miRNA Profile Assessment of Urine Exosomes from Boric Acid Treated Rats as Potential Biomarkers for Testicular Toxicity

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Abstract

The objective of this study is to identify miRNAs in urine specific to boric acid (BA) related reproductive effects in rats. Urine samples were evaluated to identify differentially expressed testicular-specific miRNAs in BA treated rats using next-generation sequencing (NGS). Boric acid was administered to male Sprague Dawley rats via oral gavage at a dose level of 500 mg BA/kg bw/day for 28 days, a dose level known to produce fertility effects in male rats with minimal overt signs of toxicity. At the end of 28-days urine was collected over 24 hours. Urine samples were shipped to QIAGEN Genomic Services for exosomes isolation and miRNA analysis. Histopathology was conducted on testes and epididymis confirming BA treatment related effects. Test substancerelated findings included lower epididymis weights, smaller epididymides, and microscopic findings of cellular debris and decreased spermatid cellularity in the epididymis and tubular degeneration/atrophy and atypical residual bodies in the testis. Tubular degeneration/atrophy in the test substance-treated group was characterized by decreased numbers of germ cells with degeneration of spermatocytes and spermatids. Urine exosomes and miRNA were isolated with QIAseq 52 Spike-ins through exoRNeasy. miRNA sequencing was performed using an Illumina NextSeq500. The miRNA library preparation was completed using the QIAseq miRNA Library Kit. Several miRNAs were identified in rat urine as possible biomarkers for BA related effects on male reproductive system: miR-34c-5p, miR-449a-5p and miR-122-5p were decreased in BA treated rats compared to untreated controls. These miRNAs have also been identified to be decreased in humans with sertoli cell related spermatogenic failure. BA has been shown to affect Sertoli Cells in the testes of rats. Additionally, let-7-5p (decreased), mir-141-3p (increased) and miR21-5p (increased) were differentially expressed in BA treated rats. These miRNAs have been identified as potential biomarkers for human male non-obstructive azoospermia. Also, miR-27b-3p levels decreased in BA treated rats shown to be associated with asthenozoospermia in humans. These results provide the basis for the potential application of miRNAs as biomarkers for BA related testicular toxicity and highlight the value of urine exosome miRNA NGS as a discovery tool.

Background

The basis of the current classification of boric acid is developmental and reproductive effects in laboratory animals. However, similar fertility effects have not been seen in highly exposed workers. Extensive evaluations of sperm parameters in highly exposed workers in Turkey and China have demonstrated no effects on male fertility (5-6). The studies included semen analysis, the most sensitive test for testicular toxicity in humans. Workers in boron mining and processing industries represent the maximum possible human exposure. Mechanistic data is needed to justify a change in classification of boric acid from a Repr. 1B to Category 2. Data from these studies will provide in part mechanistic data to support a change in the classification of borates.

Methods and Materials

Boric acid was administered to male Sprague Dawley rats via oral gavage at a dose level of 500 mg/ BA/kg bw/day for 28 days, a dose level known to produce fertility effects in males with minimal overt signs of toxicity. A control group of male Sprague Dawley rats not administered boric acid treated in the same manner with the exception of dosing with boric acid. At the end of 28-days the rats were terminated; urine was collected and sent to QIAGEN Genomic Services for exosomes isolation and miRNA analysis using next-generation sequencing (NGS). Testes and epididymis were weighed, and histopathology was conducted on testes and epididymis to confirm treatment related effects. RNA was isolated from 2.0mL urine with proprietary RNA isolation protocol optimized for serum/plasma (no carrier added). Total RNA was eluted in ultra-low volume. The library preparation was done using the QIAseq miRNA Library Kit (QIAGEN). A total OF 5ul total RNA was converted into microRNA NGS libraries. Adapters containing UMIs were ligated to the RNA. Then RNA was converted to cDNA. The cDNA was amplified using PCR (22 cycles) and during the PCR indices were added. After PCR the samples were purified. Library preparation QC was performed using either Bioanalyzer 2100 (Agilent) or TapeStation 4200 (Agilent). Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar ratios. The library pool(s) were quantified using the qPCR ExiSEQ LNA™ Quant kit (Exiqon). The library pool were then sequenced on a NextSeq500 sequencing instrument according to the manufacturer instructions. Raw data was de-multiplexed and FASTQ files for each sample were generated using the bcl2fastq software (Illumina inc.). FASTQ data were checked using the FastQC tool.

Results

Test substance-related effects in the epididymis consisted of lower epididymis weights, gross observations of small epididymis, and histologic observations of cellular debris and decreased spermatid cellularity. Test substance-related effects in the testis consisted of tubular degeneration/atrophy and atypical residual bodies.

Several miRNAs were identified in rat urine as possible biomarkers for BA related effects on male reproductive system:

- miR-34c-5p, miR-449a-5p and miR-122-5p were decreased in BA treated rats compared to untreated controls. These miRNAs have also been identified to be decreased in humans with sertoli cell related spermatogenic failure. BA has been shown to affect Sertoli Cells in the testes of rats. (1-3)
- let-7-5p (decreased), mir-141-3p (increased) and miR21-5p (increased) were differentially expressed in BA treated rats. These miRNAs have been identified as potential biomarkers for human male non-obstructive azoospermia. (3)
- miR-27b-3p levels decreased in BA treated rats shown to be associated with asthenozoospermia in humans. (4)

Figure 1. Heat map and unsupervised hierarchical clustering by sample and genes were performed on the listed samples using the 50 microRNAs that have the largest coefficient of variation based on TMM counts. Data is based on samples from the EGME, Control, and BoricAcid groups. Based on Normalized TMMs for each microRNA for each sample.

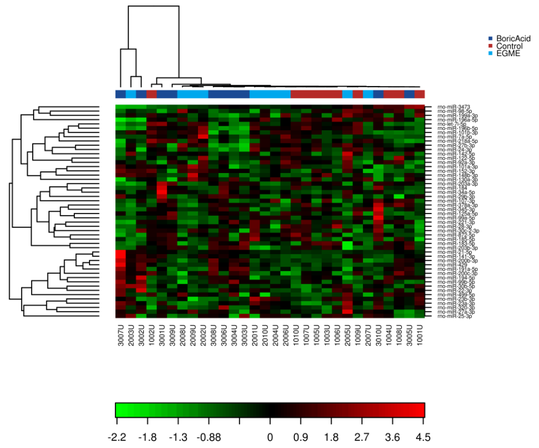


Figure 2. Principal component analysis (PCA) plot for EGME, Control, and BoricAcid. The PCA was performed on all samples passing QC using the 50 microRNA that have the largest coefficient of variation based on TMM counts. Each circle represents a sample.

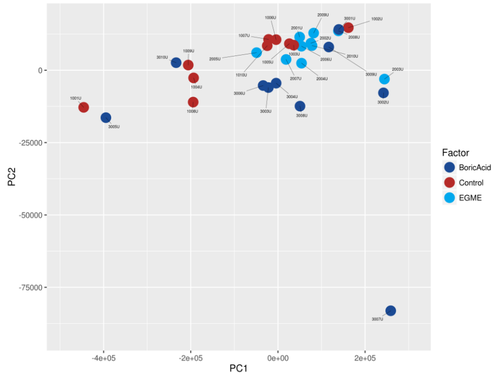
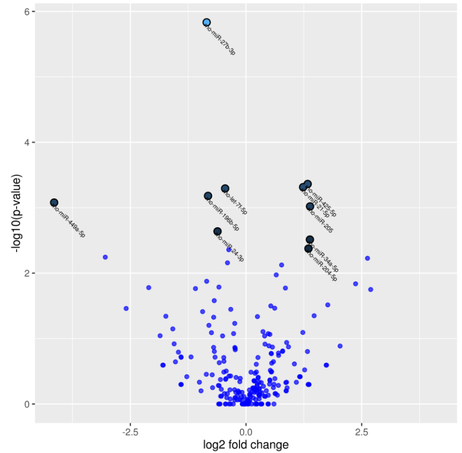


Table 1. 25 most significantly differentially expressed microRNA and annotation, with log fold change (logFC) between groups "BoricAcid" and "Control" raw p-values, Benjamini-Hochberg FDR corrected p-values as well as the average TMM values per group.

Name	LogFC	BoricAcid TMM	Control TMM	P-value	FDR
rno-miR-27b-3p	-0.85	6465	11578	<0.0001	0.0004
rno-miR-425-5p	1.33	568	213	0.0004	0.0337
rno-miR-21-5p	1.23	25379	10793	0.0005	0.0337
rno-let-7f-5p	-0.45	67061	91692	0.0005	0.0337
rno-miR-196b-5p	-0.82	4421	7827	0.0007	0.0349
rno-miR-449a-5p	-4.15	10	357	0.0008	0.0362
rno-miR-205	1.38	540	206	0.0010	0.0362
rno-miR-24-3p	-0.62	2079	3191	0.0023	0.0761
rno-miR-34a-5p	1.38	1461	549	0.0031	0.0903
rno-miR-204-5p	1.35	436	156	0.0042	0.1057
rno-miR-26b-5p	-0.37	18196	23544	0.0044	0.1057
rno-miR-34c-5p	-3.04	55	529	0.0057	0.1208
rno-miR-147	2.63	120	8	0.0059	0.1208
rno-let-7e-5p	-0.40	4968	6572	0.0070	0.1317
rno-miR-429	0.77	6107	3580	0.0075	0.1325
rno-miR-191a-5p	0.65	5339	3406	0.0106	0.1762
rno-miR-98-5p	-0.85	300	574	0.0133	0.2049
rno-miR-324-3p	2.37	98	13	0.0146	0.2049
rno-miR-196a-5p	-0.59	1482	2201	0.0163	0.2049
rno-miR-760-3p	-2.10	19	118	0.0166	0.2049
rno-miR-141-3p	0.85	5139	2852	0.0169	0.2049
rno-miR-142-3p	-1.09	162	357	0.0172	0.2049
rno-let-7b-3p	2.69	64	0	0.0178	0.2049
rno-miR-101b-3p	-0.69	912	1461	0.0263	0.2906
rno-miR-196c-5p	1.77	152	40	0.0306	0.3232

Figure 3. Volcano plot showing the relationship between the p-values and the fold change in normalized expression between the experimental groups "Boric Acid" and "Control". The 10 microRNAs with the lowest p-values are marked with names on the plot.



Conclusion

Several miRNAs were identified in rat urine as possible biomarkers for BA related effects on male reproductive system. miR-34c-5p, miR-449a-5p and miR-122-5p were decreased in BA treated rats compared to untreated controls. These miRNAs have also been identified to be decreased in humans with sertoli cell related spermatogenic failure. BA has been shown to affect Sertoli Cells in the testes of rats. Additionally, let-7-5p (decreased), mir-141-3p (increased) and miR21-5p (increased) were differentially expressed in BA treated rats. These miRNAs have been identified as potential biomarkers for human male non-obstructive azoospermia. miR-27b-3p levels was decreased in BA treated rats shown to be associated with asthenozoospermia in humans. These results provide the basis for the potential application of miRNAs as biomarkers for BA related testicular toxicity and highlight the value of urine exosome miRNA NGS as a discovery tool.

References

(1) Munoz et al (2015) Altered miRNA Signature of Developing Germ-cells in Infertile Patients Relates to the Severity of Spermatogenic Failure and Persists in Spermatozoa. Scientific Reports 5:17991
(2) Wang & Xu (2015). Role of microRNAs in mammalian spermatogenesis and testicular germ cell tumors. Reproduction Review 149:R127-R137.
(3) Kotaga N (2014). MicroRNAs and spermatogenesis. Fertility and Sterility Vol. 101 6:1552-1562.
(4) Zhou et al. (2104). The Expression of Cysteine-Rich Secretory Protein 2 (CRISP2) and Its Specfiic Regulatory miR-27b in the Spermatozoa of Patients with Asthenozoospermia. Biology of Reproduction 92(1):28, 1-9.
(5) Duydu et al. (2011). Reproductive toxicity parameters and biological monitoring in occupationally and environmentally boron-exposed persons in Bandırma, Turkey. Arch Toxicol. 2011 Jun;85(6):589-600.
(6) Scialli et al. (2010). An overview of male reproductive studies of boron with an emphasis on studies of highly exposed Chinese workers. Reproductive Toxicology 29: 10 – 24