Small RNAs in Rat Sperm Are a Predictive and Sensitive Biomarker of Exposure to the Testicular Toxicant Ethylene Glycol Monomethyl Ether

Angela R. Stermer,¹ Gerardo Reyes, Susan J. Hall, and Kim Boekelheide

Department of pathology and laboratory medicine, Brown University, Providence, Rhode Island 02912

¹To whom correspondence should be addressed at Department of pathology and laboratory medicine, Brown University, Box G, Providence, RI 02912. Fax: 401-863-9008; E-mail: angela_stermer@brown.edu.

ABSTRACT

Testicular histology and semen parameters are considered the gold standards when determining male reproductive toxicity. Ethylene glycol monomethyl ether (EGME) is a testicular toxicant with well-described effects on histopathology and sperm parameters. To compare the predictivity and sensitivity of molecular biomarkers of testicular toxicity to the traditional endpoints, small RNAs in the sperm were analyzed by next generation RNA-sequencing (RNA-seq). Adult rats were exposed to 0, 50, 60, or 75 mg/kg EGME by oral gavage for 5 consecutive days. Testis histology, epididymal sperm motility, and sperm small RNAs, including microRNAs (miRNAs), mRNA fragments, piwi-interacting RNAs (piRNAs), and tRNA fragments (tRFs), were analyzed 5 weeks after cessation of exposure. Testicular histology showed a significant dose-dependent increase in retained spermatid heads (RSH), while sperm motility declined with increasing dose. RNA-sequencing of sperm small RNAs was used to identify significant dose-dependent changes in percent mRNA fragments (of total reads), percent miRNAs (of total reads), average tRF length, average piRNA length, and piRNA and tRF length-distributions. Discriminant analysis showed relatively low predictivity of exposure based on RSH or motility compared to the average read length of all assigned RNAs. Benchmark dose (BMD) modeling resulted in a BMD of 62 mg/kg using RSH, whereas average read length of all assigned RNAs resulted in a BMD of 47 mg/kg. These results showed that sperm small RNAs are sensitive and predictive biomarkers of EGME-induced male reproductive toxicity.

Key words: biomarkers; testicular toxicity; small RNA; sperm epigenetics; preconception exposures.

Male reproductive toxicity poses a regulatory challenge due to the lack of simple robust analytical methods to detect adverse effects ([Sasaki et al., 2011](#)). The most reliable method to evaluate the effects of toxic compounds on the male reproductive tract is histological examination of testicular tissue. Other existing markers of reproductive toxicity, such as plasma testosterone and inhibin B levels, are of poor prognostic value ([Dere et al., 2013](#)). Semen parameters have high inter- and intra-individual variability ([Jarow et al., 2013](#); [Redmon et al., 2013](#)). Functional assessments that determine reproductive capacity are required for medical substances ([International Conference on Harmonisation, 2005](#)), and continuous breeding strategies have been used to quantify the fertility effects of environmental exposures ([Environmental Protection Agency, 1996](#)). Reproducible

isolation protocols of sperm RNA ([Bianchi et al., 2018](#)) and the availability of low-cost, high-throughput next generation sequencing have created an opportunity to use advanced techniques to identify highly predictive, sensitive molecular biomarkers of male reproductive tract toxicity within sperm.

The contents of sperm are unique in many ways, and reflect the developmental process of these specialized cells. Germ cells develop into sperm under the influence of the surrounding somatic cells, including Sertoli cells, peritubular myoid cells, testicular macrophages, and Leydig cells. Sperm retain many RNA species from progenitor germ cells. Sperm lack 18S and 28S ribosomal RNAs ([Cappallo-Obermann et al., 2011](#)) and contain a large number of small non-coding RNAs ([Ostermeier et al., 2005](#)), including tRNA fragments (tRFs), piwi interacting RNAs

(piRNAs), and microRNAs (miRNAs) (Krawetz et al., 2011; Peng et al., 2012; Schuster et al., 2016).

These small RNAs differ in their size, biogenesis, and cellular origin. The miRNAs are generally approximately 22 nucleotides (nt) long (Lee et al., 2003) whereas piRNAs are approximately 24–31 nt long (Yin and Lin, 2007) in their mature, biologically active form. The miRNAs and piRNAs in sperm originate within the developing germ cells. The miRNA and piRNA expression profiles differ based on the stage of differentiation of the germ cells (Aravin et al., 2007; Smorag et al., 2012). Mature tRFs in sperm are quite different from other small RNAs in sperm; they are approximately 28–34 nt long and taken up by way of fusion of epididymosomes with sperm in the epididymis (Sharma et al., 2016).

Sperm RNAs are of significant scientific interest from a toxicological perspective because they reflect testicular and epididymal changes that may occur due to exposure. Ethylene glycol monomethyl ether (EGME) is a well-described testicular toxicant (reviewed in Hardin, 1983). It has been used heavily as an industrial solvent. Ethylene glycol monomethyl ether is metabolized to the active compound methoxyacetic acid (MAA) by alcohol dehydrogenase (reviewed in Welsh, 2005). The testicular toxicity of EGME is characterized by an initial loss of pachytene spermatocytes through apoptosis (Creasy and Foster, 1984). Exposure of rats to 100 mg/kg EGME by oral gavage showed reproducible loss of this population of germ cells; higher exposures caused apoptosis in additional germ cell populations (Creasy and Foster, 1984), resulted in multinucleated germ cells, and caused Sertoli cell vacuoles (Chapin et al., 1984). Loss of this germ cell population through apoptosis leads to a dramatic reduction in epididymal sperm and sperm motility, and increased abnormal sperm 5 weeks later (Chapin et al., 1985), the approximate length of time it would take for pachytene spermatocytes to develop into epididymal sperm in rat. The sperm effects resulted in decreased reproductive performance of males, resulting in fewer pregnancies and siring fewer pups 5 weeks after exposure to EGME (Chapin et al., 1985).

Studies to determine molecular mechanisms in the Sertoli cells and germ cells were performed at exposure levels in excess of 500 mg/kg EGME or MAA (Clark et al., 1997; Tirado et al., 2003; Tirado et al., 2004; Wang and Chapin, 2000; Wade et al., 2008). High exposures (2 g/kg) in rats and (300 mg/kg) in monkeys to EGME cause increased mRNA and miRNAs in the testis (Fukushima et al., 2005; Fukushima et al., 2011; Sakurai et al., 2015). The toxicity of EGME at low doses has not been explored in detail. It is hypothesized that small RNAs will be changed after EGME exposure. Here, traditional measures of EGME-induced testicular toxicity are compared with the effects on small RNAs as biomarkers at low levels of exposure.

MATERIALS AND METHODS

Animal care and EGME exposure paradigm. Fischer rats (11–12-weeks-old, strain code 002) were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed in the Brown University animal care facility under controlled temperature (25°C–28°C) and humidity (30%–70%) with a 12L:12D cycle and received Purina Rodent Chow 5010 (Farmer's Exchange, Framingham, MA) and water *ad libitum*. All procedures for care and use of animals were approved by the Brown University Institutional Animal Care and Use Committee in accordance with U.S. Public Health Service requirements and the *Guide for the Care and Use of Laboratory Animals*. The rats were allowed to acclimate for 1 week prior to experimentation. Ethylene glycol monomethyl ether (Fisher Scientific) was diluted in sterile tap

water and given at 2 ml/kg volume daily for 5 days as previously described (Chapin et al., 1985). A preliminary dose-range finding experiment showed a steep response between 50 mg/kg giving no histopathological effects, and near total loss of sperm after exposure to 100 mg/kg (Supplementary Figure 1). Because of these results, we narrowed the dose response range to 0, 50, 60, and 75 mg/kg per day for 5 days. Two groups at 5 and 13 weeks after EGME exposure were collected ($n=10$ per exposure per group per collection, total $n=80$).

Tissue collection. Rats were killed by CO₂ asphyxiation followed by thoracotomy then weighed. Testes were trimmed of excess tissue and weighed. The left testis was fixed in Davidson's Fixative, and the right testis was cut in half with one half snap frozen (liquid nitrogen) in optimum cutting temperature compound (OCT compound, Tissue Tek), and the other snap frozen in an Eppendorf tube. The caput epididymides were fixed in Davidson's Fixative, whereas the cauda epididymides were prepared as below for sperm RNA extraction. Sperm from a section of the vas deferens proximal to the prostate was dissected and incubated in 1% BSA warmed to 37°C for 5 min for sperm motility videos.

Sperm RNA extraction. Total sperm RNA was isolated fresh on the day of collection, as previously described in detail (Bianchi et al., 2018). In brief, cauda epididymides were repeatedly punctured with 18 and 22 G needles then placed in pre-warmed 37°C PBS for 10 min. Epididymal tissue was discarded and cell suspension was centrifuged, then the pellet was resuspended in somatic cell lysis buffer (0.05% SDS 0.25% Triton X in ddH₂O) for 10 min on ice. This suspension was centrifuged then the pellet was subject to sperm head lysis using the lysis buffer, with 100 µl stainless steel microbeads (0.2 mm, Next Advance, Inc) homogenized for 5 min, then incubated at 65°C for 5 min. DNA was removed by incubating with RNase-free DNase I (50 Kunitz, Qiagen) for 10 min. The lysate was then subject to Acid-Phenol Chloroform phase separation and then RNA isolation using the mirVana total RNA isolation kit (Invitrogen). Ammonium acetate (5M) precipitation was used to clean and concentrate the RNA. RNA quality and quantity were assessed using the Nanochip on the Bioanalyzer (Agilent), which showed lack of 18S and 28S peaks, and quantitative PCR of intact PRM2 (Bianchi et al., 2018). One sample (Rat 37, 60 mg/kg exposure group) did not have enough RNA to process for RNA-seq.

Sperm motility. Sperm motility was assessed from videos taken on a EOS Rebel T3i camera (Nikon) mounted on a Axiovert 35 microscope (Zeiss). Ten microliters of sperm suspension from the vas deferens was placed on a Petroff-Hausser Counting Chamber (Hauser Scientific), and 3 different areas were recorded for at least 10 s each. The videos were converted in to a series of pictures using Photoshop 2016 (Adobe), and those pictures were analyzed by an in-house customization of a CASA-like plugin for ImageJ available on the web (Wilson-Leedy and Ingermann, 2007, <https://imagej.nih.gov/ij/plugins/casa.html>; Accessed March 18, 2019). The total sperm analyzed was used to generate the number of sperm in the sample (for small RNA per sperm), and the percent motility was recorded from the plugin's generated output.

Testis histology. Fixed testes were embedded in plastic and 3 micron sections were stained with Hematoxylin Periodic Acid-Schiff stain. Slides were randomized, blinded, and scanned using the Aperio Scanscope (Leica Biosystems). Histological scoring was performed, where retained spermatid heads (RSH) in stages IX–XI were counted and normalized to the number of

Figure 1. Exposure paradigm for sperm biomarkers of a germ cell specific toxicant. Ethylene Glycol monomethyl ether (EGME) targets pachytene spermatocytes, a specific sub-type of germ cell. To be able to look at biomarkers in sperm after EGME exposure, adult rats were given 0, 50, 60, or 75 mg/kg for 5 days, causing specific injury to the pachytene spermatocytes. Then, 5 weeks later, those pachytene spermatocytes have developed into sperm, and the sperm are collected for analysis. Thirteen weeks later, a whole second round of spermatogenesis has occurred, and a second group of rat sperm samples were collected.

Table 1. Body Weight, Testis Weight, and Epididymis Weights of 5 and 13 Weeks After EGME Exposure

Exposure (mg/kg)	Body Weight (g)	Testis Weight (g)	Epididymis Weight (g)
<i>5-week recovery</i>			
0	274 ± 7.19	1.48 ± 0.03	0.44 ± 0.011
50	270 ± 3.86	1.51 ± 0.02	0.42 ± 0.009
60	272 ± 2.96	1.50 ± 0.02	0.41 ± 0.007
75	268 ± 3.53	1.48 ± 0.03	0.39 ± 0.013
<i>13-week recovery</i>			
0	330 ± 6.52	1.61 ± 0.02	0.48 ± 0.008
50	333 ± 6.27	1.56 ± 0.04	0.47 ± 0.017
60	333 ± 6.17	1.60 ± 0.02	0.48 ± 0.006
75	330 ± 5.49	1.55 ± 0.02	0.47 ± 0.006

Body weight, testis weight, and epididymal weights were recorded for each exposure group 0, 50, 60, and 75 mg/kg at each collection time, 5 and 13 weeks after exposure. Values are reported as mean ± SEM. No statistical differences were found using a one-way ANOVA test and Dunnett's post-hoc analysis. A linear regression showed a non-zero association of epididymal weight with exposure at 5 weeks ($p = .003$).

tubules counted. After scoring the slides were decoded and compared across the dose-response curve.

Small-RNA cloning. Ten nanograms small RNA was used as input for the cDNA library creation. Ion total RNA seq kit v2 (ThermoFisher Scientific) was used to construct the library according to manufacturers' instructions for small RNA. Briefly, 3' and 5' adapters were hybridized and ligated to the RNA. The RNA was reverse transcribed into cDNA, then purified and size selected via the proprietary magnetic bead cleanup procedure. The cDNA libraries were amplified and barcoded simultaneously, then purified again by the magnetic bead procedure. Molar concentration and fragment size were determined using the Agilent Bioanalyzer. Thirty-nine libraries were multiplexed into 15 chips (3 libraries per chip), where each multiplexed sample on the same chip represented a different exposure to prevent bias. Single-end sequencing was performed by the IonProton sequencer (ThermoFisher Scientific) at Boston University. Average number of reads per library was 16 million. The average read length was 30 nt.

Small-RNA-seq data processing. Reads were aligned using the—very-sensitive-local setting of Bowtie (Langmead et al., 2009) to

each of the following; piRNAs (RNA central v10, <https://rncentral.org/>, species: *Rattus norvegicus*, RNA type: piRNA; Accessed March 18, 2019), mRNA fragments (Ensembl Rnor_6.0 version 93.6, <https://useast.ensembl.org/info/data/ftp/index.html>, bio-type: protein coding; Accessed March 18, 2019), and tRFs (UCSC Table browser, rn6, <http://genome.ucsc.edu/cgi-bin/hgTables>, repeatmasker track, repClass: tRNA; Accessed March 18, 2019). For miRNA, reads were aligned to the hairpin miRNA gene annotation (miRBase release 22, <http://www.mirbase.org>; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011; Kozomara and Griffiths-Jones, 2014) using Bowtie 2, with custom sensitivity settings (-D 20 -R 3 -N 1 -L 10 -i S, 1, 0.50; Langmead and Salzberg, 2012). The alignment summary was used to determine population level changes in RNA type, only the aligned reads to each RNA type were counted to determine RNA characteristics such as RNA length and length distribution. Reads aligned to each type of RNA for each sample were merged using SAMtools (Li et al., 2009) to obtain “all assigned RNAs.”

Fragment histograms. The number of reads were counted for each read length for an increasing number of nt in an integer/discrete fashion from 8 to >200 nt for each sample. Reads that fell

Figure 2. Photomicrographs of cross-sections of control and 75 mg/kg exposed rat testis. Testicular cross-sections of stage IX seminiferous tubules from control (A) and 75 mg/kg ethylene glycol monomethyl ether (EGME)-exposed rat testis (B). RSH (Retained Spermatid Heads—arrows), missing germ cell layers (asterisk), and a smaller diameter seminiferous tubule are seen after EGME exposure (B). PASH staining; scale bar, 60 μ m.

between 15 and 40 nt (biologically active small RNAs) were included for further analysis. A weighted average was taken for each sample for each RNA type, to find the average RNA length. The distribution of all assigned RNAs were found to have bimodal distribution, therefore the small nt length peak divided by the large nt length peak was calculated for each sample (the peak ratio) to quantify shifts in length distribution due to exposure. The small nt length peak was defined as RNA's between 22 and 27 nt long, and the large nt length peak was defined as RNA's between 28 and 33 nt long.

Statistics. All statistics were analyzed using JMP pro 13 software (SAS Institute, Cary, North Carolina). Mean $\pm$ SEM or quartile box plots were used to show the spread of the data. For endpoints that were normally distributed, a one-way ANOVA across exposure with Dunnett's post-hoc analysis was run to determine dose-dependent changes from control (0 mg/kg). Motility, small RNA per sperm, percent mRNA fragments, and mRNA fragment peak ratio were not normally distributed so a non-parametric Wilcoxon/Kruskal-Wallis (Rank sums) test and Steel comparison with control was performed. A discriminate analysis was run on both the traditional toxic endpoints (RSH and motility) and molecular biomarker endpoints (RNA per sperm, RNA population percentages, average RNA length, and RNA peak ratio). The receiver operator characteristic (ROC) curve plots specificity and 1-sensitivity of each characteristic (endpoint) for predicting category (exposure). The area under the curve (AUC) value is calculated from the ROC curve (reviewed in Lasko et al., 2005), and gives the probability of the characteristic to correctly predict category (DeLong et al., 1988). A Chi-squared test was run in R version 3.5.1 (R foundation for statistical

Figure 3. RSH, motility, and small RNA per sperm quantitation over the dose-range of EGME. Quartile type box-plots of: retained spermatid heads (RSH) normalized to the number of total tubules counted (A), sperm motility as percent motile sperm per sperm counted (B), and small RNA (fg) per sperm for samples exposed with 0, 50, 60, 75 mg/kg exposure after 5 weeks (C). * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$, found by one-way ANOVA followed by Dunnett's post-hoc analysis for RSH and RNA, Wilcoxon rank-sum followed by Wilcoxon each-pair tests for motility.

Table 2. Percent of Total Reads Assigned to tRNA, piRNA, miRNA, and mRNA Annotations for each exposure level

RNA Type	0 mg/kg	50 mg/kg	60 mg/kg	75 mg/kg
miRNA	4.4% ± 0.8%	4.6% ± 0.4%	6.4% ± 1.0%	8.6% ± 0.7%***
mRNA fragments	4.7% ± 0.5%	4.9% ± 0.3%	8.0% ± 1.5%	10.5% ± 0.9%**
piRNA	30.4% ± 1.8%	32.9% ± 0.8%	31.5% ± 1.7%	32.2% ± 2.2%
tRFs	51.4% ± 4.5%	54.8% ± 4.1%	65.6% ± 1.6%	55.0% ± 3.5%

Percent mapped reads to each of the tRNA (tRFs), piRNA, miRNA, and mRNA (fragments) annotations for 0, 50, 60, and 75 mg/kg exposure groups. Mean ± SEM are reported, * $p \leq .05$, ** $p \leq 0.01$, *** $p \leq .001$, found by One-way ANOVA followed by Dunnett's post-hoc analysis. Significant results are bolded and italicized.

Table 3. Average RNA Length by Type for Each Exposure level

RNA Type	0 mg/kg	50 mg/kg	60 mg/kg	75 mg/kg
All assigned RNAs	26.48 ± 0.13	26.82 ± 0.14	27.31 ± 0.09***	27.67 ± 0.14***
miRNA	17.71 ± 0.05	17.61 ± 0.03	17.70 ± 0.08	17.71 ± 0.07
mRNA fragments	22.82 ± 0.22	23.10 ± 0.16	23.07 ± 0.21	22.93 ± 0.33
piRNA	27.24 ± 0.11	27.86 ± 0.32	28.22 ± 0.21***	28.85 ± 0.20***
tRFs	26.41 ± 0.14	26.63 ± 0.14	27.43 ± 0.19***	28.48 ± 0.24***

The average RNA size for each RNA type and each exposure was calculated using a weighted average approach. Mean ± SEM are reported, * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$, found by One-way ANOVA followed by Dunnett's post-hoc analysis. Significant results are bolded and italicized.

computing, <https://www.r-project.org>, Accessed March 18, 2019) on the all assigned RNA size-distribution between 15 and 40 nt across the exposure groups.

Benchmark dose model fitting and endpoint comparison. Benchmark dose (BMD) software from the EPA was used to calculate the BMD and BMDL (lower 95% confidence limit of the BMD) (BMDS 2.7, <https://www.epa.gov/bmds/download-benchmark-dose-software-bmds>). Benchmark dose model fitting was performed on endpoints that were both normally distributed, and significantly changed with exposure found using a one-way ANOVA. These data were z-transformed to account for different units across multiple endpoints. Dose group, number of samples per group, mean and standard deviation per group for each endpoint were entered into BMDS Wizard. The exponential M2, exponential M3, exponential M4, exponential M5, Hill, linear, power, polynomial 2°, and polynomial 3° models were fit to these continuous-type data. The lowest Akaike information criterion was used to determine best fit when comparing models (Hogan et al., 2012). Benchmark dose model summaries for all fit models are included in the supplemental information (Supplementary Tables 1–8). The BMD curves for the best-fit model are included in the supplemental information (Supplementary Figures 2–9).

RESULTS

Rats were exposed to 0, 50, 60, or 75 mg/kg EGME via oral gavage daily for 5 consecutive days. The male reproductive tract was evaluated 5 weeks later, the approximate length of time required for the EGME-exposed pachytene spermatocytes to develop into sperm (Figure 1). Sperm were collected from a second group of rats 13 weeks after EGME exposure to determine whether the effects of EGME persisted beyond direct exposure to the developing germ cells. There was a linear association between EGME exposure and decreased epididymal weights at 5 weeks; however, no significant differences in body, testis, or epididymal weights (Table 1) were seen at either time point or at any dose.

Testicular cross-sections (Figure 2) showed a dose-dependent increase in RSH per tubule, and the 75 mg/kg exposure group had significantly more RSH per tubule than the control group (Figure 3A, $p = .012$). After 13 weeks, this histological abnormality was no longer present. Sperm motility (Figure 3B) decreased with increasing EGME exposure at 5 weeks and was significantly lower in the 75 mg/kg exposure group compared to control ($p = .013$). Sperm motility returned back to normal after 13 weeks. The amount of small RNA per sperm dose-dependently increased at 5 weeks and was significantly increased in the 75 mg/kg exposure group compared to the control group (Figure 3C, $p < .0001$).

Sperm small RNA-sequencing was performed on the 5-week samples. In the control samples, the total amount of small RNA identified was distributed among tRFs (50%), piRNAs (30%), miRNAs (5%), and mRNA fragments (5%) (Table 2). There was a significant, 2.2-fold increase in mRNA fragments with EGME exposure (75 mg/kg, $p = .001$, Table 2). There was also a significant, 2-fold increase in miRNAs after exposure to EGME (75 mg/kg, $p = .0006$, Table 2) compared to control.

The average RNA-read size of all assigned RNAs (between 15 and 40 nt) increased with exposure (60 mg/kg, $p = .0002$; 75 mg/kg, $p = .0001$, Table 3). When the reads were segregated by RNA type, the average size of piRNAs and tRFs increased with exposure. Both tRFs and piRNAs were significantly longer in the 60 mg/kg ($p = .001$, $p = .011$, respectively) and 75 mg/kg exposure groups ($p < .0001$, $p < .0001$, respectively) (Table 3).

A histogram of small RNA size (in nt) by number of RNAs of each length showed a distinct bimodal length-distribution, with the largest numbers of RNAs being 22–27 nt long and 28–33 nt long (Figure 4). A Chi-square test determined that there were significant differences between the length-distributions of total small RNA size among the exposure groups ($p = 2.2 \times 10^{-16}$). To capture the change in the bimodal length-distribution, a peak ratio was calculated comparing the RNAs of size ranges of nucleotides across doses. In the control samples, there were 2-fold more RNAs in the small nucleotide length peak (22–27 nt) than in the large nucleotide length peak (28–33 nt) (Figure 4A). With increasing EGME exposure, fewer RNAs in the small nucleotide peak

Figure 4. Number of RNAs of each length (in nt) by RNA type over the dose-range of EGME. RNA size distribution changes with exposure level of EGME, shown by (A, C, E, G) histograms of counts per read length for each type of RNA with exposure, and (B, D, F, H) quartile box plots of the peak ratio with exposure. A and B, all assigned RNAs. C and D, piRNAs. E and F, tRFs. G and H, mRNA fragments. Different lines illustrate average response of all samples in the same exposure level. Peak ratio was calculated from the number of RNA's between 22 and 27 nt, divided by the number of RNA's between 28 and 33 nt. * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$, found by one-way ANOVA followed by Dunnett's post-hoc analysis.

Figure 5. Discriminant analysis of gold standard endpoints and RNA biomarkers. Discriminant analysis that shows the sensitivity and specificity of endpoints to predict exposure is shown in receiver operator characteristic (ROC) curves for: retained spermatid heads (RSH) (A), motility (B), and all assigned RNAs for each exposure group (C). Each curve has a corresponding area under the curve (AUC) to demonstrate the accuracy of the endpoint to predict exposure.

and more RNAs in the large nucleotide peak were found. This caused the peak ratio of all assigned RNAs to be significantly different in both the 60 mg/kg ($p < .0001$) and 75 mg/kg ($p < .0001$) exposure groups (Figure 4B).

The RNA length-distribution was determined for each type of RNA in each exposure. Each RNA type had a distinct pattern of length distribution that changed with exposure. The miRNAs were on average too small (17 nt) to calculate a peak ratio. In control samples, there was approximately the same number of piRNAs 22–27 nt in length as piRNAs 28–33 nt long (Figure 4B); however, there were 2-fold more tRFs 22–27 nt in length than tRFs 28–33 nt long (Figure 4C). The peak ratio changed in a dose-dependent manner for both piRNAs and tRFs (Figs. 4F–G); these small RNAs had significantly smaller peak ratios at 60 mg/kg (piRNAs $p = .0029$, tRFs $p < .001$) and 75 mg/kg (piRNAs $p < .0001$, tRFs $p < .0001$) compared to control. The mRNA fragments were mostly 22–25 nt long (Figure 4D). There were significantly fewer mRNA fragments of this size, where the peak ratio increased significantly with exposure (Figure 4H, 60 mg/kg $p = .0048$, 75 mg/kg $p < .0005$); however, this ratio did not take into consideration the increasing number of mRNA fragments below 20 nt.

A discriminant analysis was performed to determine how well each type of biomarker could determine exposure based on response. Retained spermatid heads had a poor probability of predicting exposure level, where all of the AUC values were less than or equal to 0.76 (Figure 5A). Sperm motility had a poor probability of predicting exposure as well, where at best motility had an AUC of 0.83 at 75 mg/kg exposure, whereas the 60 mg/kg exposure group only had 0.40 AUC on the ROC curve (Figure 5B). Of all the endpoints, the average read length of all assigned RNAs had the best probability of discriminating between exposures with AUC values above 0.76 for all exposures (Figure 5C).

Benchmark dose modeling was used as another way to compare these endpoints. The BMDs had a range of values that differed by a factor of 1.5, depending on endpoint (Table 4). Retained spermatid heads resulted in the highest BMD (62 mg/kg) compared to NGS-derived average RNA length of all assigned reads, with the lowest BMD (47 mg/kg) (Table 4).

DISCUSSION

The results indicate that small RNAs are good biomarkers of testicular toxicity in this rat EGME exposure model. The no observable adverse effects level (NOAEL) and the lowest observable adverse effects level (LOAEL) are used in non-carcinogenic risk assessment to determine the safety index for exposure to potentially toxic compounds. Endpoints that result in lower NOAEL/LOAEL values are considered to be more susceptible to alterations by the toxicant at lower exposures, and represent more sensitive biomarkers of toxicity. NOAEL and LOAEL for effects of EGME on RSH, motility, and RNA per sperm are 60 and 75 mg/kg, respectively (Figure 3). Conversely, the NOAEL and LOAEL of the small RNA length and distribution for all assigned RNAs, piRNAs, and tRFs were 50 and 60 mg/kg, respectively (Table 3, Figure 4). Our results are consistent with historical EGME toxicity data that show a steep dose-dependent response for exposures of between 50 and 100 mg/kg for several consecutive days in rats (Chapin et al., 1985; Creasy and Foster, 1984; Foster et al., 1983; Foster et al., 1984). Benchmark dose modeling has been developed to more accurately estimate points of departures than the NOAEL/LOAEL point estimates.

Table 4. Model Fit for Each Significant Histopathological and Molecular Endpoint With BMD and BMDL Determination

Endpoint	Model	GOF	BMD _{1SD}	BMDL _{1SD}
RSH	Polynomial 3°	0.422	62.0	37.0
miRNA percent	Polynomial 3°	0.504	59.2	49.7
piRNA length distribution	Polynomial 3°	0.343	52.8	48.2
tRF average length	Polynomial 3°	0.206	51.3	41.5
All assigned RNA length distribution	Polynomial 3°	0.224	48.2	36.9
tRF length distribution	Polynomial 3°	0.126	48.0	39.0
piRNA average length	Polynomial 2°	0.938	47.8	32.2
All assigned RNA average length	Power	0.169	46.9	34.7

RSH = retained spermatid heads, GOF = goodness of fit p-value, BMD_{1SD} = benchmark dose (mg/kg) causing 1 standard deviation in response from the control value, BMDL_{1SD} = lower 95% confidence limit of the benchmark dose. The endpoints are arranged in order of sensitivity, where the least sensitive endpoints with the highest BMD are at the top and the most sensitive endpoint with the lowest BMD is at the bottom. Benchmark dose model fitting was performed on the z-transformed data for each endpoint.

The BMD is the dose that causes 1 standard deviation in response rate of, and adverse effect relative to, the background response rate of the endpoint. The BMD harmonizes cancer and non-cancer risk assessment approaches (Hogan et al., 2012). The highest BMD resulted from RSH histology with a BMD of 62 mg/kg, whereas the lowest BMD resulted from NGS-derived average read length of all assigned RNAs with a BMD of 47 mg/kg. All assigned read lengths not only generated the lowest BMD, but also returned the best AUC values for each exposure after discriminant analysis (Figure 5). Taking the NOAEL/LOAEL approach, BMD approach and the discriminant analysis approach together, the small RNA endpoints identify lower points of departure for EGME adverse effects than histology and sperm parameters.

Drawing conclusions from this comparison across points of departure is complex. Normality is difficult to achieve across four exposure levels, and the endpoints measured acted differently across the dose-response curve. Motility acted in all or nothing response resulting in a bimodal distribution, and mRNA fragment percent increasingly skewed the distribution to one side with exposure. Of all the endpoints that were not generated using next generation sequencing, RSH was the only endpoint to qualify as normally distributed for BMD model fitting. This allowed only a limited comparison between molecular and traditional testicular toxicity endpoints. Additional limitations upon interpreting the data resulted from differences in the variability across endpoints. Retained spermatid heads had a 10-fold greater coefficient of variation of the z-transformed data than many of the NGS-based endpoints (Supplementary Table 9). This indicates that the lower BMD values calculated for NGS endpoints may be due to the increased accuracy of their measurement. It remains uncertain why the molecular endpoints resulted in lower BMDs. The lack of corresponding fertility data, embryonic development of offspring, and the use of a single model toxicant indicate that there is a limited application for molecular biomarkers at this time. However, the results in this manuscript indicate that small RNA in sperm change comparably to traditional endpoints in the EGME model, encouraging future research into such molecular biomarkers of testicular toxicity.

It is important to investigate further whether molecular changes occur at low doses of exposure for understanding male reproductive toxicity. Strong evidence exists of apoptosis of pachytene spermatocytes at high doses of EGME exposure in the rat (Ku et al., 1995). Lower dose exposures may initiate the mechanism of EGME toxicity, however, the cell could remain

viable and maintain the capacity to differentiate into sperm carrying altered small RNAs resulting from exposure. Apoptosis of germ cells is a common response to a number of testicular stressors (reviewed in Boekelheide, 2005; Richburg, 2000). Low doses of other testicular toxicants have been shown to result in abnormal sperm, carrying altered molecular contents. For example, X-irradiation is a testicular injury model that targets spermatogonia and spermatocytes for apoptosis (Henriksen et al., 1996). Apoptosis is a result of DNA damage in these cells after exposure. Abnormal chromatin structure in sperm is found after low doses of X-irradiation (Sailer et al., 1995). It is clear that meaningful toxic effects can occur at low exposure levels that are insufficient to produce germ cell death.

The highly sensitive changes occurring in the tRFs and piRNAs within sperm following EGME-induced testicular toxicity raises concern regarding the preconception exposure window. In mouse models of stress and metabolic disease, tRFs in sperm are likely the drivers of, or play an integral role in, epigenetic inheritance from sperm (Chen et al., 2016; Gapp et al., 2014). The tRFs interfere with mRNA synthesis directly in the proximal regulatory regions of the gene (Chen et al., 2016) and repress transposable element-driven genes (Sharma et al., 2016) that are necessary for early embryogenesis. The piRNAs also control transposable elements in the germ line (Newkirk et al., 2017); however, they coordinate suppression of transposable elements through DNA methylation and histone modification (reviewed in Peng and Lin, 2013). Whether the EGME-induced changes seen here lead to subsequent embryonic effects is unknown. The effects of other male reproductive toxicants on embryonic development have been explored. Phthalate metabolites, measured in urine of male clinical subjects, show modest associations with blastocyst quality in assessment of in vitro fertilization (Wu et al., 2017). The results taken together indicate that low-dose EMGE exposures could pose a preconception threat to offspring of exposed fathers.

We conclude that small RNAs in sperm are useful biomarkers of EGME-induced testicular toxicity. In this study, the total amount of RNA is increased (Figure 3), and the mRNA fragment population is increased (Table 2). These results relate to previous work that indicate specific mRNAs in sperm are changed after exposure to other model testicular toxicants such as 2, 5-hexanedione, cyclophosphamide, and carbendazim (Dere et al., 2016; Pacheco et al., 2012). Future studies will test whether specific mRNA fragments are changed in response to EGME, and determine common mechanisms that alter RNA content in sperm across testicular toxicants.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

DECLARATION OF CONFLICTING INTERESTS

K. Boekelheide is an occasional expert consultant for chemical and pharmaceutical companies and owns stock in ExxonMobil. K. Boekelheide and S.J. Hall own stock in Semma Therapeutics, an early stage biotechnology company developing a therapeutic for type 1 diabetes. These activities are unrelated to the current work but are mentioned in the spirit of full disclosure.

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