Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses

Putita Saetun, Tistaya Semangoen, and Visith Thongboonkerd

1Medical Proteomics Unit, Office for Research and Development, and 2Department of Immunology and Immunology Graduate Program, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

Submitted 10 December 2008; accepted in final form 26 March 2009

Saetun P, Semangoen T, Thongboonkerd V. Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses. Am J Physiol Renal Physiol 296: F1346–F1354, 2009. First published April 1, 2009; doi:10.1152/ajprenal.90736.2008.—One of the obstacles in analyzing frozen urine samples is the formation of uncharacterized precipitates. Frequently, these precipitates are discarded before analysis. Some laboratory data may be erroneous if these precipitates contain important compounds. In the present study, we examined urinary sediments precipitated after overnight storage at −20°C. Although cells and debris were removed before freezing, the precipitates remained, whereas storing the centrifuged urine overnight at 4°C did not result in precipitate formation. There were no significant differences observed among 10 healthy individuals (5 men and 5 women). EDTA (5 mM) could efficiently reduce the amount of precipitates to ~25% of the initial amount. The addition of exogenous CaCl2, but not sodium oxalate and NaCl, significantly increased the amount of precipitates in a dose-dependent manner. Linear regression analysis revealed a significant correlation between endogenous urinary calcium level and the amount of precipitates. Frequently, these precipitates are discarded before analysis. Some laboratory data may be erroneous if these precipitates contain important compounds. In the present study, we therefore characterized urinary sediments precipitated after freezing and demonstrated their effects on urinary protein and chemical analyses.

MATERIALS AND METHODS

Urine collection, removal of cells/debris, and storage. Random midstream urine specimens were collected from 10 normal healthy individuals (5 men and 5 women) who were 25–30 yr old and had neither recent illness nor medication. All of the women had no menstrual cycle at the time of collection. The study was approved by the Institutional Ethical Committee. Each urine sample was then divided into many aliquots (40 ml each). Two aliquots were centrifuged, and one was immediately stored at −20°C. Other aliquots were centrifuged at 1,000 g for 5 min to remove cells and debris before storage at −20°C. One of the centrifuged aliquots and another uncentrifuged aliquot were pooled with the corresponding aliquots obtained from other subjects to make the centrifuged and uncentrifuged urine pools, respectively. These pooled samples were then further divided into several 40-ml subaliquots before storage at −20°C.

Examination of the freezer-induced urinary sediments by phase-contrast microscopy. After overnight storage at −20°C, the samples were thawed at room temperature (RT). The flocculated sediments were isolated by centrifugation at 2,000 g for 5 min. Thereafter, the sediments were examined and imaged using a phase-contrast microscope (Olympus CKX41, Olympus, Tokyo, Japan).

Effect of EDTA on type and quantity of the freezer-induced urinary sediments. To determine the type of crystal components in urinary sediments, EDTA, a common chelator of divalent cations, was employed. After overnight storage at −20°C, the centrifuged urine samples were thawed at RT and the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min. One milliliter of 5 mM EDTA or dH2O was then added to the sediments. After mixing, the remaining sediments were isolated by centrifugation at 2,000 g for 5 min, and were then open-air dried, weighed, and examined under a phase-contrast microscope (Olympus).

Effect of exogenous (additional) calcium on the amount of the freezer-induced urinary sediments. Calcium chloride (CaCl2) was added to the centrifuged urine samples obtained from all 10 individuals to make the final concentrations of 0, 12.5, 25, 50, 100, and 200 mg/dl.
overnight storage at \(-20^\circ\text{C}\) overnight. Thereafter, the samples were thawed at RT and the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min, open-air dried, and weighed. In parallel, sodium oxalate (NaOx; at final concentrations of 0, 1.25, 2.5, 5, 10 and 20 mM) and sodium chloride (NaCl; at final concentrations of 0, 12.5, 25, 50, 100 and 200 mM) were also challenged to the individual centrifuged urine samples. Note that the concentrations of NaOx added were 10 times less than those of CaCl\(_2\) because the oxalate level in the normal urine was about one-tenth of the normal urinary calcium level (22).

Correlation of endogenous urinary calcium concentration and the amount of the freezer-induced urinary sediments. The individual centrifuged urine samples (40 ml each) were collected from 10 healthy subjects. Before storage, 1-ml aliquots were taken from individual samples to measure urinary calcium levels by colorimetric assay using a Roche/Hitachi analyzer (Roche Diagnostics, Indianapolis, IN), whereas the remaining samples were stored at \(-20^\circ\text{C}\) overnight. After a complete thaw at RT, the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min, open-air dried, and weighed. Correlation of endogenous urinary calcium concentrations and the amounts of the freezer-induced urinary sediments was determined by linear regression analysis.

Effect of temperature on precipitation of urinary sediments. The pooled uncentrifuged and pooled centrifuged urine samples were stored at either \(-20\) or \(4^\circ\text{C}\) overnight. After a complete thaw at RT, the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min. Thereafter, the sediments were examined and imaged using a phase-contrast microscope (Olympus).

Effect of pH on precipitation of urinary sediments. The pH of individual centrifuged urine samples was adjusted by either HCl or NaOH to make their final pH levels of 5.0, 5.8, 6.6, 7.4, and 8.2 before overnight storage at \(-20^\circ\text{C}\) (the control was the unadjusted sample). After a complete thaw at RT, the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min, open-air dried, and weighed. In parallel, the pH of the pooled centrifuged urine samples was also adjusted as for the individual samples. After pH adjustment and freeze/thaw processes, the urinary sediments were isolated and examined under a phase-contrast microscope (Olympus).

Determination of depletion of proteins and calcium in the urine by the freezer-induced urinary sediments. To determine the loss of urinary proteins, two pooled centrifuged urine samples were stored at \(-20^\circ\text{C}\) overnight. After a complete thaw at RT, one sample was fractionated by centrifugation at 2,000 g for 5 min, and the supernatant was isolated from the sediment-enriched fraction. The other (unfractionated) sample was vigorously shaken and vortexed until the flocculated sediments were dissolved. In addition, a pooled urine sample stored at 4°C (without sediments) served as the control. Proteins in the control, supernatant, sediment-enriched fraction, and unfractionated sample after vigorous shaking (1 ml each) were then precipitated by 75% ethanol. The protein pellets were isolated by centrifugation at 12,000 g for 5 min and resuspended in 30 μl of 1× Laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromphenol blue, and 0.0625 M Tris-HCl). The recovered proteins were then simultaneously resolved in 12% SDS-PAGE using SE260 Mini-Vertical Electrophoresis Unit (GE Healthcare, Uppsala, Sweden) with the current of 20 μA/gel for 2 h. The resolved proteins were finally visualized by Coomassie brilliant blue G-250 stain (Fluka Chemica, Buchs, Switzerland). Band intensity was measured by ImageQuant TL software (GE Healthcare). Selected bands were excised and identified by quadrupole time-of-flight mass spectrometry as previously described (30, 33).

To determine the loss of urinary calcium, other two pooled centrifuged urine samples were stored at either 4 or \(-20^\circ\text{C}\). After overnight storage, the samples kept at 4°C remained clear, whereas the frozen sample had the flocculated sediments. After isolation of these sediments, the supernatant was saved and measured for total calcium level compared with the nonfrozen sample, based on a colorimetric assay using the Roche/Hitachi analyzer (Roche Diagnostics).

Statistical analyses. All statistical analyses were performed using SPSS software (version 13.0). The change in amounts of urinary sediments by EDTA and depletion of urinary calcium by the freezer-induced urinary sediments were determined using a Mann-Whitney test. Effects of CaCl\(_2\), NaOx, NaCl, and pH were examined using ANOVA with Tukey’s post hoc multiple comparisons test. Distribution of the data was evaluated by a Kolmogorov-Smirnov test. Correlation of urinary calcium concentration and the amount of the

Fig. 1. Sediments in individual uncentrifuged urine samples after overnight storage at \(-20^\circ\text{C}\). Random midstream urine specimens collected from healthy individuals were stored at \(-20^\circ\text{C}\) overnight and then thawed at room temperature (RT). Their urinary flocculated sediments were isolated by centrifugation at 2,000 g for 5 min and imaged under a phase-contrast microscope. Original magnification power was \(\times 100\) for all panels.
freezer-induced urinary sediments was determined by linear regression analysis. \( P \) values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Urine samples almost always had the flocculated sediments after being frozen overnight or longer. Figure 1 shows that the uncentrifuged urine samples obtained from individual healthy subjects had a similar pattern and type of the freezer-induced urinary sediments, which were composed mainly of calcium oxalate dihydrate (COD) crystals with typical bipyramidal shape (32, 34, 35), amorphous crystals that were much larger than COD crystals, bladder epithelial cells, and debris. Our results were consistent with findings obtained from routine urinalysis, which commonly reveals “microscopic sediments” including bladder epithelial cells, crystals, and physiological casts in the normal urine, whereas pathological casts, renal tubular epithelial cells, erythrocytes, and leukocytes are found in the diseased urine (4). Additionally, the most common type of calcium-containing crystals found in normal urine is COD (11, 14), which is also associated with hypercalciuria, but not with hyperoxaluria in which the predominate crystal type is calcium oxalate monohydrate (COM) (8). However, overnight storage in the freezer yielded a much greater amount of urinary “macroscopic sediments” compared with that of microscopic sediments found in the fresh normal urine.

Because bladder epithelial cells and debris were accompanied by crystal components, we thus examined whether removal of cells and debris could prevent the formation of freezer-induced urinary sediments. Figure 2 shows that although cells and debris were eliminated before freezing, typical COD and amorphous crystals remained in the centrifuged urine, indicating that the freezer-induced urinary sediments were composed mainly of crystals and did not depend on cells and debris.

Fig. 3. Effect of EDTA on type and quantity of the freezer-induced urinary sediments. Two 40-ml aliquots of the centrifuged urine pool were stored at \(-20^\circ\text{C}\) overnight. After a complete thaw at RT, the flocculated urinary sediments were isolated by centrifugation at 2,000 \( g \) for 5 min. Thereafter, the sediments isolated from the first aliquot were added with 1 ml of \( \text{dI water (A)} \), whereas those isolated from the second were added with 1 ml of 5 mM EDTA (B). After mixing, the urinary sediments were isolated by centrifugation at 2,000 \( g \) for 5 min and were then open-air dried, weighed, and examined under a phase-contrast microscope. Original magnification \( \times 400 \) for both panels.
Fig. 4. Effect of exogenous (additional) calcium on the amount of the freezer-induced urinary sediments. Before overnight storage at −20°C, the individual centrifuged urine samples (40 ml each) were added with various concentrations of CaCl₂ (A), sodium oxalate (NaOx; B), or NaCl (C). ANOVA with Tukey’s post hoc multiple comparisons was performed to compare the amounts of sediments obtained after overnight storage at −20°C (Kolmogorov-Smirnov test confirmed the normal distribution of these data before ANOVA). Significant P values are in bold and underlined. Data are reported as means ± SD (n = 10 individual samples for each bar).
To characterize the huge amorphous crystals (with a size >20 times the size of COD) predominant in the samples, we added EDTA to the sediments and examined whether EDTA could affect the type and amount of the freezer-induced urinary sediments. Our data demonstrated that EDTA could eliminate both COD and huge amorphous crystals (Fig. 3, A and B). In addition, EDTA could efficiently reduce the amount of these freezer-induced sediments (to ~25% of the initial amount) (Fig. 3C). EDTA is a well-known chelator for divalent cations (i.e., Ca$^{2+}$ and Mg$^{2+}$) and has been used widely in treatment of some diseases related to the excess of these cations (20, 23). These data therefore suggested that the huge amorphous crystals were indeed (non-oxalate) calcium-containing crystals.

To confirm that the majority of the freezer-induced urinary sediments were calcium-containing crystals, we examined the effect of exogenous or additional calcium on the amount of these sediments. Figure 4A shows that the addition of exogenous CaCl$_2$ significantly increased the amount of the freezer-induced sediments in a dose-dependent manner, whereas the addition of exogenous NaOx had no effect on the amount of such sediments (Fig. 4B). This implied that the majority of crystals consumed most of free calcium ions in the urine. Therefore, the addition of exogenous calcium could increase the formation of calcium-containing crystals (both COD and amorphous types), whereas the addition of exogenous NaOx could not enhance the formation of COD because of an insufficiency of free calcium ions left in the urine.

An argument might be made that the concentrations of NaOx used were much less (~one-tenth) than those of CaCl$_2$. We intentionally used this series of NaOx concentrations in relation to the normal level of urinary oxalate, which is usually one-tenth of the normal urinary calcium level (22). CaCl$_2$ was added to the urine samples by varying concentrations from 12.5 to 200 mM, which were 2.5-40 times greater than the upper limit of the normal urinary calcium level (~5 mM) (22). Normally, oxalate concentration in serum is very low (~2 μM), whereas its concentration progressively increases in the nephron and reaches the critical level for supersaturation (~0.1–0.5 mM) somewhere between distal renal tubules and collecting ducts (21). Oxalate is excreted into the urine with a very small amount in healthy subjects (0.1–0.45 mmol/day). Its excretion is slightly more in idiopathic CaOx stone formers (0.45–0.65 mmol/day) and is significantly greater in patients

---

**Fig. 5.** Correlation of endogenous urinary calcium concentration and the amount of the freezer-induced urinary sediments. Before storage, 1 ml of 40-ml aliquots of the individual centrifuged urine samples was taken to measure urinary calcium levels, whereas the remaining samples were stored at $-20°C$ overnight. After a complete thaw at RT, the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min, open-air dried, and weighed. Correlation of endogenous urinary calcium concentrations and the amounts of the freezer-induced urinary sediments was determined by linear regression analysis.

**Fig. 6.** Effect of temperature on precipitation of urinary sediments. The uncentrifuged (A and B) and centrifuged (C and D) urine pools were stored at 4 or $-20°C$ overnight. After all the samples were left at RT until the frozen samples were completely thawed, the urinary sediments were isolated by centrifugation at 2,000 g for 5 min and examined under a phase-contrast microscope.
with primary hyperoxaluria (1–3.5 mmol/day) (21). In our present study, oxalate ions (in NaOx) were added to the urine by varying concentrations from 1.25 to 20 mM, which were 2.5–40 times greater than the upper limit of its normal level in the urine (~0.5 mM) (21). Therefore, we strongly believed that the concentrations of NaOx used in our study, which were one-tenth lower than those of CaCl₂ but equivalent to 2.5–40 times greater than their respective normal values, were justified. Moreover, we also confirmed that the increased amounts of the freezer-induced urinary sediments were significantly associated with an increment of calcium, not chloride, as the addition of NaCl had no effect at all on the amount of such sediments (Fig. 4C).

We also determined the effect of endogenous urinary calcium levels on the amount of the freezer-induced urinary sediments. Figure 5 illustrates that urinary calcium concentrations (normally < 35 mg/dl) (36) in 10 healthy individuals were significantly correlated with the amounts of the freezer-

![Graph showing the effect of pH on precipitation of urinary sediments.](http://ajprenal.org)

**Fig. 7.** Effect of pH on precipitation of urinary sediments. The pH in the pooled (A) and individual (B) centrifuged urine samples were adjusted by either HCl or NaOH to make their final pH levels 5.0, 5.8, 6.6, 7.4, and 8.2 before overnight storage at −20°C (the control was the unadjusted sample). After a complete thaw at RT, the flocculated sediments were isolated by centrifugation at 2,000 g for 5 min and were then open-air dried, weighed, and examined under a phase-contrast microscope. For quantitative analysis of individual samples in B, ANOVA with Tukey’s post hoc multiple comparisons were performed (Kolmogorov-Smirnov test confirmed the normal distribution of these data before ANOVA). The data are reported as means ± SD (n = 10 individual samples for each bar).
induced urinary sediments. Linear regression analysis revealed Pearson’s correlation coefficient (r) of 0.894 (P < 0.001) for this correlation. Taken together, our data indicated that the freezer-induced urinary sediments were mainly calcium-containing crystals (both COD and amorphous types).

The effect of temperature on precipitation of urinary sediments was also evaluated. Figure 6 demonstrates that while there were typical COD and amorphous calcium crystals present in the uncentrifuged and centrifuged samples kept at −20°C, these crystals were not found in the samples kept at 4°C (only cells and debris were observed in the uncentrifuged urine, whereas there were no sediments found in the centrifuged sample). In addition, there was no difference observed in the samples stored at −20 vs. −70°C (data not shown). Moreover, vigorous shaking of the sample at RT could redissolve these precipitates. It should be emphasized that although the centrifuged samples stored at 4°C were not accompanied by such sediments, they could not be archived or stored for a long period due to a problem of bacterial contaminations (31).

The normal urine pH is ~5.0–7.0. The effect of pH on precipitation of urinary sediments was also evaluated. Figure 7 shows that adjustment of the urine pH had some effects on the type of the freezer-induced urinary sediments. COD crystals were preferably crystallized at a pH of ~5.8–6.6, whereas the amorphous calcium crystals were preferentially crystallized at other pH ranges. Our data were in accordance with the findings reported in a previous study, which indicated that COD was predominately in the normal urine and preferentially crystallized at the pH range of <5.4–6.7 (14). Moreover, the pH at 5.8 and 7.4 tended to have slightly smaller amounts of the freezer-induced urinary sediments.

Normal urine generally contains a small amount of proteins (<100 mg/day) (28). Several previous studies have reported that some proteins in the normal human urine can modulate calcium oxalate crystal nucleation, growth, and aggregation (18, 27, 39). Currently known inhibitory proteins for calcium oxalate crystals include nephrocalcin (6, 7, 24), Tamm-Horsfall protein (16, 17), uropontin (27), inter-α-trypsin inhibitor (bikunin) (2), urinary prothrombin fragment 1 (crystal matrix protein) (26), and trefoil factor 1 (TFF1) (5, 4). These modulators have similar physicochemical properties as they are mostly anionic proteins that can bind to free calcium ions (5, 19). We therefore examined whether the formation of the freezer-induced urinary sediments, which were composed mainly of COD and amorphous calcium crystals, could affect the amount of urinary proteins and/or urinary calcium levels. SDS-PAGE analysis of urinary proteins revealed that the freezer-induced urinary sediments entrapped a considerable amount of urinary proteins and diminished the amount of total protein in the urine supernatant (Fig. 8A). Some of these entrapped proteins were identified by quadrupole time-of-flight mass spectrometry (Table 1). Quantitative band intensity analysis also revealed the decreased levels of these identified proteins, which had recovered to basal (control) levels after vigorous shaking to redissolve the freezer-induced urinary sediments (Table 1 and Fig. 8A). Two of the entrapped proteins were identified as Tamm-Horsfall protein and bikunin. Our data confirmed that the stone-modulating proteins could be depleted by the freezer-induced precipitation. Another entrapped protein was identified as albumin. Our data were consistent with the findings in previous studies, which reported that urinary concentrations of albumin (12) and other proteins (10) were significantly decreased after the freezing of urine at −20°C due to an entrapment of albumin and other proteins in the precipitates. Moreover, these sediments significantly reduced urinary calcium levels by 23.6 ± 1.1% (P < 0.001) (Fig. 8B). These data therefore indicated that the freezer-induced urinary sediments significantly affect urinary protein and chemical
analyses. Moreover, depletion of calcium ions and calcium-containing crystals in the freezer-induced urinary sediments would definitely interfere with analysis of the stone type in nephrolithiasis/urolithiasis patients (1, 8).

In summary, our study reports for the first time characterizations of the freezer-induced urinary sediments. The majority of these sediments included COD and amorphous calcium crystals, whereas bladder epithelial cells and debris were found only in uncentrifuged urine. Moreover, our findings clearly indicate that these sediments could significantly affect routine analyses of the urine (i.e., measurement of urinary protein and calcium levels). Therefore, these freezer-induced precipitates must be taken into account for analyses of frozen urine, and the vigorous shaking of the samples after a complete thaw to redissolve these sediments before analyses is highly recommended.

ACKNOWLEDGMENTS

We thank Wararat Chiangjong for handful assistance and are grateful to Prof. Shui-Tein Chen and Dr. Supachok Sinchaiku for mass spectrometric analysis.

GRANTS

This study was supported by The Thailand Research Fund, Commission on Higher Education, Mahidol University, the National Research Council of Thailand, Siriraj Grant for Research and Development, and the National Center for Genetic Engineering and Biotechnology (to V. Thongboonkerd) and by a Siriraj Graduate Thesis Scholarship (to T. Semangoen).

REFERENCES


Table 1. Quantitative band intensity analysis and protein identification

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Protein Name</th>
<th>NCBI ID</th>
<th>Method of Identification</th>
<th>Identification Score (MS, MS/MS)</th>
<th>%Coverage (MS, MS/MS)</th>
<th>No. of Matching Peptides (MS, MS/MS)</th>
<th>Supernatant/ control</th>
<th>Sediment/ control</th>
<th>Band Intensity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uromodulin</td>
<td>gi 119570700</td>
<td>MS, MS/MS</td>
<td>66, 135</td>
<td>21, 8</td>
<td>12, 5</td>
<td>0.41</td>
<td>3.19</td>
<td>0.96</td>
</tr>
<tr>
<td>2</td>
<td>Albumin</td>
<td>gi 3212456</td>
<td>MS, MS/MS</td>
<td>148, 66</td>
<td>35, 4</td>
<td>18, 3</td>
<td>0.52</td>
<td>1.45</td>
<td>1.12</td>
</tr>
<tr>
<td>3</td>
<td>Bikunin/α-microglobulin</td>
<td>gi 579676</td>
<td>MS/MS</td>
<td>NA, 65</td>
<td>NA, 15</td>
<td>NA, 2</td>
<td>0.52</td>
<td>3.13</td>
<td>0.95</td>
</tr>
</tbody>
</table>

NCBI, National Center for Biotechnology Information; MS, mass spectrometry; NA, not applicable.