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

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# Equal contributions by these authors

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Abbreviations: COD, calcium oxalate dihydrate; COM, calcium oxalate monohydrate; dI, deionized water; EDTA, ethylenediaminetetraacetic acid; NaOx, sodium oxalate; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFF1, trefoil factor 1

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ABSTRACT

One of the obstacles in analyzing frozen urine samples is the formation of uncharacterized precipitates. Frequently, these precipitates are discarded prior to analysis. Some laboratory data may be erroneous if these precipitates contain important compounds. In the present study, we examined urinary sediments precipitated after an overnight storage at -20°C. Although cells and debris were removed prior to 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 males and 5 females). EDTA (5 mM) could efficiently reduce the amount of precipitates to approximately 25% of the initial amount. The addition of exogenous CaCl₂, but not NaOx 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 ($r=0.894; \ p<0.001$). Urine pH also had some effects on type and amount of precipitates. These precipitates were composed mainly of calcium oxalate dihydrate and amorphous calcium crystals. The results also showed that these precipitates could deplete urinary proteins and calcium ions (23.6±1.1 % decrease). Therefore, these freezer-induced urinary sediments significantly affect protein analysis and measurement of calcium levels in the urine. However, vigorous shaking of the sample at room temperature could re-dissolve these precipitates. Our data strongly indicate that these freezer-induced precipitates must be taken into account when the frozen urine samples are analyzed.
INTRODUCTION

Urinalysis and urine chemistry are fundamental laboratory tests for diagnosis of kidney and related disorders. Extensive studies have been conducted to search for novel non-invasive biomarkers for earlier diagnosis and monitoring therapeutic response in many kidney and other diseases (25). In the post-genomic era, urinary proteomics (9, 13, 15, 25, 28) and metabolomics (3, 37, 38) are commonly employed to search for biomarker molecules in the urine. In such study, simultaneous analysis of a large number of samples is more preferable than analysis of multiple batches of a small number of samples to avoid inter-assay and/or inter-laboratory variations. Therefore, well-designed, prospectively collected urine samples (with appropriate inclusion and exclusion criteria) are most appropriate. Frequently, these samples are stored in the freezer (at -20°C or lower) for a while prior to analyses. However, some studies have dealt with archival urine samples, which have been frozen for a long time.

One of the obstacles in analyzing frozen urine samples is the formation of uncharacterized precipitates, which almost always develop after freezing the samples overnight or for longer. Frequently, these precipitates are discarded prior to any kind of analyses. Unfortunately, there are no data available to explain what these freezer-induced precipitates are and why they always form in the frozen urine. Some laboratory data may be erroneous if these precipitates contain important compounds. In the present study, we therefore characterized urinary sediments precipitated after an overnight storage of the urine samples at -20°C 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 males and 5 females) who were 25-30 years old and had neither recent illness nor medication. All females 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 uncentrifuged and one of them was immediately stored at -20°C. Other aliquots were centrifuged at 1,000 g for 5 min to remove cells and debris prior to 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 sub-aliquots prior to storage at -20°C.

Examination of the Freezer-Induced Urinary Sediments by Phase-Contrast Microscopy

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

Effect of EDTA on Type and Quantity of the Freezer-Induced Urinary Sediments

To determine type of crystal components in urinary sediments, ethylenediaminetetraacetic acid (EDTA), a common chelator of divalent cations, was employed. After an overnight storage at -20°C, the centrifuged urine samples were thawed at RT and the flocculated sediments were isolated by a centrifugation at 2,000 g for 5 min. 1 mL of 5 mM EDTA or dI water was then added to the sediments. After mixing, the remaining
sediments were isolated by a centrifugation at 2,000 g for 5 min, and were then open-air dried, weighed and examined under a phase-contrast microscope (Olympus Co. Ltd.).

**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 mM prior to storage at -20°C overnight. Thereafter, the samples were thawed at RT and the flocculated sediments were isolated by a 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 CaCl₂ because the oxalate level in the normal urine was approximately 1/10 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. Prior to storage, 1-mL aliquots were taken from individual samples to measure urinary calcium levels by colorimetric assay using Roche/Hitachi analyzer (Roche Diagnostics; Indianapolis, IN), whereas the remaining samples were stored at -20°C overnight. After a complete thaw at RT, the flocculated sediments were isolated by a 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°C or 4°C overnight. After a complete thaw at RT, the flocculated sediments were isolated by a centrifugation at 2,000 g for 5 min. Thereafter, the sediments were examined and imaged using a phase-contrast microscope (Olympus Co. Ltd.).

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 to 5.0, 5.8, 6.6, 7.4 and 8.2 prior to an overnight storage at -20°C (the control was the unadjusted sample). After a complete thaw at RT, the flocculated sediments were isolated by a 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 Co. Ltd.).

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°C overnight. After a complete thaw at RT, one sample was fractionated by a centrifugation at 2,000 g for 5 min and the supernatant was isolated from the sediment-enriched fraction. The other (unfractionated) sample was vigorously shaked 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 a centrifugation at 12,000 g for 5 min, and resuspended in 30 μL of 1X Laemmli’s 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 AG, 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°C or -20°C. After an overnight storage, the sample kept at 4°C remained clear, whereas the frozen sample had the flocculated sediments. After an isolation of these sediments, the supernatant was saved and measured for total calcium level compared to the non-frozen sample, based on colorimetric assay using Roche/Hitachi analyzer (Roche Diagnostics).

**Statistical Analyses**

All statistical analyses were performed using SPSS software (version 13.0). Change in amounts of urinary sediments by EDTA and depletion of urinary calcium by the freezer-induced urinary sediments were determined using Mann-Whitney test. Effects of CaCl₂, NaOx, NaCl and pH were examined using ANOVA with Tukey’s post-hoc multiple comparisons. Distribution of the data was evaluated by Kolmogorov-Smirnov test. Correlation of urinary calcium concentration and the amount of the freezer-induced urinary sediments was determined by linear regression analysis. *P* values < 0.05 were considered statistically significant.
RESULTS AND DISCUSSIONS

Urine samples almost always had the flocculated sediments after freezing overnight or for longer. **Figure 1** shows that the uncentrifuged urine samples obtained from individual healthy subjects had 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 the normal urine is COD (11, 14), which is also associated with hypercalciuria, but not with hyperoxaluric in which the predominate crystal type is calcium oxalate monohydrate (COM) (8). However, the overnight storage in the freezer yielded a much greater amount of urinary “macroscopic sediments” as compared to that of “microscopic sediments” found in the fresh normal urine.

Because bladder epithelial cells and debris were accompanied with 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 prior to 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.

To characterize the huge amorphous crystals (with a size of >20 times of the size of COD) predominated 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 (**Figures**
In addition, EDTA could efficiently reduce the amount of these freezer-induced sediments (to approximately 25% of the initial amount) (Figure 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 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 (Figure 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 (approximately 1/10) 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 1/10 of the normal urinary calcium level (22). CaCl$_2$ was added into the urine samples by varying concentrations from 12.5 to 200 mM, which were 2.5 to 40 times greater than the upper limit of the normal urinary calcium level (approximately 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 to 0.45 mmol/day). Its excretion is
slightly more in idiopathic CaOx stone formers (0.45 to 0.65 mmol/day) and is significantly greater in patients with primary hyperoxaluria (1 to 3.5 mmol/day) (21). In our present study, oxalate ions (in NaOx) were added into the urine by varying concentrations from 1.25 to 20 mM, which were 2.5 to 40 times greater than the upper limit of its normal level in the urine (approximately 0.5 mM) (21). Therefore, we strongly believed that the concentrations of NaOx used in our study, which were 1/10 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 on the amount of such sediments at all (**Figure 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-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°C versus at -70°C (data not shown). Moreover, vigorous shaking of the sample at RT could re-dissolve these precipitates. It should be emphasized that although the centrifuged samples stored at 4°C were not
accompanied with 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 approximately 5.0-7.0. 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 the pH of approximately 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 predominate 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. The normal urine generally contains a small amount of proteins (< 100 mg/day) (28). Several of 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, 29). 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 (Figure 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 were then recovered to the basal (control) levels after vigorous shaking to re-dissolve the freezer-induced urinary sediments (Table 1 and Figure 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 freezing the 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) (Figure 8B). These data therefore indicated that the freezer-induced urinary sediments significantly affect urinary protein and chemistry 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/uro lithiasis 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 the 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 the frozen urine and the vigorous shaking of the samples after a complete thaw to re-dissolve these sediments prior to analyses is highly recommended.
ACKNOWLEDGEMENTS

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REFERENCES


Table 1: Quantitative band intensity analysis and protein identification.

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<td>NA, 15</td>
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FIGURE LEGENDS

Figure 1. Sediments in individual uncentrifuged urine samples after an overnight storage at -20°C. Random midstream urine specimens collected from healthy individuals were stored at -20°C overnight and then thawed at RT. Their urinary flocculated sediments were isolated by a centrifugation at 2,000 g for 5 min and imaged under a phase-contrast microscope. Original magnification power was 100X for all panels.

Figure 2. Effect of removal of cells and debris prior to storage at -20°C. The uncentrifuged (A) and centrifuged (1,000 g for 5 min to remove cells and debris) (B) urine pools were compared. After an overnight storage at -20°C, both pools were thawed at RT and their flocculated sediments were isolated by a centrifugation at 2,000 g for 5 min and examined under a phase-contrast microscope. Original magnification power was 400X for both panels.

Figure 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°C overnight. After a complete thaw at RT, the flocculated urinary sediments were isolated by a centrifugation at 2,000 g for 5 min. Thereafter, the sediments isolated from the first aliquot were added with 1 mL of 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 a centrifugation at 2,000 g for 5 min, and were then open-air dried, weighed and examined under a phase-contrast microscope. Original magnification power was 400X for (A) and (B) (N = 3 independent experiments for both panels). The quantitative data in (C) were obtained from individual centrifuged urine samples and are reported as Mean + SD (N = 10 individual samples for each bar).
Figure 4. Effect of exogenous (additional) calcium on the amount of the freezer-induced urinary sediments. Prior to an overnight storage at -20°C, the individual centrifuged urine samples (40 mL each) were added with various concentrations of CaCl$_2$ (A), NaOx (B) or NaCl (C). ANOVA with Tukey’s post-hoc multiple comparisons were performed to compare the amounts of sediments obtained after an overnight storage at -20°C (Kolmogorov-Smirnov test confirmed the normal distribution of these data prior to ANOVA). Significant $p$ values are bolded and underlined. Data are reported as Mean ± SD (N = 10 individual samples for each bar).

Figure 5. Correlation of endogenous urinary calcium concentration and the amount of the freezer-induced urinary sediments. Prior to 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 a 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.

Figure 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°C or -20°C overnight. After leaving all the samples at RT until the frozen samples were completely thawed, the urinary sediments were isolated by a centrifugation at 2,000 g for 5 min and examined under a phase-contrast microscope.

Figure 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 to 5.0, 5.8, 6.6, 7.4 and 8.2 prior to an overnight storage at -20°C (the control was the unadjusted sample). After a complete thaw at RT, the flocculated sediments were isolated by a 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 prior to ANOVA). The data are reported as Mean ± SD (N = 10 individual samples for each bar).

Figure 8. Depletion of proteins and calcium in the urine by the freezer-induced urinary sediments. (A) Two centrifuged urine pools were stored at -20°C overnight. After a complete thaw at RT, one sample was fractionated by a centrifugation at 2,000 g for 5 min and the supernatant was isolated from the sediment-enriched fraction. The other (unfractionated) sample was vigorously shaked 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 precipitated by 75% ethanol, simultaneously resolved in 12% SDS-PAGE and visualized by Coomassie Brilliant Blue G-250 stain. Selected bands (labeled with numbers) were quantified, excised and identified by quadrupole time-of-flight mass spectrometry (see Table 1). (B) Other two centrifuged urine pools were stored at either 4°C or -20°C. After an overnight storage, the sample kept at 4°C remained clear, whereas the frozen sample had the flocculated sediments after a complete thaw at RT. After an isolation of these sediments, the supernatant was saved and measured for total calcium level compared to non-frozen sample. Data are reported as Mean ± SD (N = 3 independent experiments for each bar).
Figure 1

A. Male #1  
B. Male #2  
C. Female #1  
D. Female #2
Figure 2

A. Uncentrifuged pool

B. Centrifuged pool

COD

Amorphous
Figure 3

A. Centrifuged pool (without EDTA)
B. Centrifuged pool (+ 5 mM EDTA)

C. Graph showing weight (g) comparison between Without EDTA and + 5 mM EDTA. The graph indicates a statistically significant difference with **p < 0.001.
Figure 4

**A**

![Graph showing the quantity of particles in urine versus concentration of CaCl$_2$ (mM).]

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Concentration of CaCl$_2$ (mM)

**B**

![Graph showing the weight (g) versus concentration of NaOX (mM).]

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Concentration of NaOX (mM)

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<td>100 mM</td>
<td>&lt;0.001</td>
<td>100 mM</td>
<td>&lt;0.001</td>
<td>100 mM</td>
<td>0.039</td>
</tr>
<tr>
<td>200 mM</td>
<td>&lt;0.001</td>
<td>200 mM</td>
<td>&lt;0.001</td>
<td>200 mM</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Table B**

<table>
<thead>
<tr>
<th>Versus</th>
<th>12.5 mM</th>
<th>2.5 mM</th>
<th>5 mM</th>
<th>10 mM</th>
<th>20 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 mM</td>
<td>1.000</td>
<td>Control</td>
<td>1.000</td>
<td>Control</td>
<td>1.000</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>1.000</td>
<td>2.5 mM</td>
<td>1.000</td>
<td>1.25 mM</td>
<td>1.000</td>
</tr>
<tr>
<td>5 mM</td>
<td>1.000</td>
<td>5 mM</td>
<td>1.000</td>
<td>5 mM</td>
<td>1.000</td>
</tr>
<tr>
<td>10 mM</td>
<td>1.000</td>
<td>10 mM</td>
<td>1.000</td>
<td>10 mM</td>
<td>1.000</td>
</tr>
<tr>
<td>20 mM</td>
<td>1.000</td>
<td>20 mM</td>
<td>1.000</td>
<td>20 mM</td>
<td>200 mM</td>
</tr>
</tbody>
</table>
Figure 4 (cont.)

![Graph showing the quantity of particles in urine with concentration of NaCl (mM).]

<table>
<thead>
<tr>
<th>Concentration of NaCl (mM)</th>
<th>Control</th>
<th>12.5 mM</th>
<th>25 mM</th>
<th>50 mM</th>
<th>100 mM</th>
<th>200 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versus 12.5 mM</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Versus 25 mM</td>
<td>0.999</td>
<td>1.000</td>
<td>0.982</td>
<td>0.989</td>
<td>0.969</td>
<td>0.959</td>
</tr>
<tr>
<td>Versus 50 mM</td>
<td>0.914</td>
<td>0.982</td>
<td>1.000</td>
<td>0.969</td>
<td>0.959</td>
<td></td>
</tr>
<tr>
<td>Versus 100 mM</td>
<td>1.000</td>
<td>0.989</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Versus 200 mM</td>
<td>1.000</td>
<td>0.969</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P* value for each comparison.

Control versus each concentration level at different NaCl concentrations.
Figure 5

The scatter plot shows the relationship between calcium (mg/dl) and weight (g), with a correlation coefficient of $r = 0.894$ and a p-value of $p < 0.001$. The data points are linearly aligned, indicating a strong positive correlation.
Figure 6

A. Uncentrifuged pool (saved at 4°C)

B. Uncentrifuged pool (saved at -20°C)

C. Centrifuged pool (saved at 4°C)

D. Centrifuged pool (saved at -20°C)
Figure 7

A

B

<table>
<thead>
<tr>
<th>pH</th>
<th>P value Versus pH 5.0</th>
<th>P value Versus pH 5.8</th>
<th>P value Versus pH 6.6</th>
<th>P value Versus pH 7.4</th>
<th>P value Versus pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.999</td>
<td>0.999</td>
<td>0.393</td>
<td>0.864</td>
<td>0.161</td>
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<td>pH5.0</td>
<td>0.393</td>
<td>0.619</td>
<td>0.971</td>
<td>0.864</td>
<td>0.313</td>
</tr>
<tr>
<td>pH5.8</td>
<td>0.864</td>
<td>0.971</td>
<td>0.966</td>
<td>0.866</td>
<td>0.996</td>
</tr>
<tr>
<td>pH6.6</td>
<td>0.161</td>
<td>0.313</td>
<td>0.996</td>
<td>0.782</td>
<td>0.782</td>
</tr>
<tr>
<td>pH7.4</td>
<td>0.999</td>
<td>0.973</td>
<td>0.198</td>
<td>0.643</td>
<td>0.066</td>
</tr>
<tr>
<td>pH8.2</td>
<td>0.999</td>
<td>0.973</td>
<td>0.198</td>
<td>0.643</td>
<td>0.066</td>
</tr>
</tbody>
</table>
Figure 8

A

![Image of gel electrophoresis with labeled bands]

B

![Bar chart showing urine total calcium levels with and without sediments]

- No sediments
- With sediments

**p < 0.001