Urinary MicroRNA Biomarkers of Nephrotoxicity Demonstrate Different Variability and Directionality in Exosomal Fraction Compared to Whole (Unfractionated) Urine

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Introduction

- MicroRNAs (miRNAs) are small non-coding RNA. They are stable in biofluids.
- They could be promising biomarkers of kidney injury that can complement current protein biomarkers.
- However, miRNA data obtained from unfractionated (whole) urine lacks consistency, especially in human or large animal species.
- The miRNA panel in this study was selected based on pilot data from whole urine which exhibited high animal-to-animal variability in miRNA dynamics as well as low correlation with histopathologic severity.

Hypothesis

We hypothesized that miRNAs measured in whole urine samples may differ in response to a nephrotoxicant compared to miRNAs contained only within the exosomal fraction.

Methodology

Whole urine samples were collected from *Macaca fascicularis* exposed to 200 & 300 µg/kg of test compound with mild to moderate renal toxicity confirmed by histopathology.

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Exosomes were isolated by ultracentrifugation and counted and sized using nanoparticle tracking analysis.

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Candidate miRNAs (miRs-210-3p, 423-5p, 143-3p, 130b-3p, 486-5p, 193a-3p) were measured using real-time LNA SYBR Green PCR.

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RT-qPCR data was analyzed using Ro method.

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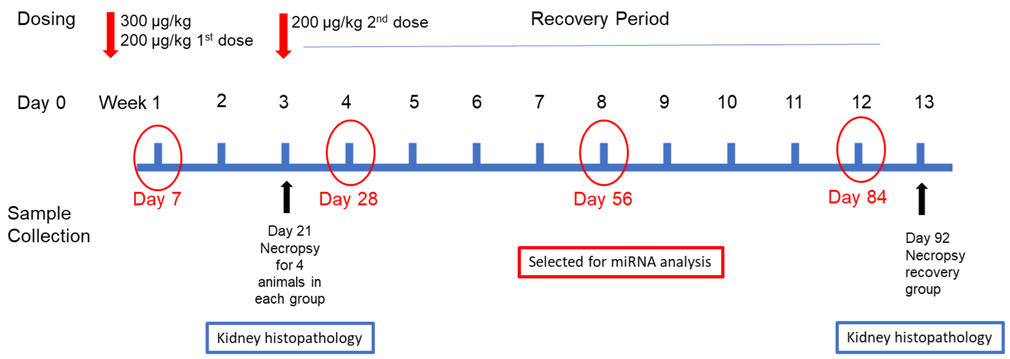
miRNA measurements were normalized to exosome counts. Exosome-normalized and whole-urine PCR samples were compared.

Experimental Design



Results

Necropsy	200ug/mL	300ug/mL
Day 21	Tubular degeneration and single cell necrosis (2/4) - minimal (2/4)	Tubular degeneration and necrosis - minimal (2/4)
Day 92 Recovery	Tubular degeneration - minimal (1/4) - moderate (2/4)	Tubular degeneration and single cell necrosis - Minimal (3/4) Multinucleated cells - minimal (2/4)

Table 1. Summary of renal histopathologic changes in monkeys treated with test article.

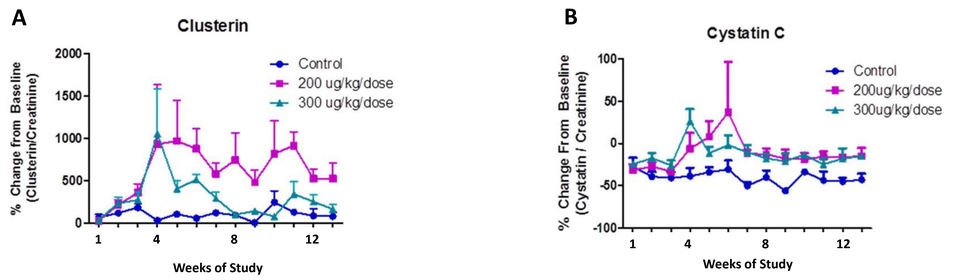


Figure 1. Histopathology and protein biomarker analysis indicates mild to moderate nephrotoxicity due to compound exposure. A) Urinary clusterin was markedly increased in both dose groups on day 28. In 300ug/kg group (single dose) clusterin retuned to control levels by day 56, while in 200µg /kg (2 doses) the increase was sustained through the end of the study. B) Cystatin C was mildly increased in both 200 and 300 µg/kg groups compared to the control starting on day 28 and the increase maintained through the end of the study. **The increase in both clusterin and cystatin C levels correlated with histopathology findings in each dose group.**

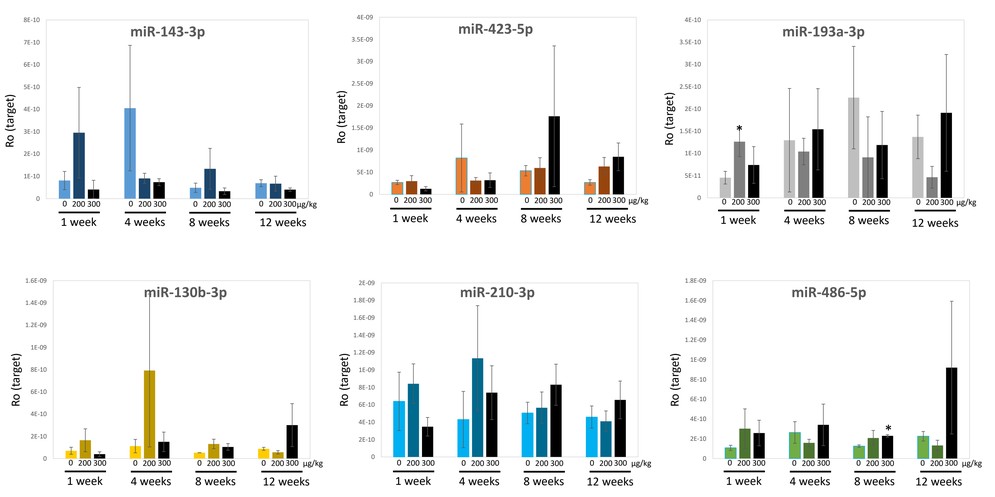


Figure 2. A panel of twenty-six miRNAs were assessed in urine at four time points in the study for both treatment groups. Six of these were selected as candidates due to trending alterations, in some cases significant. Many of these were upward trends and were marked for follow-up for exosomal measurement. Normalization to spike-in small RNAs (geometric mean of cel-miR-39-30, UniSp3 & UniSp6) and/or creatinine levels (mg/dL) did not improve CVs for targets (average for unnormalized data = 76.75; range 65.3-95.8). * = p≤0.05 Student's t-test, one tailed. Error bars = standard error of the mean. n=3.

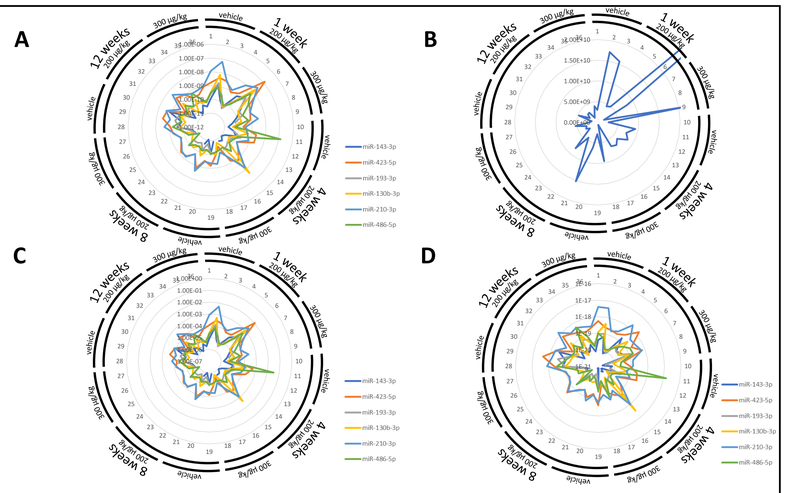


Figure 3. Sample variation is attenuated by normalizing to exosomal count and not spike-in miRNA. A) Calculated miRNA amount in each sample based on PCR amplification curves (Ro or initial fluorescence estimation). Coefficient of variation (CV) across all samples ranged from 194.5-546.4 (average 291.6). B) Exosome/ml counts across all samples. Note high variation in week 1 samples. C) CV values did not improve when miRNA estimation is normalized to spike-in miRNA (range 200.9-582.2; average 345.3). D) CV values are reduced when miRNA estimation is normalized to exosomal count (range 145.9-518.6; average 258.4).

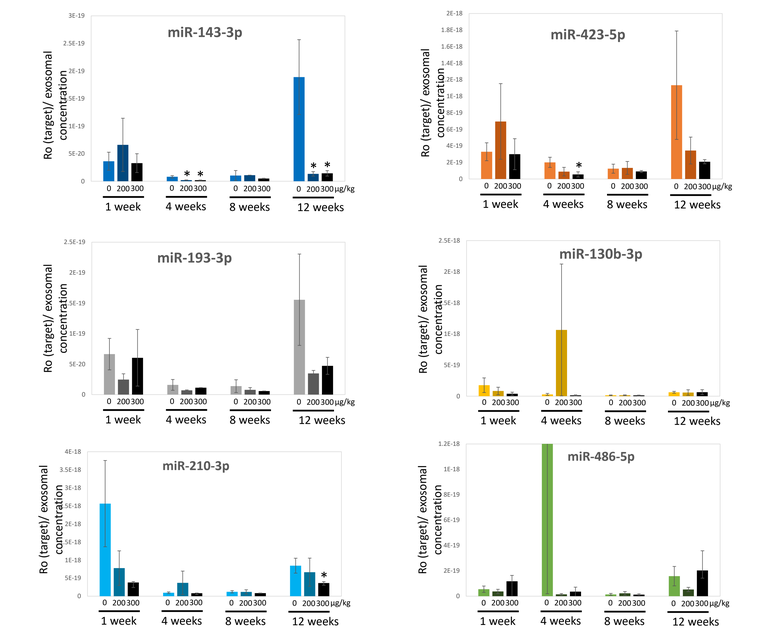


Figure 4. Significant alterations in miRNA observed in urinary exosomal fraction. Exosomal fraction miRNA values normalized to exosomal count. * = p≤0.05 Student's t-test, one tailed. Error bars = standard error of the mean. n=3.

Conclusions

- Normalization of urinary miRNAs to exosome count reduces sample to sample variability, however overall measurements were more variable than those in whole fraction.
- Despite this, we see a greater significant differences from control when measuring exosomal-derived miRNA and in a different direction, indicating an overall *reduction* in signal for some targets.
- The data suggest that exosomal packaged miRNA reflects a different mechanism than those released passively into the urine, thereby serving as different biomarkers of nephrotoxicity.

Next Steps

- Further develop a panel of miRNAs as a biomarker to predict nephrotoxicity
- Mechanistic studies *in vitro*

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