Received: 15 June 2023 | Revised: 10 October 2023 | Accepted: 20 November 2023

DOI: 10.1111/andr.13566

ANDROLOGY

WILEY

ORIGINAL ARTICLE

Alterations in sperm RNAs persist after alcohol cessation and correlate with epididymal mitochondrial dysfunction

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Funding information
NIH , Grant/Award Number: NIAAA R01AA028219; National Institute on Alcohol Abuse and Alcoholism; W. M. Keck Foundation

Abstract

Background: Chronic preconception paternal alcohol use adversely modifies the sperm epigenome, inducing fetoplacental and craniofacial growth defects in the offspring of exposed males. A crucial outstanding question in the field of paternal epigenetic inheritance concerns the resilience of the male germline and its capacity to recover and correct sperm-inherited epigenetic errors after stressor withdrawal.

Objectives: We set out to determine if measures of the sperm-inherited epigenetic program revert to match the control treatment 1 month after withdrawing the daily alcohol treatments.

Materials and methods: Using a voluntary access model, we exposed C57BL/6J males to 6% or 10% alcohol for 10 weeks, withdrew the alcohol treatments for 4 weeks, and used RNA sequencing to examine gene expression patterns in the caput section of the epididymis. We then compared the abundance of sperm small RNA species between treatments.

Results: In the caput section of the epididymis, chronic alcohol exposure induced changes in the transcriptional control of genetic pathways related to the mitochondrial function, oxidative phosphorylation, and the generalized stress response (EIF2 signaling). Subsequent analysis identified region-specific, alcohol-induced changes in mitochondrial DNA copy number across the epididymis, which correlated with increases in the mitochondrial DNA content of alcohol-exposed sperm. Notably, in the corpus section of the epididymis, increases in mitochondrial DNA copy number persisted 1 month after alcohol cessation. Analysis of sperm noncoding RNAs between control and alcohol-exposed males 1 month after alcohol withdrawal revealed a ~100-fold increase in mir-196a, a microRNA induced as part of the nuclear factor erythroid 2-related factor 2 (Nrf2)-driven cellular antioxidant response.

Discussion and conclusion: Our data reveal that alcohol-induced epididymal mitochondrial dysfunction and differences in sperm noncoding RNA content persist after alcohol withdrawal. Further, differences in mir-196a and sperm mitochondrial DNA copy number may serve as viable biomarkers of adverse alterations in the sperm-inherited epigenetic program.

KEYWORDS
alcohol, epididymis, epigenetics, mitochondrial dysfunction, paternal epigenetic inheritance, sperm

1 | INTRODUCTION

Preconception exposures are an emerging area of interest in our efforts to understand the developmental origins of birth defects, disease, and neurological dysfunction.[1,2] Although researchers have long recognized the importance of preconception maternal health in pregnancy and child developmental outcomes, paternal exposures have only recently emerged as significant modifiers of placental function and offspring development.[3,4] Indeed, researchers now recognize that sperm contain a vast suite of epigenetic information[5,6] and that a wide range of different stressors, including nutritional deficiencies or excess, inflammation, drugs of abuse, environmental toxicants, and psychological trauma, each modifies the sperm-inherited epigenome, with adverse impacts on offspring health.[7–12] Nevertheless, although we now recognize these influences, the biochemical mechanisms by which the epigenetic memories of paternal experiences and stressors influence fertility and transmit to offspring remain almost completely undefined.

One of the key outstanding questions in the field of paternal epigenetic inheritance concerns the resilience of the male reproductive tract and the germline's capacity to recover and correct sperm-inherited epigenetic errors after stressor withdrawal. Previous studies demonstrate that while some stressors exert transient impacts on overall male fertility, others permanently affect sperm production and fecundity. For example, male exposures to anabolic steroids, heat stress, and COVID-19 infection each induce transient reductions in fertility that reverse after approximately one spermatogenic cycle.[13–15] Similarly, cessation from smoking and chronic alcohol use also correlate with improvements in fertility, although the duration of recovery may extend across multiple spermatogenic cycles, depending on the severity of drug use.[16–18] In contrast, infertility induced by male chemotherapy and radiotherapy treatments may persist for several years or be permanent.[19,20] However, although stressor or toxicant withdrawal generally correlates with improvements in macro measures of male fertility, whether these exposures induce lasting changes to the sperm-inherited developmental program or if exposure-induced epigenetic errors self-correct after cessation remains unknown.

In the United States, 70% of men drink, and 40% engage in repetitive binge drinking.[21,22] Moreover, men are likelier than women to engage in risky alcohol use patterns and less likely to modify their preconception behaviors when considering fatherhood.[23] Clinical studies provide conflicting data on the impacts of alcohol intake on male fertility, with some studies suggesting modest declines, while others report no observable effects.[24–29] Nonetheless, clinical studies examining high-level, chronic exposures demonstrate adverse impacts on overall health, increases in systemic oxidative stress, and decreases in fertility.[30] Notably, a small number of studies suggest that the varied effects of alcohol on fertility across human populations may link to genetic differences in the capacity to mitigate oxidative stress, specifically polymorphisms in Glutathione S-transferase.[24] However, even in cases of heavy chronic alcohol use disorder, patients demonstrate improvements in overall fertility after withdrawal.[16,17]

Using a mouse model, our group has demonstrated that chronic preconception paternal alcohol exposures induce dose-dependent changes in placental patterning, defects in craniofacial development, and long-term effects on postnatal glucose homeostasis.[31–40] In these previous studies, we did not observe any differences in sperm count, morphology, or offspring litter size.[31–33,35,37,39] However, using an in vitro fertilization (IVF) system to model the impacts of paternal alcohol use on early embryonic development, we observed that chronic ethanol exposures reduce embryo development and pregnancy success rates in a dose-dependent manner.[38] These intergenerational effects on fertility and offspring development correlate with alcohol-induced alterations in sperm-inherited noncoding RNAs and histone structure but do not associate with any significant changes in DNA methylation.[31,33,36] Significantly, the Homanics group has also identified alcohol-induced changes in sperm noncoding RNAs using a vapor chamber exposure model, reinforcing the assertion that paternal ethanol exposures affect sperm small noncoding RNA abundance.[41] These reports join a growing body of clinical and preclinical studies indicating paternal alcohol use induces heritable epigenetic changes in offspring phenotypes that correlate with fetal alcohol spectrum disorder (FASD) behavioral, neurological, and structural defects.[2,42] However, whether, like clinical studies examining measures of overall fertility, alcohol withdrawal ameliorates the observed epigenetic changes in sperm is not known.

Small RNAs play a central role in the epigenetic transmission of environmental information from parents to offspring.[6] Two previous reports, including work from our group, identified alcohol-induced changes in sperm-derived microRNAs (miRNAs) and select

tRNA-derived sequences.[33,41] Herein, we examined the impacts of cessation from chronic alcohol exposure on sperm small noncoding RNA abundance. Packaging of mammalian noncoding RNAs into sperm initially occurs during testicular spermatogenesis but is modified during epididymal transit.[43] In mice, large-scale changes in sperm-inherited miRNAs and tRNA-derived sequences occur during epididymal transit,[43] which occurs over a 10-day period.[44] However, previous studies examining mouse models of binge drinking demonstrate that the negative impacts of alcohol withdrawal, including anxiety- and depressive-like disturbances and molecular alterations in neurological activity, last for at least 3 weeks.[45,46] Therefore, we hypothesized that cessation for 1 month would allow for the normalization of alcohol-induced changes in the sperm noncoding RNA profile. Instead, our analyses reveal that chronic alcohol exposures induce a lasting molecular signature of mitochondrial dysfunction. Notably, even after 1 month of abstinence, elements of this signature remain in the corpus segment of the epididymis, and significant differences in the sperm noncoding RNA profile persist. These data suggest that, like neurological models examining alcohol withdrawal, the male reproductive tract and sperm-inherited epigenetic program continue to exhibit evidence of alcohol-induced disturbance after toxicant removal.

2 | MATERIALS AND METHODS

2.1 | Animal studies and ethanol exposures

We designed our study following ARRIVE guidelines and conducted all experiments following IACUC regulations, with prior approval by the Texas A&M University IACUC, under protocol number 2020-0211. We utilized male C57BL/6J strain mice (RRID:IMSR_JAX:000664), which we derived from a breeder nucleus and housed in the Texas A&M Institute for Genomic Medicine. We maintained males on a standard chow diet (Catalog# 2019; Teklad Diets, Madison, WI, USA) with free water access and a reverse 12-h light/dark cycle (lights off at 8:30 AM). As in our previous studies, we added shelter tubes (catalog# K3322; Bio-Serv, Flemington, NJ, USA) and additional nestlets to minimize animal stress and enhance cage enrichment.

Beginning on postnatal day 90, we individually caged males and initiated the control and ethanol (**EtOH**) treatments using a prolonged version of the drinking in the dark model.[47] Using published methods,[37,38] we exposed males to control (water alone), 6% or 10% (w/v) EtOH (catalog# E7023; Millipore-Sigma, St. Louis, MO, USA) treatments, with exposures beginning 1 h after the beginning of the active (dark) cycle and lasting for 4 h. To ensure identical handling, we simultaneously exchanged water bottles across the control and EtOH treatments. Each week, during the regular cage change, we recorded the weight of each mouse (kg) and the total weekly fluid consumption (g), then calculated their weekly fluid consumption as grams of fluid consumed/kg body weight. To analyze tissues and sperm derived from active drinkers, we sacrificed Cohort 1 ($n = 8$ per treatment) after 10 weeks of constant EtOH exposure using CO_2 asphyxiation followed by cervical dislocation. For Cohort 2, we ceased the control and EtOH treatments and left the mice undisturbed for an additional 4 weeks before sacrifice using CO_2 asphyxiation and cervical dislocation, followed by tissue collection and sperm isolation. We refer to this latter group as the EtOH-cessation treatment.

2.2 | Isolation of mouse sperm

After sacrifice, we surgically isolated the male reproductive tract and separately placed the left and right cauda, with approximately 1 cm of the vas deferens, into one well of a 12-well plate containing 1 mL of warmed (37°C) phosphate-buffered saline (PBS). We extruded sperm from the vas deferens using dissection forceps and made four to five small incisions into the caudal epididymis to allow sperm to swim out. We incubated plates at 37°C for 30 min, then pelleted the sperm using centrifugation (3000 *g* for 5 min). Next, we washed sperm samples in PBS, pelleted the samples again, incubated sperm in somatic cell lysis buffer (SCLB: 0.1% SDS, 0.5% Triton X-100) on ice for 30 min, pelleted samples by centrifugation (3000 g for 5 min at 4°C) and conducted a second wash in SCLB. Next, we diluted a 10 μL aliquot 1:50 in diH_2O, confirmed sample purity by microscopy, and determined sperm concentration using a Neubauer chamber slide. Lastly, we centrifuged isolated sperm at 3000×*g* at 4°C for 5 min, washed samples in PBS, snap-froze the sperm pellets, and stored them at −80°C.

2.3 | Nucleic acid isolation—tissues

We isolated DNA from tissue samples using the DNeasy Blood and Tissue kit (Catalog# 69506; Qiagen, Germantown, MD, USA) and RNA using the RNeasy Plus mini kit (Catalog# 74136; Qiagen, Germantown, MD, USA), following the manufacturer-recommended protocol.

2.4 | RNA isolation—sperm small RNAs

We isolated sperm RNAs following the Mansuy lab protocol[48] with modest modifications. After thawing the sperm pellet on ice for 15 min, we resuspended the pellet in 100 μL of Buffer RLT (Catalog# 74136; Qiagen, Germantown, MD, USA) fortified with 100 mM of 2-Mercaptoethanol (Catalog# M3148; Millipore-Sigma, St. Louis, MO, USA). After verifying the complete resuspension of the sperm cells, we added 900ul of Trizol (Catalog# 15596018; Thermo-Fisher, Waltham, MA, USA) fortified with 100 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Catalog# C4706; Millipore-Sigma, St. Louis, MO, USA) and vigorously vortexed samples until we could no longer visualize cellular clumps. We then added 200 μL of chloroform-isoamyl alcohol (Catalog# 25666; Millipore-Sigma, St. Louis, MO, USA) to the samples, repeatedly mixed by inversion for 30 s, followed by a rest at room temperature for 3 min. We then centrifuged the samples at 12,000×*g* for 15 min at 4°C, then carefully removed the aqueous phase to a fresh RNase-free tube. Next, we added 10 μL of glycogen (Catalog# R0551; Thermo-Fisher, Waltham, MA, USA) to the isolated aqueous

phase and mixed the samples using inversion. We added one volume of 2-propanol (Catalog# I9616; Millipore-Sigma, St. Louis, MO, USA), incubated the tubes for 10 min at room temperature, then centrifuged the samples at 12,000×*g* for 15 min at 4°C to precipitate the RNA and then discarded the supernatant. We washed precipitated RNA pellets with 75% ethanol twice, centrifuging after each wash at 12,000×*g* for 5 min at 4°C. After the final wash, we air-dried the pellet and resuspended samples in 50 μL of RNase-free water. To improve the purity of isolated RNA and perform DNAse digestion, we used the Zymo RNA Clean and Concentrator Kit (Catalog# R1013; Zymo Research, Irvine, CA, USA), following the manufacturer-recommended protocol.

2.5 | Informatic analysis epididymal tissues

We isolated total RNA from the caput portion of the epididymis and sent samples to Quick Biology (Pasadena, CA, USA) for deep sequencing. We used the open-source, web-based Galaxy[49] server (www.usegalaxy.org) to process and analyze our data files. We used FastQC and MultiQC[50] to perform the initial quality control analysis of the raw paired-end, total RNA sequence files and then used Trimmomatic[51] to remove the Illumina sequencing adapters. We used RNA STAR[52] to map the reads to the *Mus musculus* reference genome (UCSC version GRCm39/mm39) and featureCounts[53] to determine the read abundance for all genes, followed by annotation versus M27 GTF (GENCODE, 2020). Next, we used DESeq2[54] to generate the PCA plots and the volcano plot function to produce graphical representations of the (log2FC, q-value < 0.05) gene expression levels. Finally, we increased the number of differentially expressed genes by reducing the stringency of our analysis to no longer account for the false discovery rate (log2FC, p-value < 0.05) and exported this expanded list into the Ingenuity Pathway Analysis software package to conduct pathway enrichment analysis.[55,56]

2.6 | Informatic analysis sperm RNAs

We sent sperm small RNA samples to Quick Biology (Pasadena, CA, USA) for deep sequencing analysis. Small RNA libraries were constructed using the Qiaseq miRNA library kit (Catalog# 331502; Qiagen, Germantown, MD, USA) and sequenced to a depth of approximately 15 million raw reads per sample. We trimmed the raw fastq files using Trimmomatic[51] to remove the Qiagen small RNA adapters and the TESmall package[57] to map and count small RNA reads. We exported files generated by TESmall into R (version 4.2.1) and performed differential gene expression analysis using the DESeq2[54] package. Finally, we used GraphPad Prism 9 to generate the volcano plots.

2.7 | Quantitative Polymerase Chain Reaction (PCR) analysis

We measured mitochondrial DNA copy number using primer sequences described previously[58] and the AzuraView GreenFast qPCR Blue Mix LR kit. We measured the mitochondrial D-loop region [D-Loop2 Fwd CCCTTCCCCATTTGGTCT D-Loop2 Rev TGGTTTCACGGAGGATGG; D-Loop3 Fwd TCCTCCGTGAAACCAACAA; D-Loop3 Rev AGCGAGAAGAGGGGCATT] and normalized measures of mtDNA to genomic DNA by measuring the abundance of the nuclear Tert gene, encoding the catalytic subunit of the telomerase complex [Tert Fwd CTAGCTCATGTGTCAAGACCCTCTT; Tert Rev GCCAGCACGTTTCTCTCGTT]. We describe the data normalization and handling procedures below.

2.8 | Data management

We managed the data generated in this study using a detailed data management plan that prioritizes safe and efficient data handling. We have stored all data on Google Drive for long-term storage, retrieval, and preservation. We have archived the sequencing files generated from this project in the GEO database under accession number GSE234535.

2.9 | Statistical analysis

Using previously observed variation in physiologic (male placental weights) and qPCR-based measures,[35] we used R (version 4.2.1) to perform a power analysis (*pwr.anova.test*(k = 3, f = 1.25, *sig.level* = 0.05, power = 0.99)) and determined our required sample sizes fell between 6–8 observations. We initially collected alcohol consumption data and physiological measures for each exposed male by hand and then transcribed these data into Google Sheets or Microsoft Excel, where we collated the data. For qPCR analysis of mitochondrial copy number, we imported the replicate cycle threshold (Ct) values for the DLoop region into Excel, then normalized measures to the nuclear *Tert* gene. 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, USA), 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. We describe the statistical tests underlying each figure in Table 1.

3 | RESULTS

Here, we set out to determine if measures of the sperm-inherited epigenetic program revert to match the control treatment after withdrawing the ethanol-exposed animals from their daily alcohol treatments. To this end, we utilized our limited access model to expose adult C57BL/6J males to alcohol for 10 consecutive weeks, encompassing

TABLE 1 Description of statistical testing for each presented figure.

Graph	Statistical test	Sample size
Figure 1B,C	Two-way ANOVA followed by Tukey's post hoc test.	$n = 8$ males per treatment
Figure 1D	Kruskal–Wallis one-way ANOVA, followed by Dunn's multiple comparisons test.	$n = 8$ males per treatment
Figure 3A	Kruskal–Wallis one-way ANOVA, followed by Dunn's multiple comparisons test.	Caput 8, Corpus 8, Cauda 7
Figure 3B	*t*-Test with Welch's correction	Control 7, EtOH 8
Figure 3C	Mann–Whitney test	Control 8, EtOH 8
Figure 3D	*t*-Test	Control 8, EtOH 7
Figure 3E	Mann–Whitney test	Control 8, EtOH 8
Figure 3F	*t*-Test with Welch's correction	Control 7, EtOH 8
Figure 3G	One-way ANOVA followed by a Fisher's exact test	Control 5, 6% EtOH 6, 10% EtOH 6
Figure 3H	One-way ANOVA followed by a Fisher's exact test	Control 6, 6% EtOH 6, 10% EtOH 6
Figure 3I	Kruskal–Wallis one-way ANOVA, followed by Dunn's multiple comparisons test.	Control 6, 6% EtOH 6, 10% EtOH 6
Figure 4A,B	*t*-Test	Control 6, 6% EtOH 6,
Figure 5C	Mann–Whitney test	Control 4, EtOH 4
Figure 5D	Mann–Whitney test	Control 4, EtOH 4

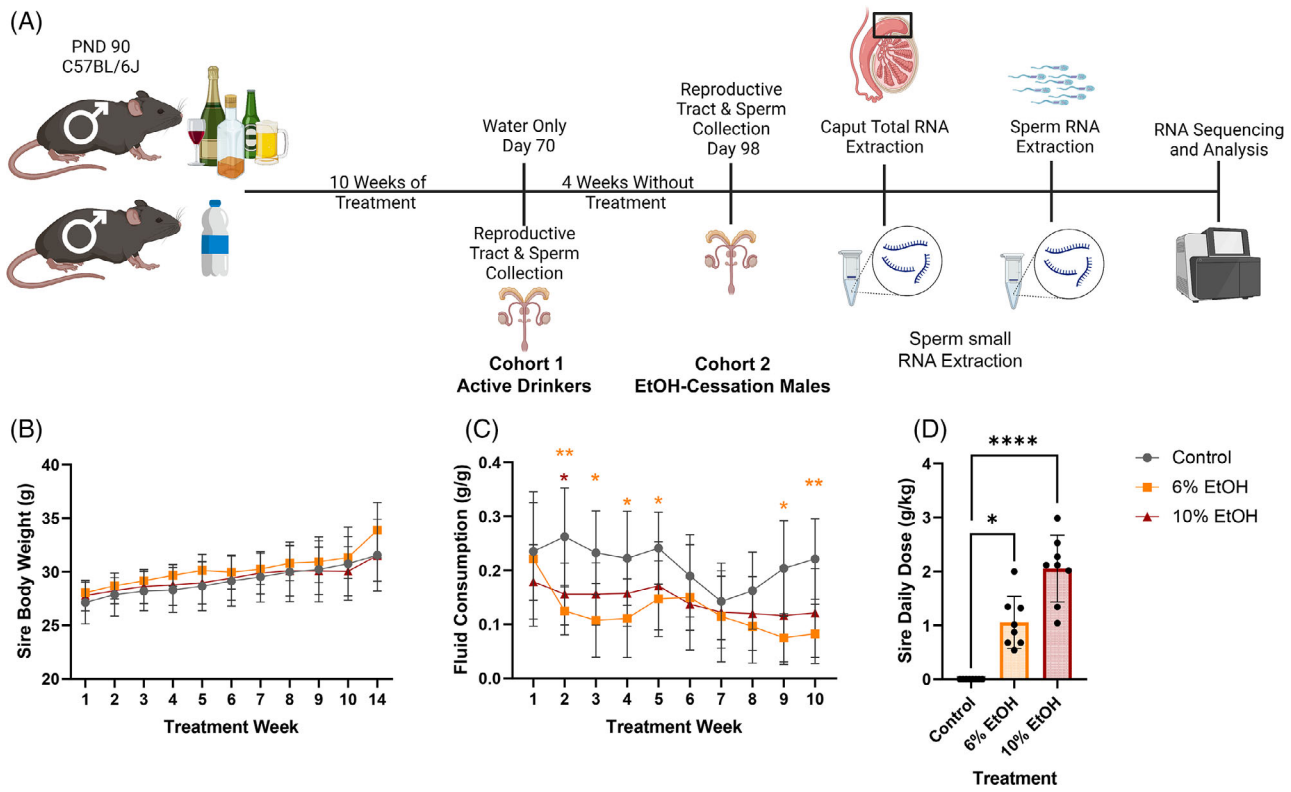

FIGURE 1 A mouse model to determine the capacity of the sperm epigenome to recover 1 month after the cessation of alcohol exposures. (A) Experimental design: we exposed C57BL6/J males to 6% and 10% alcohol for 10 weeks, then collected tissues from a cohort of active drinkers (Cohort 1). We then ceased the alcohol exposures, allowed males to recover for four weeks, collected tissues and sperm (Cohort 2), then used RNA-sequencing to compare RNA profiles between treatments. Comparison of male (B) average weekly weight gain between treatment groups, (C) average weekly fluid consumption, and (D) average daily dose of ethanol between treatment groups ($n = 8$). We compared treatments using either a two-way ANOVA followed by Tukey's post hoc analysis or a Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparisons test. For C, Asterix denote significant differences compared to the control treatment. Error bars represent the standard error of the mean, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

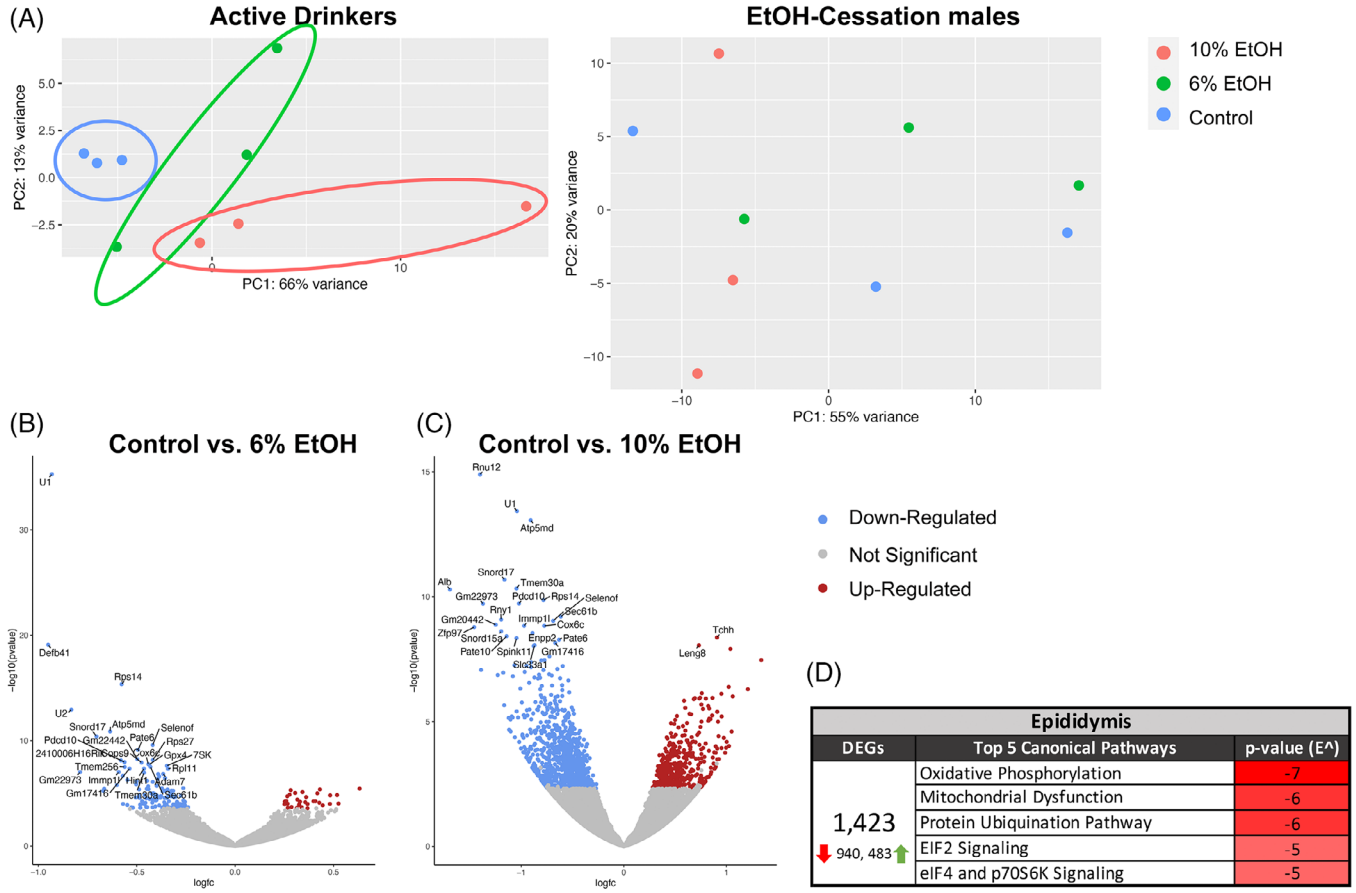

FIGURE 2 Chronic alcohol exposure induces altered gene expression patterns in the caput section of the epididymis. (A) Principal component analysis of gene expression between the caput epididymis isolated from Control, 6% EtOH, and 10% EtOH-treated males (left Cohort 1 active drinkers, right Cohort 2 EtOH-cessation males). Volcano plot contrasting down- and upregulated differentially expressed genes between (B) control vs. 6%EtOH-treated males and (C) control vs. 10%EtOH-treated males (log2FC, q-value < 0.05). (D) Ingenuity pathway analysis of differentially expressed genes identified in the 10% EtOH treatment group (log2FC, p-value < 0.05). $n = 3$ males per treatment for each cohort.

approximately two murine spermatogenic cycles.[44] We exposed males to three treatments, consisting of 6% and 10% ethanol (vol/vol EtOH) exposure groups, while we exposed the control group to water alone. After 10 weeks, we sacrificed a cohort of exposed males ($n = 8$), which we labeled active drinkers, and collected tissues and sperm. We then stopped the EtOH and control treatments and left the second cohort of males undisturbed for 4 weeks, then sacrificed the males and collected tissues and sperm. We refer to this second cohort as EtOH-cessation males (Figure 1A). We did not observe any differences in the body weights of exposed males across the treatment course (Figure 1B). Although we observed instances of treatment-specific differences in sire weekly fluid consumption across the 10-week treatment (Figure 1C), we did not observe any differences in the average daily EtOH dose between the 6% and 10% EtOH treatments (Figure 1D).

Our group and others have reported differences in the small RNA content of sperm induced by chronic alcohol exposure.[33,41] To further understand the physiological basis of these changes, we isolated RNA from the caput epididymis of active drinkers and EtOH-Cessation males across the three treatment groups and conducted deep sequencing analysis of the transcriptome ($n = 3$). During dissections, we flash-froze the caput section of the epididymis while we used the corpus and cauda sections to collect sperm. Therefore, we focused our RNA-seq analysis on the caput section. Principal component analysis revealed a clear clustering of each treatment group within Cohort 1 (Figure 2A). In contrast, samples derived from EtOH-cessation males (Cohort 2) displayed a wide dispersion with no overt clustering of treatment groups (Figure 2A). Using DESeq2,[54] we identified the differentially expressed genes (log2FC, q-value < 0.05) between treatment groups within each cohort. We observed a dose-dependent increase in the number of differentially expressed genes in active drinkers, with 329 differentially expressed genes in comparisons of Controls to the 6% treatment group and 1,423 in comparisons of control males to the 10% treatment (Figure 2B,C). However, we did not observe any significant (log2FC, q-value < 0.05) differentially expressed genes in comparisons between the 6% and 10% treatment groups, likely due to the high intersection of these two populations (data not shown). We then decreased the stringency of our analysis to no longer account for the false discovery rate (log2FC, p-value < 0.05) and obtained a larger number of differentially expressed genes, which we used to conduct gene pathway analysis. Pathway analysis of differentially expressed genes in the 10%

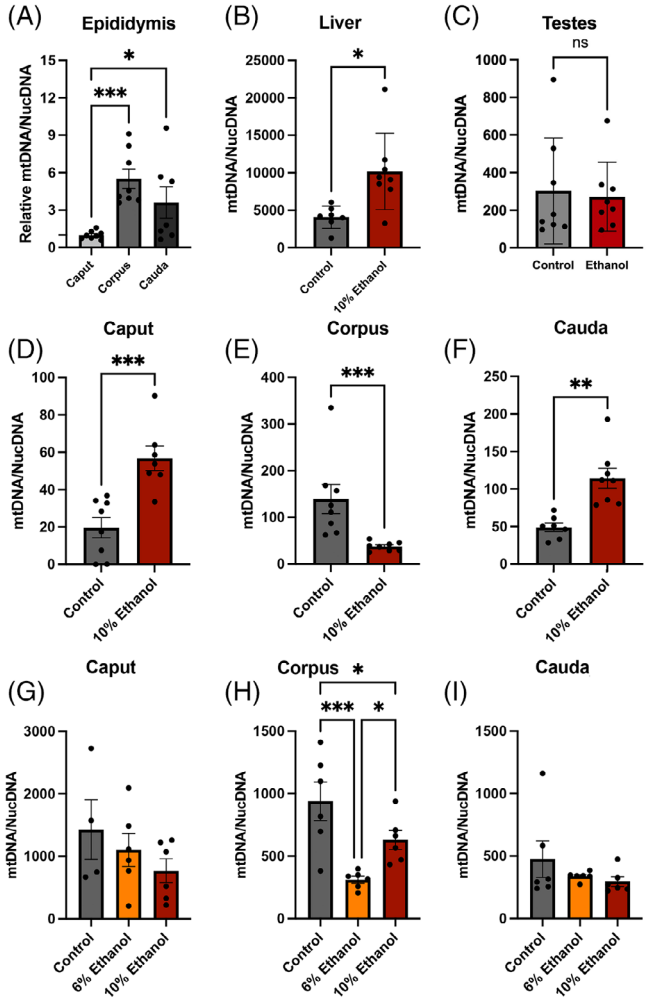

FIGURE 3 Chronic alcohol exposure induces lasting changes in mitochondrial DNA copy number within the epididymis. (A) Quantitative PCR analysis of mitochondrial DNA copy number (mtDNAcn) across the caput, corpus, and cauda sections of the epididymis isolated from control males ($n = 8$). For ease of comparison, we normalized qPCR ratios to the caput section. Comparison of mtDNAcn in the (B) liver and (C) testis isolated from control and 10% EtOH males in the cohort of active drinkers (Cohort 1; $n = 8$). Alcohol-induced alterations in mtDNAcn across the (D) caput, (E) corpus, and (F) cauda sections of the epididymis isolated from control and actively drinking 10% EtOH males (Cohort 1; $n = 8$). Segment-specific differences in mtDNAcn across the (G) caput, (H) corpus, and (I) cauda sections of the epididymis 4 weeks after the cessation of alcohol (Cohort 2; $n = 8$). We compared the impacts of alcohol treatment on mtDNAcn using either a Kruskal–Wallis one-way ANOVA, followed by Dunn's multiple comparisons test, a Student's *t*-test with Welch's correction, a Mann–Whitney test, or a one-way ANOVA followed by a Fisher's exact test, depending on treatment and the normality of the dataset. Error bars represent the standard error of the mean, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

treatment compared to the control treatment identified alterations in processes linked to mitochondrial dysfunction, oxidative phosphorylation, and the generalized stress response (EIF2, EIFI4, and p70S6k signaling) (Figure 2D).

In contrast to our analyses of active drinkers, our comparisons of EtOH-cessation males identified minimal to no differentially expressed genes. For example, in comparisons between the control and 6% treatments, we only identified three differentially expressed genes (*WAP Four-Disulfide Core Domain 13* (*Wfdc13*) and *Defensin Beta 123* and *128* (*Defb23*, *Defb28*)), while we did not identify any significant differentially expressed genes between the control and 10% treatments (data not shown). These observations suggest that epididymides of active drinkers exhibit alterations in processes related to mitochondrial dysfunction, oxidative phosphorylation, and the generalized stress response but that these differences revert after the cessation of alcohol use. Notably, we previously identified differential expression of genetic pathways regulating oxidative phosphorylation and mitochondrial function in the early embryo and placentae of offspring derived from alcohol-exposed males,[35,38] suggesting this transcriptional signature may transmit to the early offspring through sperm.

Alterations in the architecture, function, and number of mitochondria are one of the main hallmarks of alcohol-related liver disease.[59] Recent studies demonstrate that in the liver, alcohol exposure modulates mitochondrial DNA copy number (mtDNAcn) and transcription, potentially driving the sequela of this disorder.[60] However, no studies have determined whether alcohol adversely impacts the mitochondrial function in other organ systems, including the male reproductive tract. The extrachromosomal mitochondrial genome encodes 37 critical bioenergetic genes, is present in hundreds of copies per cell, and changes in mitochondrial DNA abundance serve as a proxy measure for the disease-associated mitochondrial dysfunction.[61] Therefore, using previously described methods,[58] we assayed mtDNAcn in the male reproductive tracts of active drinkers and cessation males.

Previous studies demonstrate differential expression of several mitochondrial genes across the three macro sections of the epididymis, with the corpus segment exhibiting the highest mitochondrial content.[62] Consistent with these studies, we identified a significant increase in mtDNAcn across the corpus and cauda sections compared to the caput (Figure 3A). We next compared the liver, testes, and the three sections of the epididymis isolated from control and active drinkers for alterations in mtDNAcn. As the 10% treatment exhibited the greatest number of differentially expressed genes, we focused our analyses on these tissues. Consistent with studies examining alcohol-related liver disease,[60] our analyses identified alterations in hepatic mtDNAcn (Figure 3B). In contrast, we did not observe any changes in mtDNAcn in the testes (Figure 3C). Comparisons of mtDNAcn across the epididymis identified segment-specific changes, with the caput and cauda each exhibiting alcohol-induced increases in mtDNAcn, while the corpus segment exhibited a significant decline (Figure 3D–F). Notably, when we examined epididymal segments isolated from EtOH-cessation males, we did not identify any differences in mtDNAcn in the liver (data not shown). However, we identified persistent mtDNAcn changes in the corpus segment across both the 6% and 10% EtOH treatments, while in contrast, we did not identify any significant differences in the caput or cauda segments (Figure 3G–I). These observations reveal that chronic alcohol exposure induces alterations

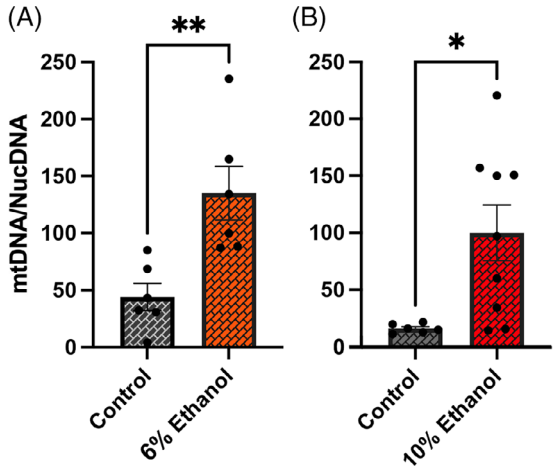

FIGURE 4 Analysis of mitochondrial copy number in alcohol-exposed sperm. (A) Quantitative PCR analysis of mitochondrial DNA copy number (mtDNAcn) in cryopreserved sperm used in our previously published IVF experiments[38] ($n = 6$). Analysis of mtDNAcn in fresh sperm isolated from control and 10% EtOH-treated actively drinking males (Cohort 1; $n = 8$). We used a Student's *t*-test to compare treatments. Error bars represent the standard error of the mean, $^{*}p < 0.05$, $^{**}p < 0.01$.

in mtDNAcn in the liver and epididymis, but not the testis, and that aspects of this signature persist 1 month after alcohol cessation.

In clinical studies examining IVF success rates, increases in sperm mtDNAcn correlate with lower odds of oocyte fertilization and reduced rates of high-quality Day 3 and transfer quality Day 5 embryos.[63] Previously, we observed dose-dependent reductions in IVF embryo survival and pregnancy success rates, with the pregnancy success rate of the 10% EtOH treatment falling to half those of controls.[38] Therefore, we isolated mitochondrial DNA from a selection of the cryopreserved sperm samples we used in these previous experiments and compared mtDNAcn between the control and 6% treatments. Consistent with clinical observations, we also identified a significant increase in mtDNAcn in cryopreserved samples derived from the 6% treatment group compared to the controls (Figure 4A). As the 10% treatment group required twice the number of IVF transfers, we did not have enough remaining cryopreserved samples to reliably assay mtDNAcn using this treatment cohort. However, when we compared mtDNAcn between the control and 10% treatment using fresh sperm samples, we observed a significant increase in mtDNAcn in alcohol-exposed sperm (Figure 4B). These observations indicate that similar to clinical studies examining IVF patients, the alcohol-induced reductions in IVF outcomes we previously reported correlate with increased sperm mtDNAcn.

We next determined if the cessation of alcohol exposure would rescue ethanol-induced changes in the small RNA content of sperm.[33,41] Previously, we found preconception paternal alcohol exposures induce dose-dependent changes in offspring fetoplacental growth.[37] To avoid the confounding influences of varying exposure levels, we selected cessation males experiencing a similar average daily EtOH dose. The median daily EtOH dose for cessation alcohol-exposed males across both the 6% and 10% treatment groups was 1.34 g/kg. Therefore, we isolated sperm small RNAs from alcohol-exposed males with an average daily dose around this population median ($n = 4$; 1.04, 1.32, 1.34, and 1.35 g/kg) and conducted deep-sequencing analysis. We sequenced sperm small RNAs to a depth of approximately 15 million raw reads per sample and obtained read lengths ranging from 16 to 40 nucleotides in length. Like previous examinations of the small RNA profiles of mouse sperm, we found that most small RNA reads mapped to structural RNAs (~40% structural RNAs, including snoRNAs, snRNAs, tRNA fragments, and vault RNAs[57]) and Piwi-interacting RNAs (~20% piRNAs) (Figure 5A). Notably, we identified significant differences in the percentages of small RNAs mapping to microRNAs (miRNAs) and exonic regions, with cessation-EtOH males exhibiting proportionally fewer miRNAs than cessation-controls and a greater percentage of exonic-derived fragments (Figure 5B–D). Whether these exonic-derived sequences represent small RNAs or differentially expressed genes from earlier in sperm production that fragment during differentiation remains unknown.

Principal component analysis of miRNA, piRNA, and tRNA-derived sequences did not identify clustering between cessation-control and EtOH-exposed males (miRNA data shown in Figure 5E). However, we did observe modest clustering of exonic- and transposable element (TE)-derived sequences (Figure 5F,G). Analysis of small RNA reads using DESeq2 (log2FC, *q*-value < 0.05) identified multiple differentially enriched miRNA, structural RNA (predominantly tRNA-derived), and exonic sequences (Figure 5H–J). Finally, we identified one differentially enriched TE (LINE:L1:L1ME3A_sense_TE). Significantly, many differentially enriched miRNAs and tRNA-derived sequences associate with oxidative and cellular stress responses,[64–68] similar to the genetic pathways identified in our transcriptomic analysis of the epididymis above (Figure 2D). These data reveal that sperm from EtOH-cessation males retain a distinct small RNA signature compared to unexposed cessation-controls.

4 | DISCUSSION

Chronic preconception male alcohol exposures alter the sperm-inherited developmental program and transmit an epigenetic memory to offspring, inducing FASD-like phenotypes.[2] Previous studies by our group and the Homanics lab have identified alcohol-induced changes in sperm-inherited microRNAs and tRNA fragments,[33,41] which we can correlate with transcriptional alterations in the genetic pathways regulating oxidative phosphorylation, mitochondrial function, and the generalized stress response in the early embryo and placentae.[35,38] As previous studies examining mouse models of binge drinking demonstrate that the negative impacts of alcohol withdrawal persist for 3 weeks,[45,46] we hypothesized that alcohol cessation for 1 month would allow for the normalization of this EtOH-induced epigenetic change. However, after 1 month of abstinence, we still identified alcohol-induced changes in sperm small RNAs and evidence of altered mitochondrial biology in the corpus segment of the epididymis. These observations suggest that some aspects of alcohol-induced

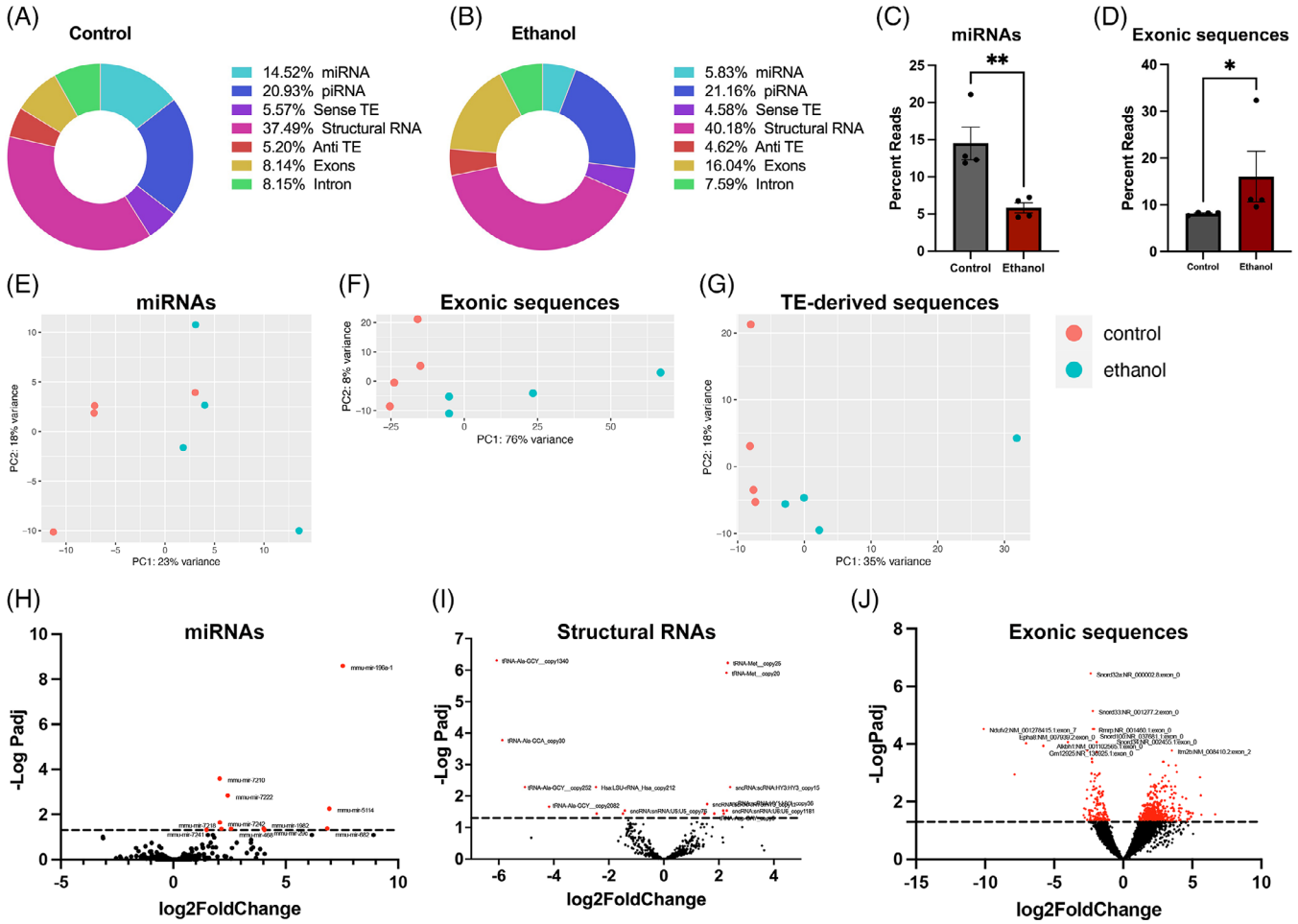

FIGURE 5 Sperm isolated from alcohol-exposed EtOH-Cessation males retain a distinct small RNA signature. Comparison of total small RNA species in sperm isolated from (A) cessation-control and (B) EtOH-exposed males in the EtOH-cessation cohort after 4 weeks of abstinence from alcohol ($n = 4$). Differential enrichment of sequenced (C) microRNAs (miRNAs) and (D) exonic sequences between EtOH-cessation-control and alcohol-exposed males ($n = 4$). Principal component analysis comparing enrichment of sperm (E) miRNA, (F) exonic, and (G) transposable element (TE)-derived sequences between cessation-control and alcohol-exposed males ($n = 4$). Volcano plot comparing differentially enriched (H) miRNA-, (I) structural RNA-, and (J) exonic-derived sequences between cessation-control and EtOH-exposed males after 4 weeks of abstinence from alcohol (log2FC, q-value < 0.05; $n = 4$). We used a Mann–Whitney test to compare the percentages of miRNA and exonic-derived sequences between treatments. Error bars represent the standard error of the mean, $^{*}p < 0.05$, $^{**}p < 0.01$.

mitochondrial dysfunction remain and that significant differences in the noncoding RNA signature of sperm persist during alcohol withdrawal. As we discovered that alterations in sperm ncRNAs persist, future studies will prolong the cessation period and determine if normalization of the sperm noncoding RNA signature correlates with the recovery of offspring fetoplacental phenotypes.

Previous studies examining alcoholic liver disease induced by chronic EtOH exposures identified mitochondrial fragmentation, defective mitophagy, decreased abundance of cellular antioxidants, and a significant increase in mitochondrial reactive oxygen species (ROS).[60] Consistent with these observations, in active drinkers, we identified a transcriptional signature of mitochondrial dysfunction in the caput segment of the epididymis and evidence of altered mtDNAcn across all segments of the epididymis but, notably, not in the testis. Previous studies describe segment-specific differences in epididymal mitochondrial content, with the corpus displaying significantly higher levels than the caput and cauda portions.[62] Our qPCR-based measures of mtDNAcn agree with these previous observations and confirm that the corpus and cauda sections contain a greater mtDNA enrichment than the caput. Further, our data demonstrate that chronic alcohol exposure induces segment-specific changes across the epididymis, some of which persist even 1 month after removing the toxicant, and that this signature also appears in alcohol-exposed sperm. Significantly, alterations in mitochondrial DNA copy number are an emerging area of interest across the DOHaD field, correlating with long-term alterations in health[69] and reduced IVF pregnancy rates.[63] We speculate that mitochondrial differences across the epididymis enable this organ to sense the surrounding environment, including cellular metabolic and redox states, and then communicate this information to spermatozoa in the form of noncoding RNAs.

In support of this assertion, our previous studies examining the miRNA content of sperm isolated from active drinkers identified an

increased abundance of microRNA miR-30a and decreased miR142. miR-30a enhances activation of the master transcriptional regulator controlling the cellular antioxidant response, Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2), by inhibiting its negative regulator Keap1.[70] In contrast, miR-142 is a direct suppressor of NRF2.[71] Therefore, the balance we previously identified in active drinkers favors miRNA-mediated activation of NRF2-driven genetic pathways responding to oxidative stress. Here, we identified differentially enriched miRNAs and tRNA-derived sequences in EtOH-cessation males that also link with oxidative and cellular stress responses.[64–68] Most notably, we identified a ~100-fold increase in mir-196a, an NRF2-controlled miRNA[65] that enhances the expression of antioxidant proteins, like heme oxygenase 1, by inhibiting their negative regulators.[64] Significantly, although not a top candidate, Rompala et al. also identified increased enrichment of this miRNA in sperm using a vapor chamber exposure model (supplemental information of reference[41]). Finally, presumed tRNA fragments, including tRNAMet identified here and tRNAGlu detected by the Homanics group,[41] selectively accumulate in the nucleus as part of the generalized stress response induced by mitochondrial dysfunction.[67] From these data, we postulate that alcohol-induced changes in epididymal ROS lead to NRF2 activation and the packaging of miRNAs in sperm that reinforce the cellular antioxidant response. However, further experimentation is required to validate this assertion and to determine how this epigenetic memory impacts mitochondrial function in the developing offspring.

Although compelling, there are several limitations to this study. First, we did not generate offspring using the cessation males. Therefore, we do not know if the sperm noncoding RNA signature we identified correlates with changes in offspring fetoplacental growth or if the resulting offspring would develop normally. However, as significant differences in the ncRNA signature of EtOH-cessation sperm and epididymal mtDNAcn remained, we speculate that abstinence for 1 month is insufficient for the epigenetic memory of paternal alcohol exposure to abate, likely due to the ongoing stress associated with alcohol withdrawal.[45,46] Furthermore, we acknowledge that our analysis does not distinguish between changes in sperm ncRNAs that are causal drivers of altered epigenetic programming in the next generation versus abnormalities that are merely additional symptoms of alcohol-induced stress. However, while the individual miRNAs vary between studies, the potential interactions with NRF2 remained consistent. We also acknowledge that our analyses do not identify specific epididymal cell types impacted by alcohol or discern gene expression differences in the corpus or cauda sub-segments. Finally, we note that mtDNAcn is a widely used but crude proxy for the mitochondrial function. However, the alignment of our data with clinical studies examining the correlations between reduced male fertility and alterations mtDNAcn[63] is compelling. Future studies will explore mitochondrial dynamics in the male reproductive tract and sperm of alcohol-exposed males and their offspring.

Obstetricians do not routinely consider paternal influences on child health, and all alcohol messaging targets women. Therefore, there is a critical need to expand alcohol messaging to include men and educate both prospective parents on the reproductive dangers of alcohol use. Central to this effort, researchers must identify the length of time required for the paternal epigenome to recover from toxicant exposures so that patients may know how long in advance of trying to conceive they need to begin abstaining from alcohol. Our data suggest that the epididymis retains an alcohol-induced signature of mitochondrial dysfunction 1 month after cessation, indicating a more extended recovery period is required after toxicant removal. Furthermore, our observations suggest that NRF2-related miRNAs, particularly mir-196a, and increases in sperm mtDNAcn may serve as viable biomarkers of paternal alcohol use and adverse alterations in the sperm-inherited epigenetic program.

AUTHOR CONTRIBUTIONS

Conceptualization: ANR and MCG. *Methodology*: ANR, SSB, and MCG. *Investigation*: ANR, SSB, SLH, DDD, AB, KNT, and MCG. *Formal analysis & Visualization*: ANR, SSB, and MCG. *Funding acquisition & Supervision*: MCG. *Writing*: ANR and MCG.

ACKNOWLEDGEMENTS

This work was supported by a medical research grant from the W.M. Keck Foundation and NIH Grant NIAAA R01AA028219.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/, reference number GSE234535.

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How to cite this article: Roach AN, Bhadsavle SS, Higgins SL, et al. Alterations in sperm RNAs persist after alcohol cessation and correlate with epididymal mitochondrial dysfunction. *Andrology*. 2023;1-12. https://doi.org/10.1111/andr.13566