Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery

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Urinary exosomes containing apical membrane and intracellular fluid are normally secreted into the urine from all nephron segments, and may carry protein markers of renal dysfunction and structural injury. We studied methods for collection, storage, and preservation of urinary exosomal proteins. We collected urine from healthy volunteers, added protease inhibitors, and stored urine samples at 4, −20, and −80°C for 1 week or 7 months. Samples were thawed with and without extensive vortexing, and three fractions were isolated: urinary sediment, supernatant, and exosome fraction. Protein concentration, electrophoresis patterns, and abundance of seven exosome-associated proteins were measured. Exosome-associated proteins were not detected in sediment or supernatant fractions. Protease inhibitors prevented degradation of exosome-associated proteins. Freezing at −20°C caused a major loss in exosomes compared to fresh urine. In contrast, recovery after freezing at −80°C was almost complete. Extensive vortexing after thawing markedly increased exosome recovery in urine frozen at −20 or −80°C, even if frozen for 7 months. The recovery from first and second morning urine was similar. The abundance of cytosolic exosome-associated proteins did not decrease during long-term storage. We concluded: (1) protease inhibitors are essential for preservation; (2) storage at −80°C with extensive vortexing after thawing maximizes the recovery of urinary exosomes; (3) the difference between first and second morning urine exosome-associated protein was small, suggesting minimal protein degradation in the urinary tract/bladder; (4) urinary exosomes remain intact during long-term storage. These urine collection, storage, and processing conditions may be useful for future biomarker discovery efforts.

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Urine is an ideal non-invasive source of biomarkers to diagnose and classify kidney diseases. New urinary biomarkers will likely help speed the laboratory and clinical development of new treatments for renal diseases.[1] Exosomes containing vesicular membranes and intracellular fluid are normally secreted into the urine from all nephron segments, and contain proteins that may be altered in abundance or physical properties in association with various renal diseases. Pisitkun *et al.*[2] successfully isolated exosomal membrane proteins in fresh human urine by differential centrifugation and demonstrated the presence of several disease-related proteins. A previous study found that urinary Na+/H+ exchanger isoform 3 (NHE3), a typical membrane protein, increases in patients with acute renal failure.[3] Thus, urinary exosomal proteomics may provide an avenue for the discovery of urinary biomarkers useful for early detection of kidney diseases and for monitoring of treatment.[4] However, how to store and preserve urinary exosomes remains unclear. The aim of this study is to clarify effective methods for the collection, storage, and preservation of urinary exosomal proteins.

RESULTS

Effects of protease inhibitors

Samples obtained from healthy individuals were prepared with and without protease inhibitors and processed as shown in Figure 1. Western blot analysis of Na–K–Cl cotransporter isoform 2 showed that the samples without protease inhibitors had no signal or decreased signal compared to the samples with protease inhibitors (Figure 2a and b).

Effects of storage and vortexing

Samples were pooled from three individuals, and then processed in five different ways (see Materials and Methods, Figure 1). Freezing at −20°C caused a major loss of exosome-associated protein measured by bicinchoninic acid protein assay (27.4% recovery compared to urine stored at 4°C). In contrast, the recovery after freezing at −80°C was 86%. However, extensive vortexing after thawing resulted in 87.4% and 100% recovery in urine frozen at −20°C and −80°C, respectively (Figure 3a). These changes in exosome-associated protein were verified using gel electrophoresis and

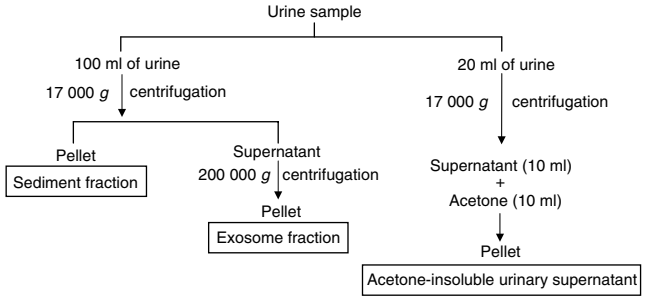

Figure 1 | The isolation of three urinary fractions. Urinary sediment (17 000 *g* pellet), exosome fraction (200 000 *g* pellet), and acetone insoluble supernatant (17 000 *g* supernatant precipitated by acetone) were isolated as described in Materials and Methods.

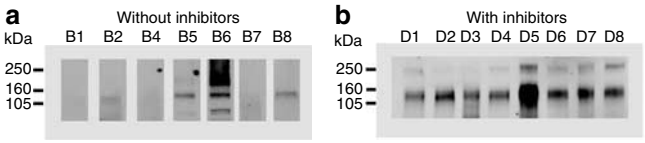

Figure 2 | The effect of protease inhibitors. Eight fresh urine samples were collected (**a**) without and (**b**) with protease inhibitors, and exosome fractions were prepared and evaluated by Western blotting for Na–K–Cl cotransporter isoform 2. Sample loading was normalized by urine creatinine. B1–B8 and D1–D8 represent two sets of different volunteers.

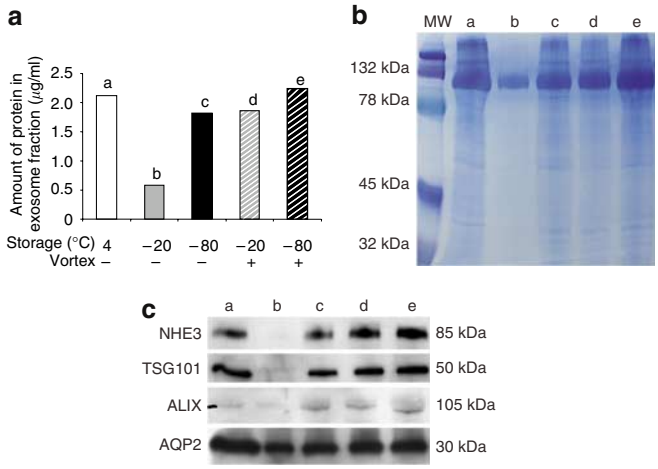

Figure 3 | The effect of storage and vortexing. Samples were pooled from three individuals. (**a**) Amount of urinary exosome-associated protein (200 000 *g* pellet), (**b**) Coomassie blue-stained gel of equal fraction volume of protein, (**c**) Western blot (10 μl aliquot) for NHE3, TSG101, ALIX, and AQP2. a, b, and c: urine samples stored at 4, −20, and −80°C without vortexing; d and e: urine samples stored at −20 and −80°C with vortexing after thawing.

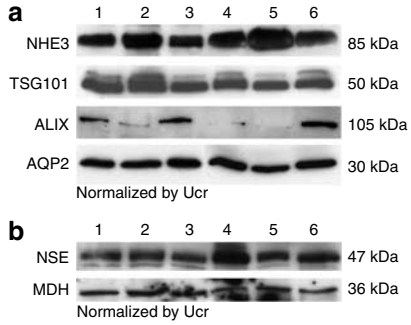

Figure 4 | Effect of long-term storage. (**a**) Western blot of NHE3, TSG101, ALIX, and AQP2 abundance in exosome fraction normalized by urine creatinine from 10 ml freshly collected urine samples (lanes 1–3) or after long-term storage (−80°C for 7 months; lanes 4–6) from three different individuals. Lanes 1 and 4, lanes 2 and 5, lanes 3 and 6 are from the same volunteer. (**b**) Western blot of cytosolic exosome-associated proteins (NSE and MDH) normalized by urine creatinine in fresh (lanes 1–3) and long-term stored (−80°C for 7 months; lanes 4–6) urine samples from three different individuals. Lanes as in (**a**).

Coommassie-blue staining (Figure 3b). Western blot analysis showed that normal urine had easily detectable amounts of NHE3, tumor susceptibility gene (TSG101), apoptosis-linked gene-2 interacting protein X (ALIX), and aquaporin 2 (AQP2). In contrast, NHE3, TSG101, and ALIX could not be detected, and less AQP2 was present in urine samples frozen at −20°C without vortexing after thawing. Freezing at −80°C preserved almost all of the specific urinary exosome-associated proteins. Extensive vortexing resulted in the complete recovery of the above four specific urinary exosome-associated proteins in pooled urine samples after freezing at either −20°C or −80°C (Figure 3c).

To test if the exosme fraction could be isolated from clinically relevant samples, we examined abundance of these four specific urinary exosome-associated proteins in a much smaller volume (10 ml) of fresh first morning urine from three individual volunteers. Samples were normalized by urinary creatinine (Ucr). We found that 10 ml of urine was sufficient to detect these four specific urinary exosome-associated proteins by Western blot analysis (Figure 4a lanes 1–3).

To determine the effect of long-term storage on the recovery of the urinary exosome fraction, we examined the abundance of the above four specific urinary exosome-associated proteins (normalized by Ucr) in the individual urine samples stored at −80°C for 7 months. The results showed that extensive vortexing also could result in almost complete recovery of the above four specific urinary exosomes compared to fresh urine samples (Figure 4a lanes 4–6). However, recovery of ALIX showed some variablity in individual urine samples (Figure 4a).

To investigate whether storage at −80°C can cause urinary exosomes to rupture or remain intact, we examined the abundance of two cytosolic proteins, neuron-specific enolase (NSE) and malate dehydrogenase (MDH), which were isolated from the exosome fraction in fresh urine and frozen urine (−80°C for 7 months) from three individual volunteers. We hypothesized that cytosolic (internal) exosome-associated proteins would not be detected if urinary exosomes were damaged by prolonged freezing. We found no difference in the abundance of NSE and MDH in urine stored at −80°C (for 7 months) compared to the fresh urine samples (Figure 4b).