 male and 12 female offspring). F) Placental efficiencies (gram of fetus produced per gram of placenta) compared between the male and female offspring of ethanol-exposed sires (n=10 male and 12 female offspring). Data were analyzed using either an unpaired t-test or a two-way ANOVA followed by Sidak's post hoc analysis. Error bars represent the standard error of the mean (* $p<0.05$, ** $p < 0.01$).

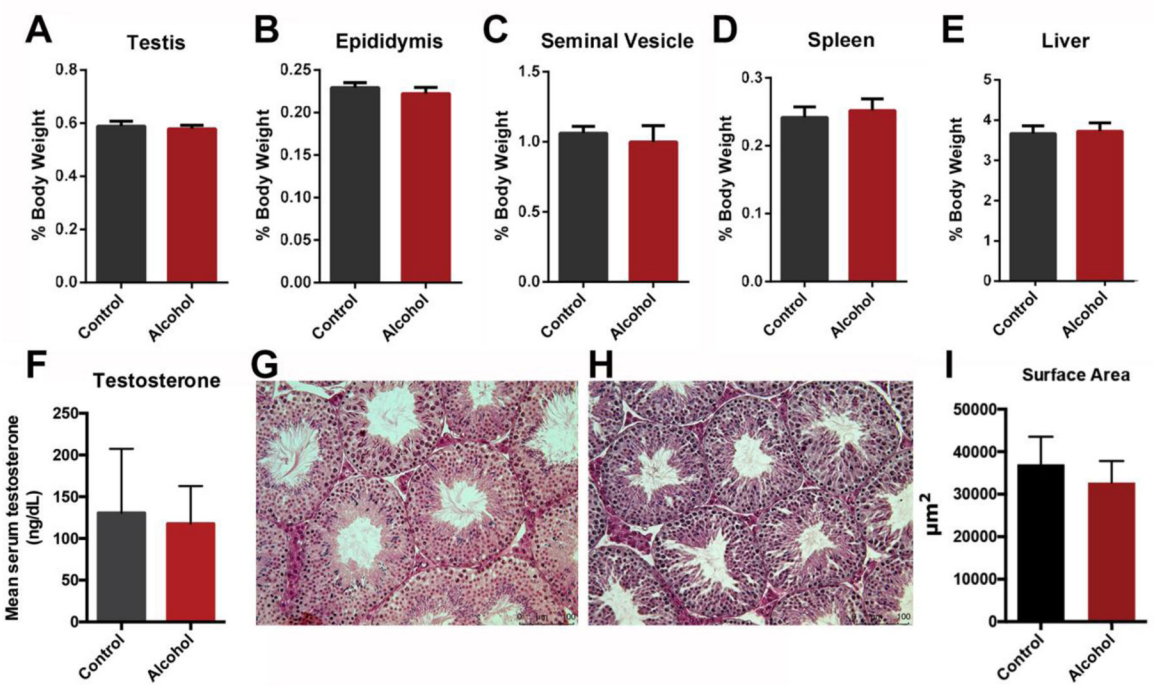

Figure 3.

Chronic alcohol exposure does not impact macro measures of male reproductive physiology. Comparisons of proportional A) testicular, B) epididymal, C) seminal vesicle, D) spleen and E) liver weights between preconception treatment groups (n=11). F) Levels of plasma testosterone were compared between preconception treatment groups (n=6). Representative hematoxylin and eosin stained sections of G) control and H) alcohol-exposed testes (n=3, with two, non-consecutive sections examined for each testis). I) Surface area of sectioned seminiferous tubules was determined using the LASX software package and compared between preconception treatment groups (n=3). Percent organ weights were arcsine transformed and an unpaired t-test with Welch's correction used to compare treatments. For all other comparisons, an unpaired t-test was applied. Error bars represent the standard error of the mean.

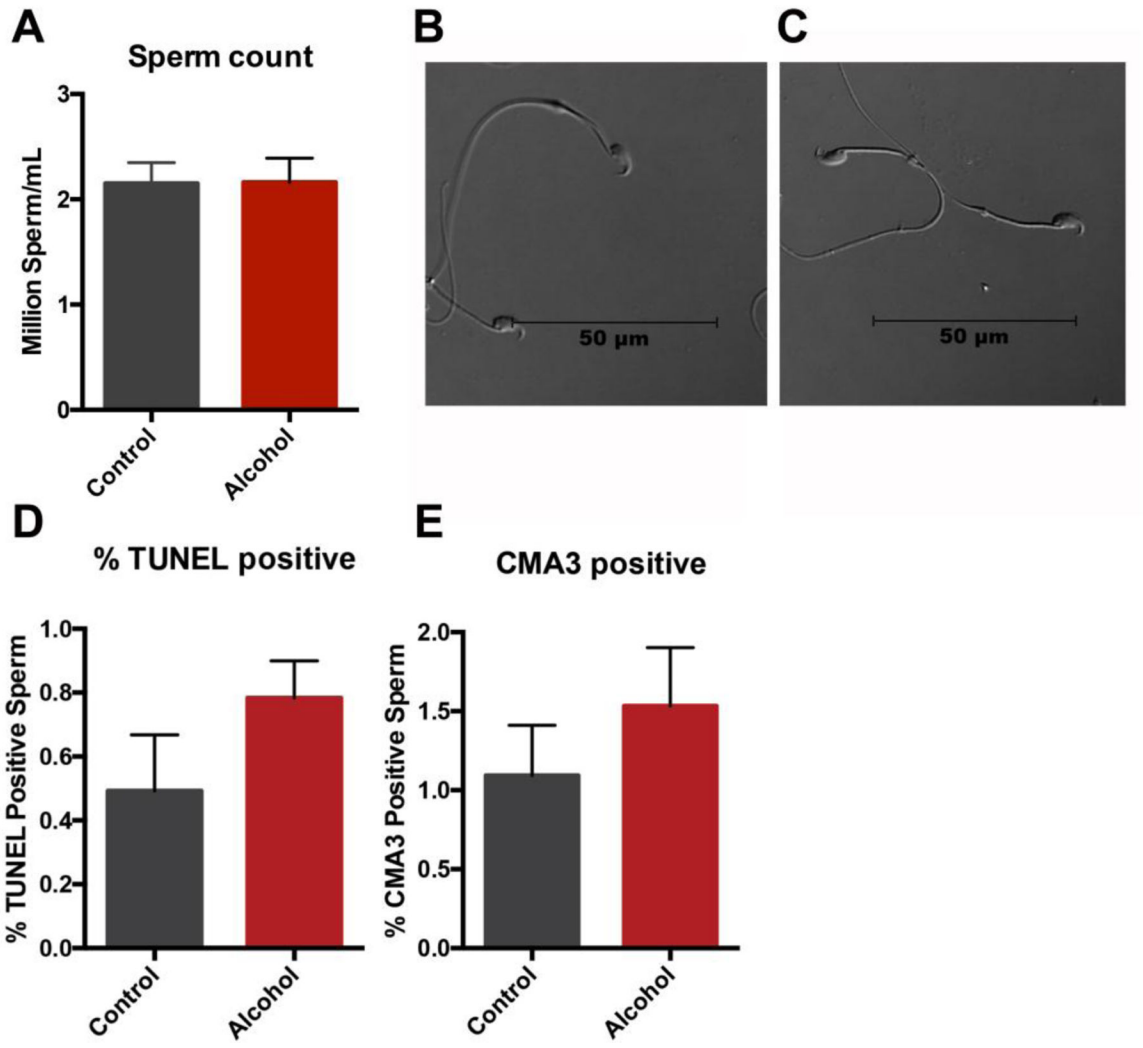

Figure 4.

Chronic paternal alcohol exposures do not measurably impede sperm production or alter either sperm DNA fragmentation or nuclear packaging. A) Total sperm counts were compared between preconception treatment groups (n=11). Representative light micrographs comparing B) control and C) alcohol-exposed sperm (n=11). The percentage of D) TUNEL positive and E) CMA3 positive sperm were compared between preconception treatment groups (n=4). Data comparing the percentage of stained sperm were arcsine transformed and an unpaired t-test with Welch's correction applied. All other comparisons were conducted using an unpaired t-test. Error bars represent the standard error of the mean.

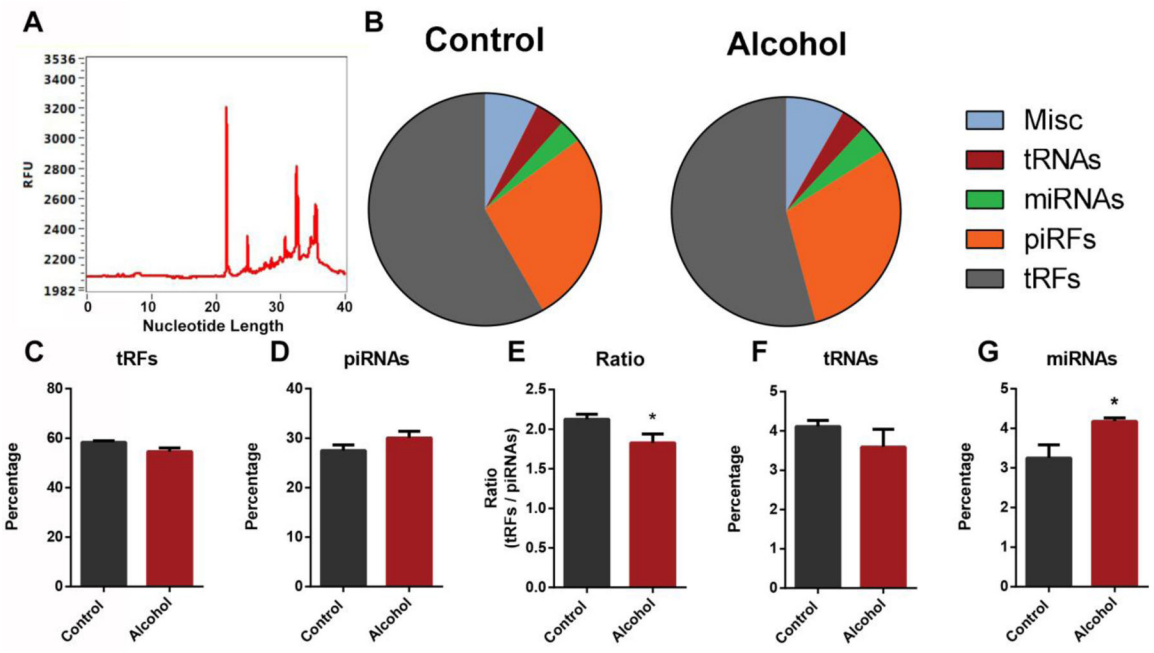

Figure 5.

Alcohol-induced alterations to the profile of sperm-inherited non-coding RNAs. A) Representative graph depicting the size distribution of RNAs isolated from sperm. B) The proportional abundance of transfer RNA-derived small RNAs (~60% tRFs) Piwi-interacting RNAs (~30% piRNAs), transfer RNAs (~5% tRNAs) and microRNAs (~5% miRNAs) between sperm derived from the two preconception treatment groups (n=4). Individual comparison of the percentage of C) tRFs and D) piRNAs mapped between preconception treatment groups. E) The ratio of tRFs:piRNAs in sperm derived from control and alcohol-exposed males. Individual comparison of the percentage of F) tRNAs and G) miRNAs mapped between preconception treatment groups. For comparison of percentages mapped, data were arcsine transformed and an unpaired t-test with Welch's correction applied. All other comparisons were conducted using an unpaired t-test. Error bars represent the standard error of the mean (* $p<0.05$).

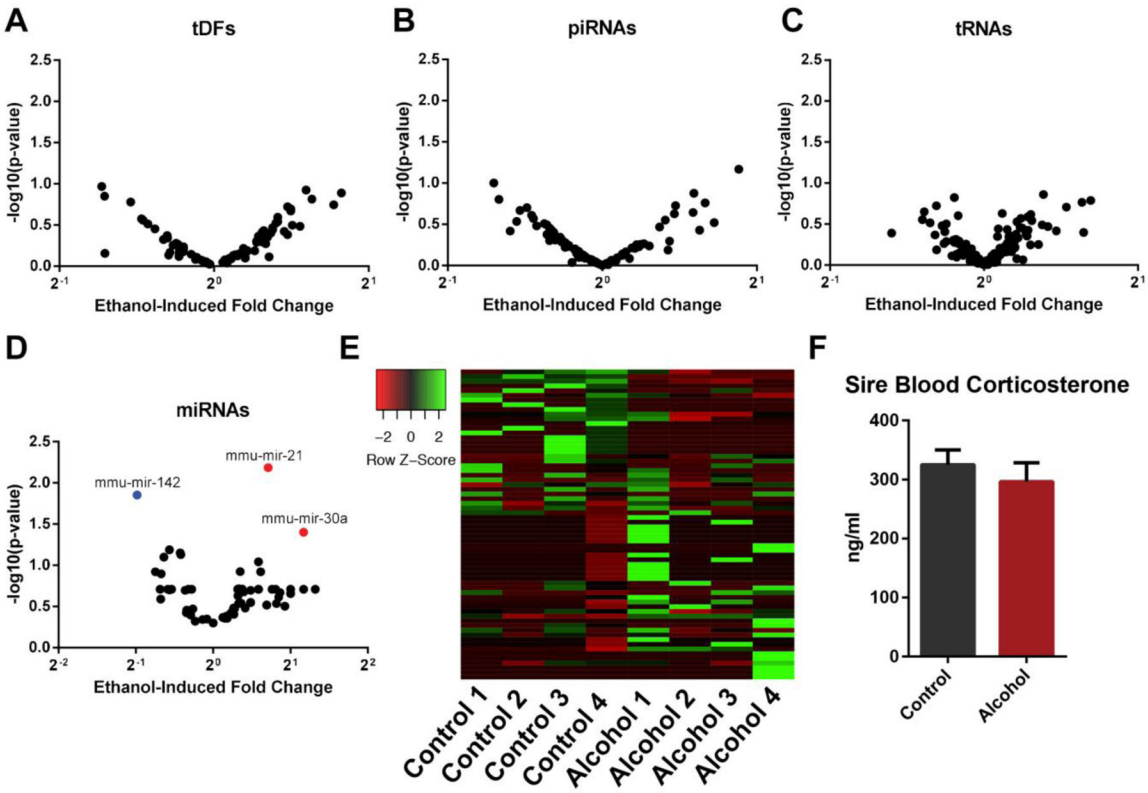

Figure 6.

Alcohol-induced changes in the abundance of sperm-inherited miR21, miR30, and miR142. Volcano plots comparing the differential enrichment of candidate A) tRFs, B) piRNAs, C) tRNAs and D) miRNAs of sperm derived from the two preconception treatment groups. E) Heatmap comparing the variation of sperm derived miRNAs between treatment groups. F) Comparison of circulating levels of corticosterone between preconception treatment groups. An unpaired t-test was applied to compare the levels of corticosterone. Error bars represent the standard error of the mean, miRNAs identified with either blue or red dots were differentially enriched ($p<0.05$).