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Ethanol-related transcriptomic changes in mouse testes

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Abstract

Background Alcohol consumption is widely known to have detrimental effects on various organs and tissues. The effects of ethanol on male reproduction have been studied at the physiological and cellular levels, but no systematic study has examined the effects of ethanol on male reproduction-related gene expression.

Results We employed a model of chronic ethanol administration using the Lieber-DeCarli diet. Ethanol-fed mice showed normal testicular and epididymal integrity, and sperm morphology, but decreased sperm count. Total RNA sequencing analysis of testes from ethanol-fed mice showed that a small fraction (~2%) of testicular genes were differentially expressed in ethanol-fed mice and that, of these genes, 28% were cell-type specific in the testis. Various *in silico* analyses were performed, and gene set enrichment analysis revealed that sperm tail structure-related genes, including forkhead box J1 (*Foxj1*), were down-regulated in testes of ethanol-fed mice. Consistent with this result, ethanol-fed mice exhibited decreased sperm motility.

Conclusion This study provides the first comprehensive transcriptomic profiling of ethanol-induced changes in the mouse testis, and suggests gene expression profile changes as a potential mechanism underlying ethanol-mediated reproductive dysfunction, such as impaired sperm motility.

Keywords Alcohol, Ethanol, Sperm motility, Spermatogenesis, Testis

Background

Alcohol is the one of the most widely consumed beverages in human history. At present, more than half of the global adult population consumes alcohol on regular basis [1]. Approximately 7.4% of the American population has been reported to be at risk for alcohol abuse or alcoholism [2]. Consequently, the detrimental effects of ethanol consumption have been extensively investigated with regard to the liver [3], psychosocial effects [4], and cardiovascular system [5]. Further, fetal ethanol spectrum disorder (FESD) can result in serious and lifelong consequences [6]. In the liver, which is the main organ of detoxication, chronic alcohol exposure can cause alcoholic liver disease, including alcoholic fatty liver, and alcoholic steatohepatitis [7].

Excessive alcohol consumption negatively affects not only hepatic function, but also male reproductive function. It has been reported to have negative effects to male reproductive function (Table 1) [8–12]. Through various ethanol administration methods, decreases in sperm count [8, 11], and sperm motility [9, 11] have been observed in ethanol- administrated rats or mice. These changes were proposed to reflect a decrease of testosterone [8, 10], but this does not fully explain the harmful effects of ethanol on the male reproductive system. Moreover, another study suggested that ethanol consumption

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Table 1 Studies on male reproductive changes induced by ethanol administration in rodents

Model species	Mouse	Rat	Rat	Rat	Rat
Ethanol administration	Intraperitoneal injection (14 days, 3 g/kg)	Intraperitoneal injection (12 days, 6 g/kg)	Oral gavage (14 days, 5 g/kg)	Self-administration (150 days, 15%)	Oral gavage (30 days, 3 g/kg)
Body weight	N.C.	N.C.	↓	N.A.	N.A.
Testis/body weight ratio	↓	N.A.	↑	N.A.	N.A.
Sperm conc.	↓	N.C.	N.C.	↓	N.A.
Sperm motility	N.A.	↓	N.A.	↓	N.A.
Testosterone level	↓	N.C.	↓	N.A.	N.A.
Histological analysis	TUNEL positive spermatogenic cells	Decreased cellular diameter and area	Atrophy and sloughing of germ cells and vacuolization	Decreased spermatogonia and exfoliation of epithelium	Decrease in the diameter of the seminiferous tubules and the number of Leydig cells
Reference	[8]	[9]	[10]	[11]	[12]

N.C., not changed

N.A., not available

↑, increase

↓, decrease

may not affect testosterone levels [9]. Clearly, further research is needed to elucidate the mechanisms underlying ethanol-induced male reproductive dysfunction. In this regard, the literature lacks a systematic transcriptomic study of the male reproductive system impacted by chronic ethanol exposure.

Previous studies to evaluating the effects of ethanol on the male reproductive system used various methods of ethanol administration (Table 1) [8–12]. Oral gavage and intraperitoneal injection of ethanol were used most often due to their ease of application. However, these methods have limitations in studies of *ad libitum* and chronic alcohol intake. To comprehensively study the effects of ethanol on male reproductive function, we generated a mouse model of chronic ethanol administration by using the Lieber-DeCarli diet [13], to ensure chronic and voluntary alcohol intake. To systematically analyze the testicular response to alcohol, we conducted what believe to be the first RNA sequencing analysis of the testicular transcriptome of ethanol-administrated mice. We identified alterations in the expression levels in a number of genes, most notably changes in sperm cilium-related genes, including forkhead box J1 (*Foxj1*). Consistently, sperm motility was found to be lower in ethanol-fed mice than in control-fed mice. Our study provides a comprehensive transcriptomic profile that may facilitate future mechanistic research on ethanol intake-induced reproductive dysfunction.

Methods

Animals

All animal experiments were conducted in accordance with Korean Food and Drug Administration (KFDA) guidelines. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Gwangju Institute of Science and Technology (GIST) (permit number: GIST-2022-049). We used 8-week-old C57BL/6 male mice (Damul Science, Daejeon, Korea) for experiments, which were performed in biological triplicate. Animals were euthanized by individually exposing them to increasing concentrations of carbon dioxide (CO_2) in darkened cages before tissue collection.

Ethanol administration

We conducted chronic ethanol administration for 16 weeks. For voluntary feeding, we provided feeding tubes (Dyets, Bethlehem, PA, USA) loaded with Lieber-DeCarli diet [13, 14] composed of Ethanol Lieber-DeCarli Regular Liquid Diet for Rodent (Dyets), distilled water, and ethanol. To allow for acclimatization, the proportion of ethanol in the diet was gradually increased from 1 to 5% w/v over 5 days. Control Lieber DeCarli diet consisted of Control Lieber-DeCarli Regular Liquid Diet for Rodent (Dyets), distilled water, and maltose dextrin (as an isocaloric ingredient replacing ethanol) [13, 14]. Daily administration of 10 ml/mouse was given to the control and 5% ethanol groups. Administration was conducted on three mice in each group.

Total RNA sequencing

After receiving ethanol or control diet for 16 weeks, mice (three mice per group) were sacrificed and testes were cryopreserved immediately, using liquid nitrogen. A TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina, San Diego, CA, USA) was used to prepare libraries. Ribosomal RNA was removed with a Ribo-Zero rRNA Removal Kit (Illumina) and random fragmentation

was performed. A HiSeq 4000 (Illumina) was used for paired-end high throughput sequencing. Expression profiles were calculated with DeSeq2 [15]. Total RNA sequencing data were uploaded to the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI) under GEO accession number GSE256349. We also used external GEO data (GSE125303) to examine the population of cell type-specific genes [16]. We defined cell type-preferential genes those exhibiting a greater abundance in a given testicular cell type compared to other testicular cell types (|fold change| > 2).

Histological analysis of testis and liver

To assess the effect of ethanol administration, we performed histological analysis of mouse testis and liver (caudate lobe) samples. Tissues were fixed and dehydrated using Bouin's solution (for testis), or 3.7% formaldehyde solution (for liver), paraffin embedded, and sectioned at 4 mm. Sections were stained with hematoxylin and eosin (H&E), and whole slides were scanned with an Aperio ScanScope CS2 System (Leica Biosystems, Wetzlar, Germany). The histological sections were analyzed from three mice per group.

Sperm motility analysis with OpenCASA

Vas deferens and Cauda epididymis were dissected, and sperm samples were collected by squeezing and incubated for 10 min at 37℃. Sperm were counted with hematocytometer and plated to a 35 mm petri dish in Whittingham media. Motile sperm were videoed using a microscope (Leica Microsystems, Wetzlar, Germany) and eXcope X9 capturing system (Dixi Science, Daejeon, Korea). Videos were preprocessed with the ImageJ [17], and the OpenCASA system [18] was used to measure multiple sperm parameters from randomly selected fields. All measurement data represent both biological and technical triplicates.

Quantitative real-time RT-PCR

To confirm the differential expression of selected genes in testis of ethanol- and control-fed mice, quantitative real-time RT-PCR (qRT-PCR) was performed. Total RNA was isolated using the Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's standard protocols and cDNA was synthesized by random hexamer and oligo(dT) priming using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). qRT-PCR was performed using the TOPreal qPCR 2X premix (Enzynomics, Daejeon, Korea) and the primers are presented in Supplementary Data 1. All amplifications were performed more than three times. Relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized with respect to the mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and ribosomal protein lateral stalk subunit P0 (*Rplp0*) transcript levels. The analyses were performed in biological and technical triplicates in each group.

Results

Effects of ethanol administration on male reproductive tissues

We fed mice with an ethanol-containing Lieber-DeCarli diet to generate a mouse model of chronic ethanol administration (see Materials and Methods). After a 16-week feeding period, mice were sacrificed and male reproductive tissues (testis and epididymis) and liver tissues were isolated (Fig. 1A). To investigate the effect of ethanol administration on the testis and liver, histological analysis of H&E-stained sections was performed. Liver sections from ethanol-fed mice had significantly larger fat globule areas (a characteristic pathology of alcoholic fatty liver) (~2.2 fold), compared to control-fed mice (Fig. 1B and Supplementary Data 2). In contrast, no histological difference was found between the testes of ethanol- and control-fed mice (Fig. 1C). These groups also did not differ with respect to the size or weight of the testis and epididymis (Fig. 1D; Table 2) or over all sperm morphology under microscopic analysis (Fig. 1E and F). However, we obtained fewer sperm from the epididymis and vas deferens of ethanol-fed mice compared to controls (Table 2). It can be assumed that the decrease in sperm count is related to cell death induced by ethanol (see Discussion).

Transcriptomic analysis of testes from ethanol-fed mice

To investigate male reproduction at the level of gene expression in ethanol-fed mice, we performed high-throughput transcriptomic analysis. Total RNA sequencing was conducted to detect testicular transcriptome changes resulting from ethanol administration to three mice per group. We observed a total of 27,347 transcribed testicular genes. Genes showing differential expression in the ethanol-fed group (ethanol-DEGs) were filtered based on |fold change| > 1.5 and *P*-value < 0.05 (Fig. 2A). A relatively small proportion of the total testicular genes were found to be ethanol-DEGs (338 up-regulated and 196 down-regulated ethanol-DEGs) among total testicular genes (1.95%) (Table 3 and Supplementary Data 3). Notably, 40.3% (215 genes) of the 534 ethanol-DEGs were annotated as long noncoding RNAs (lncRNAs), while 53.2% (284) corresponded to mRNAs. In functional enrichment analysis of ethanol-DEGs, 'extracellular', and 'immune'-related GO terms stood out (Supplementary Data 4 A and 5). The top five KEGG (Kyoto Encyclopedia of Genes and Genomes) terms included the peroxisome proliferator-activated receptor (PPAR) signaling pathway (Supplementary Data 4B and 5). We were unable

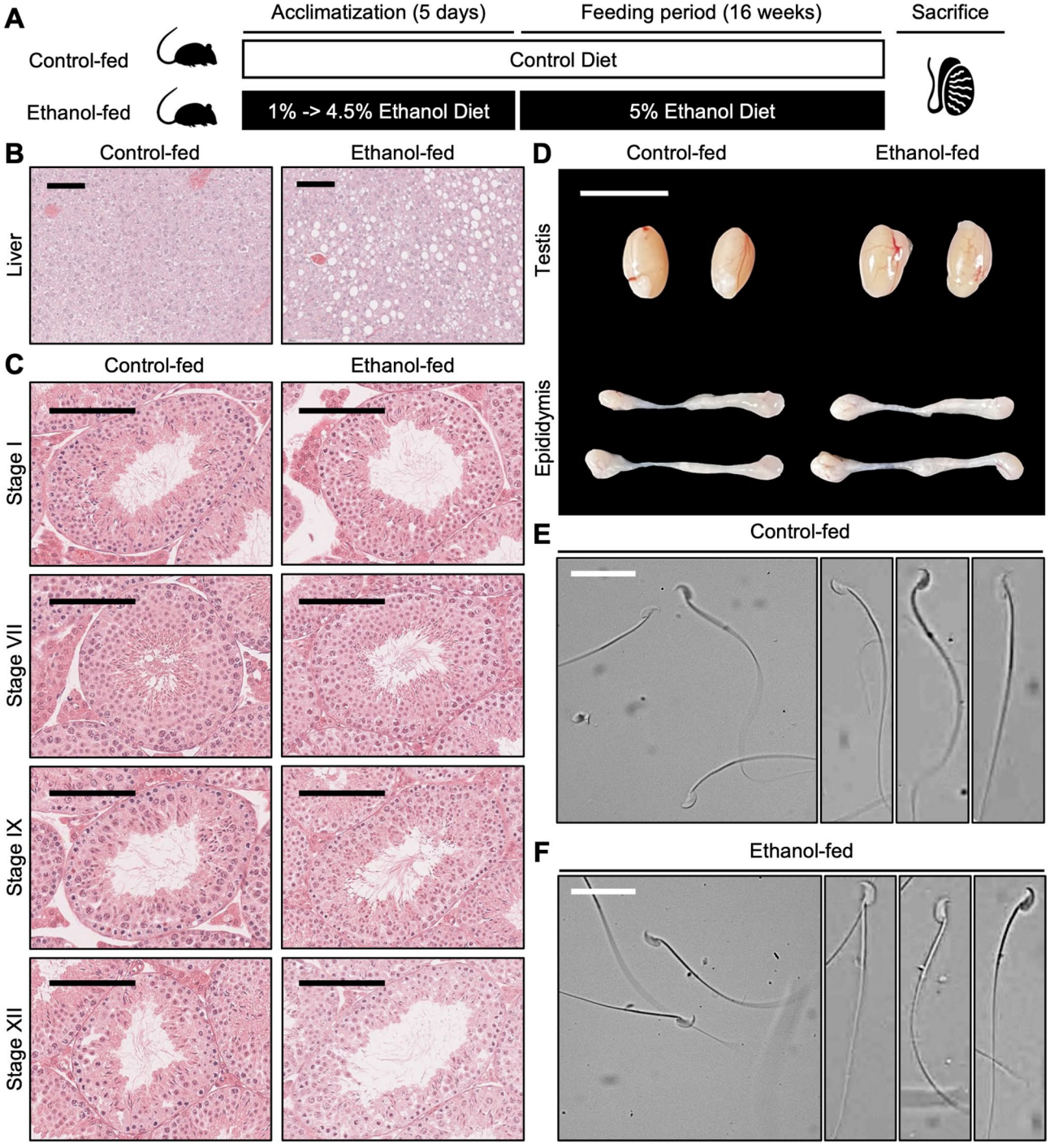


Fig. 1 Physiological changes of mouse tissues following chronic ethanol administration. (**A**) Administration schedule for using Lieber DeCarli diet to generate ethanol-fed and control-fed groups (three biological replicates in each group). (**B**) Histology (H&E staining) of liver of ethanol-fed and control-fed groups. (white scale bar = 1 cm) (**C**) Histology (H&E staining) of testis of ethanol-fed and control-fed groups (black scale bar = 100 μm). Four different spermatogenic stages (stages I, VII, IX, and XII) are shown. **(D)** Morphology of testes and epididymides of ethanol-fed and control-fed group (white scale bar = 1 cm). (**E**) Morphology of mouse sperm of control-fed group. **(F)** Morphology of mouse sperm from ethanol-fed group (white scale bar = 30 μm)

to obtain significant information about lncRNA DEGs through GO enrichment analysis.

The complex process of germ cell development requires the existence of diverse populations of cell types exist. In addition to interactive and extrinsic regulation, intrinsic regulation of spermatogenesis also plays an important role. Cell type-specific genes play important roles in successful spermatogenesis [19]. Here, we compared the

Table 2 Analyses of testis, epididymis, and sperm in control and ethanol-fed mice

Measurement	Ethanol-fed*	Control-fed*	*P*-value
Body weight (g)	28.1 ± 1.1	28.2 ± 2.4	0.9337
Testis (right) weight (g)	0.1018 ± 0.0123	0.1193 ± 0.0021	0.0725
Testis (left) weight (g)	0.1037 ± 0.0102	0.1155 ± 0.0051	0.1489
Epididymis (right) weight (g)	0.0434 ± 0.0043	0.0478 ± 0.0032	0.2251
Epididymis (left) weight (g)	0.0495 ± 0.0020	0.0478 ± 0.0090	0.7650
Average testis/body (mg/g)	3.6598 ± 0.2295	4.1763 ± 0.2599	0.0871
Average epididymis/body (mg/g)	1.6574 ± 0.1412	1.6921 ± 0.0705	0.7228
No. of vas deferens sperm (10^6 cells)	5.3 ± 0.3	7.5 ± 1.2	0.0001
No. of cauda epididymis sperm (10^6 cells)	11.4 ± 2.0	24.2 ± 6.4	0.0002

* Three mice were analyzed in each group

Table 3 Differentially expressed genes (DEGs) in testes of mice under chronic alcohol exposure

DEGs	Up	Down	Total
Total	338	196	534
Protein coding genes	196	88	284
lncRNAs	121	94	215
Pseudogenes	19	13	32
Others (scaRNA and V segment)	2	1	3

lncRNAs, long noncoding RNAs

scaRNA, small Cajal body-specific RNA

DEGs identified in ethanol-fed mice to a list of testicular cell type-specific genes (GSE125303) [16], and identified 150 cell type-specific genes among the ethanol-DEGs (23% of total DEGs) (Fig. 2B-F). Of the cell type-specific ethanol-DEGs, the largest subset corresponded to germ cell specific genes (64 genes) (Fig. 2B-F), which showed similar levels of up- and down-regulation (Fig. 2B). In contrast, most of the somatic cell-specific ethanol-DEGs were up-regulated (average up/down ratio: 5.54) (Fig. 2B).

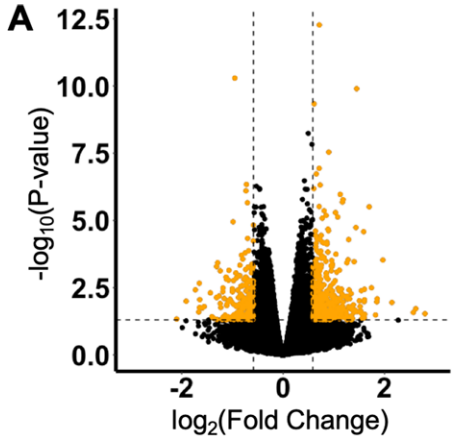
Gene set enrichment analysis (GSEA) of testes from ethanol-fed mice

In addition to GO and KEGG analyses, GSEA was performed to further examine the testicular and spermatogenic responses to ethanol consumption. We used relative log expression (RLE) normalization values of RNA sequencing data to obtain enriched gene sets. Male reproductive dysfunction due to alcohol consumption is thought to be related to ethanol metabolism and steroidogenesis [20, 21]. Therefore, we searched for such categories of enriched gene sets among the GO gene sets that showed significant and sufficient changes (FDR < 0.3, nominal p-value < 0.05; # of significant changed gene sets / # of total gene sets = 1247 / 5255) (Fig. 3A and Supplementary Data 6). We observed the following: First, the genes of the alcohol stimulus biological process-related gene sets (GO:0006066, alcohol metabolic process and GO:0097305, response to alcohol) were mostly up-regulated (Fig. 3A). Second, with respect to steroidogenesis, 'corticosteroid hormone secretion' (GO:0035930) and 'cytochrome complex assembly' (GO:0017004) gene sets were significantly up- and down-regulated, respectively (Fig. 3A). Finally, some of the gene sets ranked highly by the normalized enrichment score (NES) were directly related to germ cells; in particular two sperm tail-related gene sets (GO:0120316, sperm flagellum assembly and GO:0007288, sperm axoneme assembly) contained genes that were down-regulated by ethanol (Fig. 3A). In addition, we were able to find ciliogenesis and axoneme-related gene sets (GO:0035082, axoneme assembly and GO:0044782, cilium organization), that are highly related and ancestral gene sets of the sperm tail-related gene sets (Fig. 3A). This suggests that ethanol consumption is associated with changes in transcripts related to sperm tail movement and/or sperm tail structural organization.

Although the cellular environment of the testis differs from that of other tissues, it is unclear whether the male reproductive response to ethanol is unique to the testis. We thus compared our ethanol-induced transcriptome changes in testes with those previously reported for liver (GEO accession: GSE179648) in an ethanol-fed mouse model generated using Lieber-DeCarli diet. GSEA revealed that consistent with the idea that ethanol consumption disrupts hormone synthesis in liver and testes, certain hormone-related genes were commonly up-regulated under ethanol exposure. In contrast, we identified six prominent gene sets that were ranked in the top 20 by NES in testes, but not in liver (Fig. 3B). These sets were all related to ciliogenesis, including sperm flagellum and axoneme assembly (Fig. 3A). For example, genes in the motile cilium term (GO:0031514) in the testis were significantly changed by ethanol (Fig. 3B). Collectively, these results suggest that changes in sperm tail-related gene expression are a novel and unique feature of ethanol-related alterations in male reproduction.

Motility of sperm from ethanol-fed mice

Because the expression pattern of sperm tail-related genes was altered in ethanol-fed mice (Fig. 3A), we assessed further for changes in sperm motility using openCASA, which is an open-source computer assisted semen analysis (CASA) software program (see Materials and Methods). Sperm were incubated for 0.5 and 3 h with



B

Cell type		Up	Down	Up/Down ratio	
Germ cell		30	34	0.88	
Somatic cell	Sertoli cell	11	4	2.75	5.54
	Leydig cell	34	7	4.86	
	PTM cell	18	2	9.00	

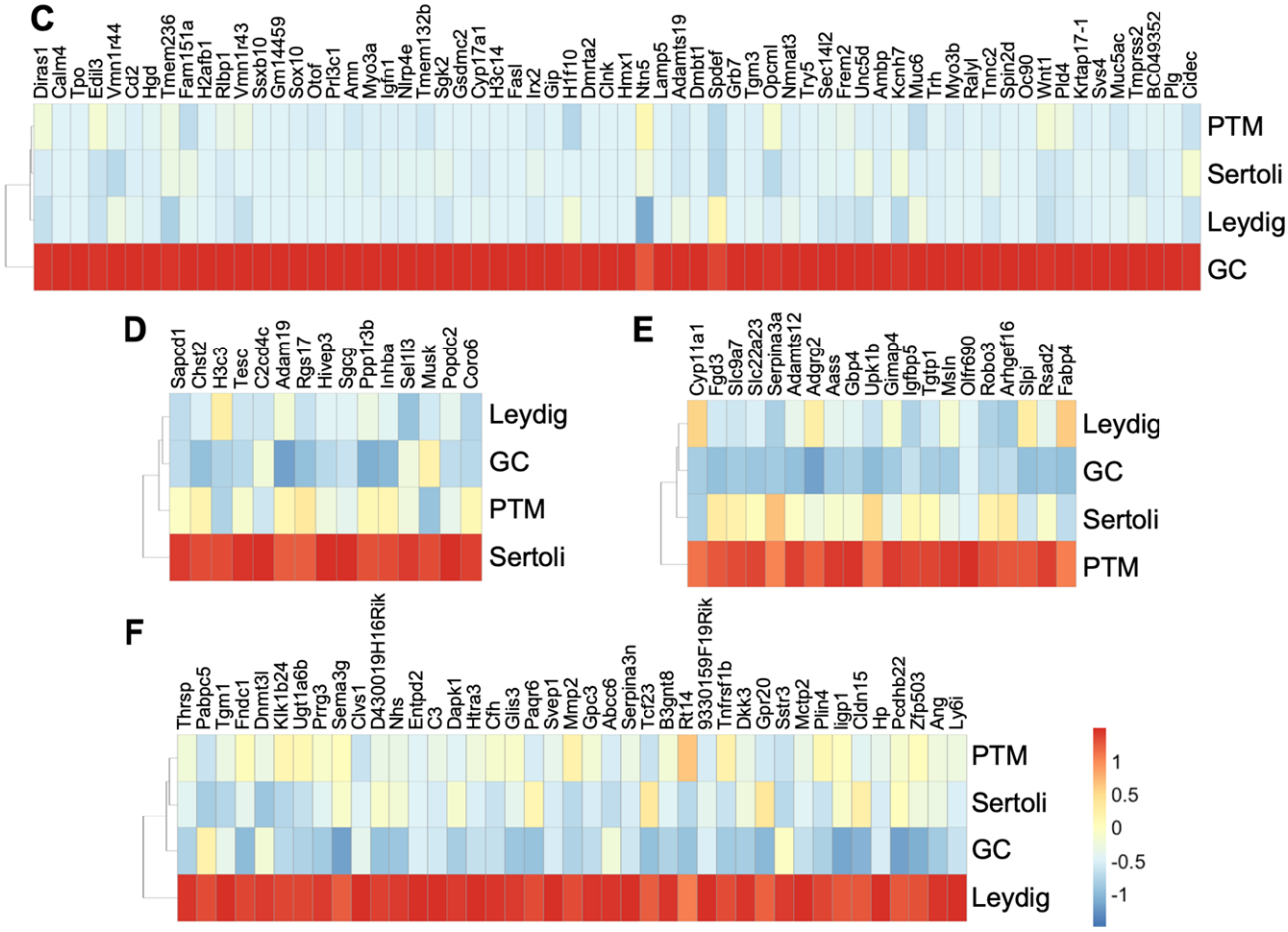


Fig. 2 Differentially expressed genes (DEGs) in testes between ethanol-fed and control-fed mice. The data represent biological triplicates for each group. (**A**) Volcano plot of testicular DEGs between ethanol-fed and control-fed mice. Orange dots show individually significant DEGs (ethanol-DEGs) with FC (fold change, ethanol-fed/control-fed) ≥ 1.5 and *p*-value < 0.05. (**B**) Numbers of ethanol-DEGs with testicular cell type specific expression patterns. (**C-F**) Heatmaps of testicular cell type specific ethanol-DEGs, including those specific to germ cells (C), Sertoli cells (D), peritubular myoid cells (F), and Leydig cells (E). GC, germ cell; PTM, peritubular myoid cell. For C-F, the colors are defined at the lower right

3% BSA and various sperm motility parameters were measured (Fig. 4). Under incubation in 3% BSA medium incubated for 0.5 h, linear velocity (VSL) and progressive motility were significantly lower in ethanol-fed mice compared to the control-fed mice (Fig. 4). Moreover, the proportion of motile sperm was lower in samples from ethanol-fed mice compared to control mice under both

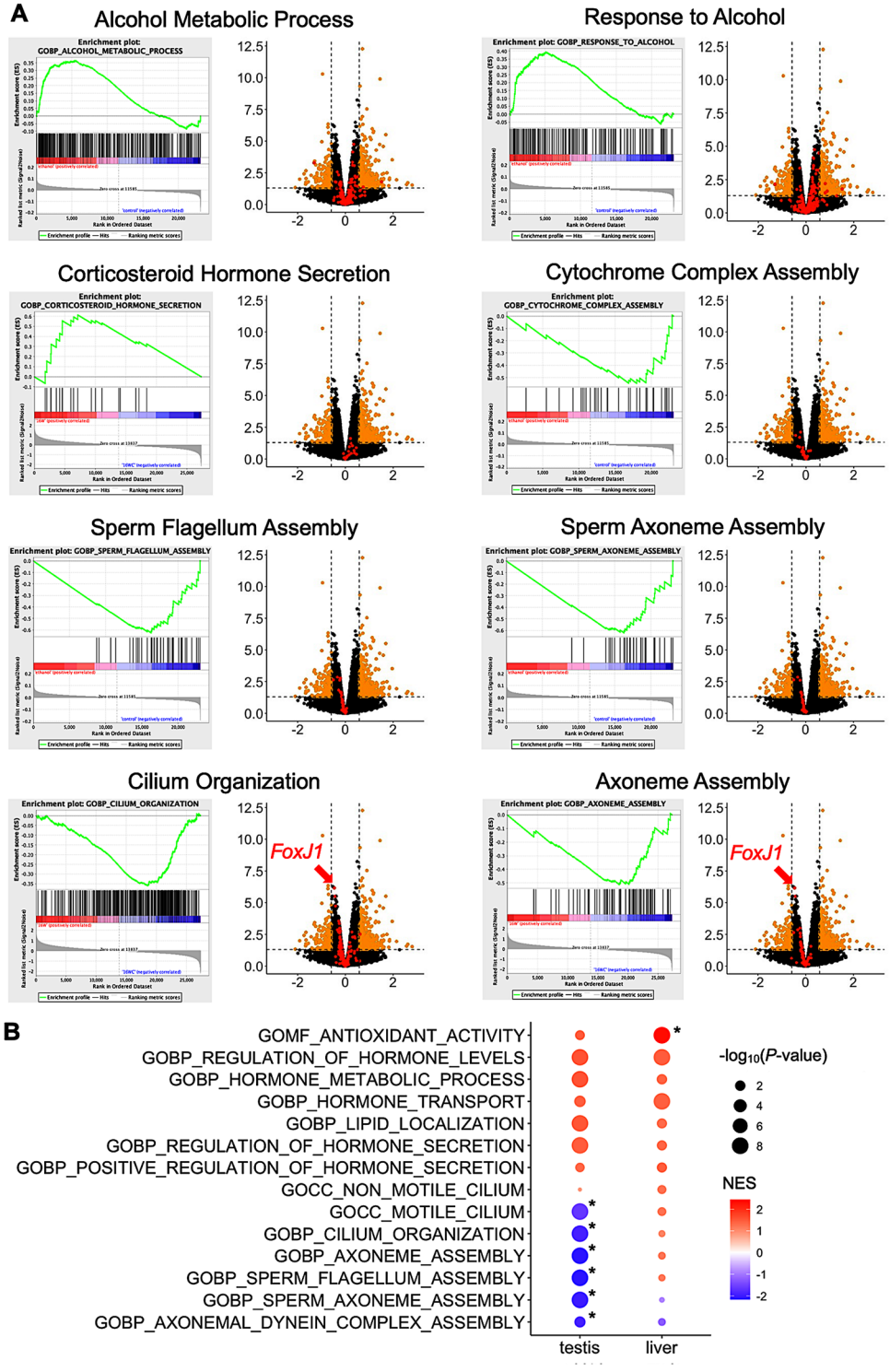


Fig. 3 Gene set enrichment analysis (GSEA) of testicular genes between ethanol-fed and control-fed mice. Three mice were analyzed per group. (**A**) Enrichment plots showing enrichment scores of expressed genes in ethanol-fed and control-fed mice. Red dots in volcano plot represent expression of gene set members. Orange dots show ethanol-DEGs. Red arrows indicate the position of *Foxj1* gene. (**B**) Bubble plot showing GSEA of from RNA sequencing data for testis and liver upon ethanol administration (* = the gene set ranked in the top 20 NES for GSEA in GSEA in each tissue.)

incubation conditions (Fig. 4). These data indicate that ethanol-fed mice exhibit abnormal sperm motility.

Given the above finding, we attempted to identify hub genes that are closely related to the sperm tail

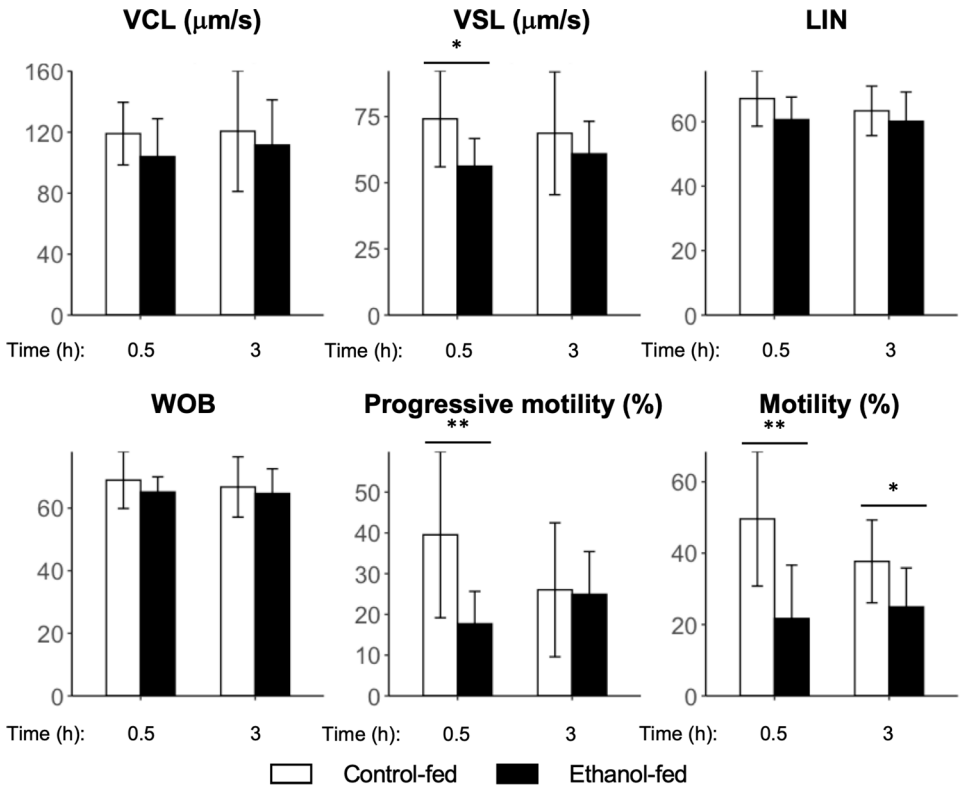


Fig. 4 Sperm motility analysis of ethanol-fed and control-fed mice. Sperm were incubated with 3% BSA for 0.5 and 3 h, and OpenCASA was used to measure sperm parameters. Three mice per group were used in the analysis, and sperm from each mouse were analyzed three times. VCL, curvilinear velocity; VSL, linear velocity; LIN, linearity; WOB, wobble coefficient. *$P < 0.05$, **$P < 0.01$

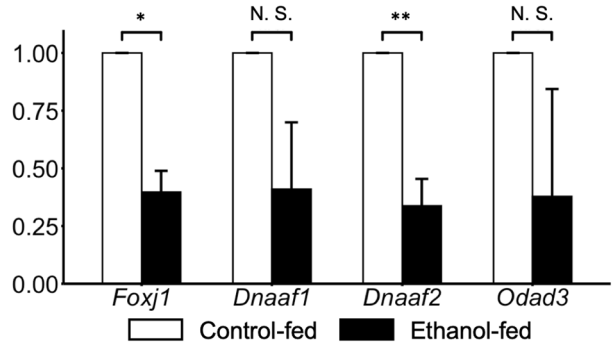
compartment and are affected by ethanol intake. One such gene was *Foxj1* (fold change = 0.725, *P*-value < 0.001), which has been well studied as a key regulator of ciliogenesis [22]. In fact, the *Foxj1* gene was identified in 'cilium organization' and 'axoneme assembly' as significantly altered gene sets in GSEA (Fig. 3A). Our qRT-PCR analysis confirmed that *Foxj1* expression was altered in the testes of ethanol-fed mice by qRT-PCR (Fig. 5A). Three other sperm tail structure-related genes were also found to be altered in ethanol-fed mouse testes, namely those encoding dynein axonemal assembly factor 1 (*Dnaaf1)*, dynein axonemal assembly factor 2 (*Dnaaf2)*, and outer dynein arm docking complex subunit 3 (*Odad3)* (Fig. 5B). qRT-PCR analysis showed that the expression levels of *Dnaaf2*, but not those of *Dnaaf1* or *Odad3*, showed a significant decrease in testes of ethanol-fed mice by qRT-PCR (Fig. 5A). Our results therefore suggest that there is a relationship between ethanol intake and changes in the testicular expression levels of genes that are important for normal sperm tail structure and motility (see Discussion).

Discussion

In the present study, we profiled and investigated transcriptional changes in mouse testis resulting from chronic ethanol consumption. Many previous studies have utilized acute administration via oral gavage or intraperitoneal injection to establish animal models of alcohol exposure [8–12]. The Liber de-Carli diet, in contrast, was developed as a milder dosing procedure that would enable researchers to study chronic alcoholic liver diseases; it has been used to generate animal models for studies of how chronic alcohol exposure impacts the liver, heart, and intestine, etc. [23]. To our knowledge, there are no studies in mice that have implemented the Liber de-Carli model to investigate how the testes are impacted by chronic ethanol administration. Here, we used the Lieber-DeCarli diet to generate a model of chronic ethanol administration in mice, and leveraged transcriptomic profiling to reveal the effects of ethanol consumption on the testis.

In our experimental setting, chronic ethanol administration triggered physiological changes, including liver histological abnormalities and, in the context of male reproductive function, changes in sperm count and motility. Consistent with previous studies on ethanol administration, the liver histology exhibited the

A



B

Analysis		*Foxj1*	*Dnaaf1*	*Dnaaf2*	*Odad3*
RNA-seq	Fold change	0.725	0.875	0.739	0.848
	P-value	<0.001	0.023	<0.001	0.001
qRT-PCR	Fold change	0.397	0.410	0.337	0.378
	P-value	0.035	0.180	0.003	0.072

Fig. 5 Expression changes of *Foxj1 and Foxj1*-related genes in ethanol-fed and control-fed mice. Three mice were analyzed per group. (**A**) Relative expression levels of *Foxj1* and *Foxj1*-related genes in ethanol-fed and control-fed mice, as measured by qRT-PCR. N.S., not significant, $^{*}P < 0.05$, $^{**}P < 0.01$. (**B**) Comparison of RNA-sequencing and qRT-PCR data on the expression level changes of *Foxj1* and *Foxj1*-related genes

presence of fat globules, which is indicative of fatty liver and similar to the findings associated with chronic liver injuries [23]. In contrast, no gross histological change was observed in the testes of ethanol-fed mice. A previous study found more apoptotic cells in the testes of ethanol-fed rats compared to control rats [24, 25]. Our use of H&E staining limited our ability to observe ethanol-induced apoptotic germ cells. Thus, we cannot be ruled out the possibility that apoptosis of spermatogenic cells may be a cause of the decreased sperm count in ethanol-fed mice.

Total RNA sequencing of testes from ethanol- and control-fed mice revealed a total 534 ethanol-DEGs. This study is the first to show that ethanol exposure has a minimal effect on overall gene expression in testes, with only a small percentage of testicular genes (approximately 2% of all testicular genes) exhibiting alcohol-related expression changes. Although, ethanol had limited effect on overall testicular gene expression, cell type-specific genes were enriched in ethanol-DEGs. GO analysis did not show any terms that could be considered relevant or noteworthy in relation to ethanol. On the other hand, KEGG analysis revealed a term related to steroid hormones, such as the PPAR pathway. In a previous study, altered expression of steroidogenesis-related proteins, such as StAR, AR, and HSP70, was found in ethanol-treated rats [26]. Additionally, ethanol consumption was reported to induce testosterone deficiency, which in turn can cause dysfunction in testicular hormone synthesis [26]. In our transcriptomic profiling, however, the genes encoding these three steroidogenesis-related genes were not found among the ethanol-DEGs, even though GSEA revealed that the ethanol-induced gene expression changes were partially associated with steroidogenesis. We observed up-regulation of genes in the hormone synthesis-related (Fig. 3B) and corticosteroid secretion-related (Fig. 3A) gene sets. Given that corticosteroid synthesis share a common precursor with androgen (17-OH-prognenolone) [27], we speculate that altered corticosteroid synthesis disrupts the hormonal environment. Cytochrome P450, which exhibited down-regulation under ethanol exposure (Fig. 3A), is known to promote the catalysis of steroids [28]. Further investigation is needed to elucidate exactly how ethanol is associated with gene expression changes leading to hormonal dysregulation during mouse spermatogenesis.

Our present results also reveal for the first time that the expression patterns of genes related to the sperm tail integrity are altered in the testes of ethanol-fed mice. Ethanol consumption is known to be associated with reduced sperm motility in humans, mice, and rats [9, 11, 29, 30]. Building on this, we herein used the OpenCASA system to confirm that sperm motility is decreased in mice fed with the Liber de Carli diet. Previous reports

have not sufficiently discussed the molecular mechanisms and relationships between spermatogenesis and reduced sperm motility in ethanol-fed model animals and men with chronic alcohol consumption [9, 29, 31]. In the present study, we aimed to elucidate the deleterious effects of ethanol on sperm parameters by examining gene expression. Our GSEA revealed that there were mild but significant expression changes in *Foxj1* and related genes (*Dnaaf1*, *Dnaaf2*, and *Odad3*) in the testes of ethanol-fed mice. *Foxj1* encodes a transcription factor that is critical for the establishment of left-right asymmetry during the development of epithelial cells with motile cilia [32, 33]. *Foxj1* and *Dnaaf2* are known to be involved in ciliogenesis and primary ciliary dyskinesia [22, 33, 34]. *Foxj1*, *Dnaaf1*, *Dnaaf2*, and *Odad3* are transcribed predominantly in the mouse testes, implying that they are involved in sperm tail formation and function [35]. In fact, *Dnaaf2* mutants have been reported to exhibit shortened sperm flagella [34]. The genes putatively targeted by FOXJ1 are related to sperm flagellum formation [34, 36]. Our qRT-PCR analyses confirmed that the transcription levels of *Foxj1* and *Dnaaf2* were decreased in the testes of ethanol-fed mice. Therefore, the ethanol ingestion-related impairment of sperm motility could be mediated by changes in the expression levels of *Foxj1*, which encodes a ciliogenesis-related transcription factor, and other genes encoding sperm tail components, including *Dnaaf2*. The transcription and protein expression levels of the another *Fox* gene encoding, forkhead box O1 (FOXO1), were found to be affected in hepatocytes under ethanol exposure [37], and in chondrocytes under oxidative stress [38]. Here, we did not find alteration of any other *Fox* genes under ethanol exposure (Supplementary Data 7). We do not know whether ethanol directly or indirectly affects the expression of germ cell genes such as *Foxj1*. Future research is needed to clarify how a small number of germ cell genes show selectivity for ethanol-induced transcriptional and phenotypic changes, while exhibiting very small degrees of expression variation. Finally, although sperm tail morphology was normal in ethanol-fed mice, we cannot rule out the possibility that the detailed structure of sperm flagellum was altered, resulting in abnormal sperm motility.

Conclusion

We herein established a mouse model chronically fed with ethanol and present the gene expression profiles of model mouse testes. Although the gross testicular and sperm morphology were normal, we observed decreases in sperm count and motility. We classified ethanol-DEGs and conducted a systematic analysis of gene expression. Our results revealed that chronic ethanol exposure induced changes in the expression patterns of genes related to sperm flagellum formation and motility, such as *Foxj1* and *Dnaaf2*. Our study newly suggests that alcohol-induced impairment of male reproductive function, i.e., reduction of sperm motility, is actually caused by changes in the expression levels of relevant genes. This study lays the foundation for research on the mechanisms linking alcohol consumption to the expression levels of these germ cell-intrinsic genes.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10696-2.

Supplementary Material 1: Title and description of data.- Supplementary Data 1. Primer pairs used in quantitative real-time PCR.- Supplementary Data 2. Analysis of hepatic fat globule areas.- Supplementary Data 4. Functional enrichment analysis of mouse testicular ethanol DEGs. - Supplementary Data 7. Expression patterns of Fox genes in ethanol-fed mice.

Supplementary Material 2: Supplementary Data 3. List of ethanol-DEGs.

Supplementary Material 3: Supplementary Data 5. Gene lists of functional enrichment analysis of mouse testicular ethanol-DEGs. In GO enrichment, terms show p-value < 0.05 and FDR < 0.3. In KEGG pathways, pathways show p-value < 0.05.

Supplementary Material 4: Supplementary Data 6. List of genes in gene sets from GSEA of ethanol-fed mice

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Not applicable.

Author contributions
C.C. conceived the study. G.H. designed the project and performed all experiments. S.J.L., S.P.H., and J.S. participated in reagent and animal preparation. G.H. analyzed the data. C.C. supervised the work. G.H. and C.C. wrote the manuscript. All authors have read and agreed to the publication of this manuscript.

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Data availability
The total RNA sequencing data can be found in the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI), under GEO accession number GSE256349.

Declarations

Ethics approval and consent to participate
All animal experiments were conducted in accordance with Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Gwangju Institute of Science and Technology (GIST) (permit number: GIST-2022-049).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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