Effects of storage time and temperature on pH, specific gravity, and crystal formation in urine samples from dogs and cats

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Objective—To determine effects of storage temperature and time on pH and specific gravity of and number and size of crystals in urine samples from dogs and cats.

Design—Randomized complete block design.

Animals—31 dogs and 8 cats.

Procedure—Aliquots of each urine sample were analyzed within 60 minutes of collection or after storage at room or refrigeration temperatures (20 vs 6°C [68 vs 43°F]) for 6 or 24 hours.

Results—Crystals formed in samples from 11 of 39 (28%) animals. Calcium oxalate (CaOx) crystals formed in vitro in samples from 1 cat and 8 dogs. Magnesium ammonium phosphate (MAP) crystals formed in vitro in samples from 2 dogs. Compared with aliquots stored at room temperature, refrigeration increased the number and size of crystals that formed in vitro; however, the increase in number and size of MAP crystals in stored urine samples was not significant. Increased storage time and decreased storage temperature were associated with a significant increase in number of CaOx crystals formed. Greater numbers of crystals formed in urine aliquots stored for 24 hours than in aliquots stored for 6 hours. Storage time and temperature did not have a significant effect on pH or specific gravity.

Conclusions and Clinical Relevance—Urine samples should be analyzed within 60 minutes of collection to minimize temperature- and time-dependent effects on in vitro crystal formation. Presence of crystals observed in stored samples should be validated by reevaluation of fresh urine. (J Am Vet Med Assoc 2003;222:176–179)

The advent of effective medical protocols to dissolve and prevent uroliths in dogs and cats has resulted in renewed interest in detection and interpretation of crystalluria. Identification of urine crystals formed in vivo may aid in detection of disorders that predispose animals to urolith formation, estimation of the mineral composition of uroliths when uroliths are not available for analysis, and evaluation of the effectiveness of medical protocols prescribed to dissolve or prevent uroliths. In vivo factors that predispose cats and dogs to crystalluria include the concentration and water solubility of crystallogenic substances in urine, urine pH, and the rate of urine flow.

Unfortunately, in vitro changes in urine composition that develop after sample collection may promote formation of the same types of crystals that form in vivo. Thus, caution must be used in clinical interpretation of crystals found in urine sediment, as these may not have been present when the sample was collected. In vitro factors that may influence formation of crystals in urine specimens include temperature, time, evaporation, urine pH, and growth of microbial contaminants that produce urease.

Examination of urine specimens within a short time after collection may minimize in vitro crystal formation and other undesirable in vitro effects that interfere with routine urinalysis. In the event that diagnostic evaluation of urine after sample collection is delayed, refrigeration (2 to 8°C [36 to 46°F]) of samples is commonly recommended. Refrigeration preserves many of the physical and chemical properties of urine as well as morphologic characteristics of urine sediment. It also minimizes in vitro growth of microbes. However, clinical observations suggest that refrigeration may enhance in vivo crystal formation. Although there is a consensus of opinion that refrigeration-associated crystal development is common, this has not been substantiated by controlled studies. The purpose of the study reported here was to determine the in vitro effects of temperature (20 vs 6°C [68 vs 43°F]) and time (6 vs 24 hours) on crystal content, pH, and specific gravity (SG) of urine specimens collected from dogs and cats.

Materials and Methods

Urine collection—Fresh urine specimens were collected from 31 dogs and 8 cats during a 24-day period. To maximize the likelihood of in vitro crystal formation, only urine specimens with SG ≥ 1.025 were evaluated. In addition, to evaluate multiple aliquots of urine under different conditions of storage, only specimens with volumes ≥ 11 mL were examined. Immediately after collection, each urine specimen was divided into 5 equal aliquots of 2 to 5 mL by use of a pipette with sterile tips. Aliquots were stored in capped centrifuge tubes to prevent evaporation. Each centrifuge tube was coded to mask the origin of the aliquot during analysis.

Experimental design—A randomized complete block design was used to evaluate the in vitro effect of temperature and time on type, number, and size of crystals in each urine specimen. One of the 5 aliquots of each specimen was randomly designated as baseline and was analyzed within 60
minutes of collection. Of the remaining 4 aliquots, 2 were randomly assigned to be stored at room temperature (20°C) and analyzed 6 or 24 hours after collection, and 2 were randomly assigned to be stored at refrigeration temperature (6°C) and analyzed 6 or 24 hours after collection.

Urine storage and analysis—All aliquots were stored at their designated temperature within 30 minutes of collection; urinalyses were performed on all aliquots within 30 minutes of completion of the specified storage period. In vitro crystal formation was defined as the presence of crystal types in stored urine aliquots that were not observed in baseline samples. Every other day during the 24-day study period, mean ± SD room (22 ± 0.7°C [72 ± 1.3°F]) and refrigeration (3 ± 0.4°C [37 ± 0.7°F]) temperatures were recorded with a thermometer.

Each urine aliquot was centrifuged; for 5 minutes at a relative centrifugal force of 400 × g. The supernatant was separated from the sediment. Aliquots of supernatant were used to determine urine SG by refractometry; urine pH was measured with a pH meter. The sediment was resuspended in 0.5 mL of urine with a transfer pipette. A drop of resuspended specimen was transferred to a urine sediment slide designed to accommodate a standard volume of urine (6.6 µL) for examination. Type, number, and size of crystals were identified with light microscopy. Crystal types were determined according to their shape and color. Amorphous crystals were classified on the basis of whether they dissolved after addition of either 1 drop of 10% acetic acid or 1 drop of solution containing 6.25 M sodium hydroxide; these treated samples were then examined by use of light microscopy. Amorphous crystals that dissolved in sodium hydroxide solution but not acetic acid solution were designated as amorphous urate; amorphous crystals that dissolved in acetic acid solution but not sodium hydroxide solution were designated as amorphous phosphate. Ten grid squares (each 0.33 mm²) on the slide were selected at random. Crystals in these 10 grids were counted at high-power magnification (450 ×), and mean number of crystals per grid was calculated. The following formula was used to determine the number of crystals per µL of urine:

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\frac{C \times V}{0.5N}
\]

where C is mean number of crystals per grid, GV is grid volume (0.011 µL), 0.5 represents the resuspended sediment volume, and V is the volume of urine centrifuged per aliquot.

By use of an ocular micrometer, the longitudinal dimension of crystals was measured in the same 10 grids. If the length of crystals in a grid appeared to vary by > 50%, all crystals in the 10 grids were measured. If the length of crystals in a grid appeared to vary by < 50%, only 5 crystals in each grid were measured. Mean crystal length in each grid was calculated. Crystal length was determined by calculation of the overall mean for the mean values of crystal length for the 10 grids.

Statistical analyses—The proportion of dogs and cats from which ≥ 1 urine aliquot had in vitro crystal formation was determined. The test for a single proportion was used to assess whether this value was significantly > 0. Analysis of variance for a randomized complete block design (animals as a block) was used to evaluate the effects of time and temperature on number and size of crystals formed in vitro, urine pH, and urine SG. Responses (in vitro crystal number and length, urine pH, and urine SG) to time (0 vs 24 hours) and temperature (room vs refrigeration) were considered the main effects. Two-way ANOVA was performed to determine effects of time and temperature for each crystal type. Response to a combination of these factors was evaluated to determine whether there was an interaction between the main effects. Assumption of normality was tested by use of residual plot analysis. Data (crystal number and length) were corrected with a square root transformation. Analyses were performed with statistical software. Values of P < 0.05 were considered significant.

Results

Urine specimens were obtained from 8 cats and 31 dogs. All samples from cats were collected by cystocentesis. Urine samples were collected from dogs by cystocentesis (n = 2) or catheterization (2) and during spontaneous voiding (27).

Frequency and type of in vitro crystal formation—In vitro formation of calcium oxalate (CaOx) crystals was observed in urine samples from 1 cat and 8 dogs. When urine was stored at room temperature, in vitro CaOx crystal formation was detected in 1 of 9 aliquots at 6 hours and 2 of 9 aliquots at 24 hours. After storage at refrigeration temperature, in vitro CaOx crystal formation was detected in 4 of 9 urine aliquots at 6 hours and in all 9 aliquots at 24 hours. In 2 dogs, in vitro formation of magnesium ammonium phosphate (MAP) crystals was observed in aliquots of urine stored for 6 and 24 hours at room and refrigeration temperatures. In vitro crystal formation in aliquots of a urine sample involved only 1 type of crystal. However, 8 of 31 dogs had crystalluria in vivo, and crystals subsequently formed in vitro in urine from 3 of these dogs. Calcium oxalate crystals developed in vitro in urine from 2 dogs with amorphous phosphate crystals in vivo, and 1 dog with MAP crystals in vivo.

Effects of time and temperature on in vitro crystal formation—The numbers of all types of crystals formed in vitro (mean ± SD, 13 ± 68/µL) in urine aliquots stored for 24 hours were significantly (P = 0.02) greater than the number of crystals formed in vitro in aliquots stored for 6 hours (mean, 2 ± 13/µL). In urine in which CaOx crystals formed in vitro, crystal number was significantly (P = 0.04) greater in aliquots stored for 24 hours (mean, 12 ± 69/µL) than in those stored for 6 hours (mean, 2 ± 11/µL). In contrast, there was no significant difference in numbers of MAP crystals formed in vitro in urine aliquots stored for 6 or 24 hours.

The numbers of all types of crystals formed in vitro in aliquots that were refrigerated (mean, 15 ± 51/µL) were significantly (P = 0.002) greater than the number of crystals formed in vitro in aliquots stored at room temperature (mean, 1 ± 5/µL). On examination of urine aliquots in which CaOx crystals formed in vitro, significantly (P = 0.003) greater crystal numbers were observed after storage at refrigeration temperature (mean, 13 ± 51/µL) than after storage at room temperature (mean, 0 ± 3/µL). However, the number of MAP crystals that developed in vitro was not significantly different between aliquots stored at refrigerator versus room temperature.

Effects of time and temperature on crystal size—Lengths of MAP and CaOx crystals formed in vitro in aliquots stored for 6 or 24 hours were not significantly different. However, mean length of all types of crystals formed in vitro in urine stored at refrigeration temperature (30 ± 46 µm) was significantly (P = 0.03) greater.
than length of crystals formed in urine stored at room temperature (mean, 27 ± 21 µm). Examination of urine in which CaOx crystals formed in vitro revealed a significant (P = 0.02) difference in crystal length between refrigerated aliquots (mean, 11 ± 5 µm) and aliquots stored at room temperature (mean, 7 ± 3 µm). Lengths of MAP crystals that formed in vitro at refrigeration and room temperatures were not significantly different.

**Interaction**—The interaction between time and temperature did not influence CaOx or MAP crystal numbers or lengths.

**Time, temperature, and urine pH**—Storage time and temperature did not have a significant effect on urine pH. Mean ± SD pH of 196 urine aliquots was 6.48 ± 0.85 (range, 4.98 to 8.62). Mean urine pH of stored aliquots varied by 0.001 ± 0.10 from baseline. Mean pH of 8 aliquots with in vitro MAP crystal formation was 6.83 ± 0.30; mean pH of 16 aliquots with in vitro CaOx crystal formation was 6.09 ± 0.65.

**Time, temperature, and urine SG**—Storage time and temperature did not have a significant effect on urine SG. Mean SG of 196 urine aliquots was 1.038 ± 0.01 (range, 1.025 to 1.064). Mean SG values of stored aliquots varied by 0.0001 ± 0.0003 from baseline. In vitro crystal formation was detected in 20 of 68 (29%) aliquots with SG ≥ 1.025 but < 1.033; in 12 of 48 (25%) aliquots with SG ≥ 1.033 but < 1.045; and 12 of 28 (43%) aliquots with SG ≥ 1.045 but < 1.055. In vitro crystal formation was not observed in aliquots (n = 12) with SG ≥ 1.055 but < 1.065. Mean SG of aliquots (n = 8) with in vitro formation of MAP crystals was 1.034 ± 0.01, and that of aliquots (6) with in vitro formation of CaOx crystals was 1.041 ± 0.01.

**Discussion**

Refrigeration is an excellent method to preserve many physical and chemical properties of urine as well as morphologic characteristics of urine sediment. For urine specimens that cannot be analyzed within 60 minutes of collection, refrigeration is frequently recommended to preserve in vivo characteristics of the sample. In routine urinalyses, refrigeration is preferred over use of chemical preservatives because it is less likely to affect results of reagent strip tests. Refrigeration also minimizes in vitro proliferation of bacteria. However, as confirmed by findings of our study, refrigeration of urine samples may enhance in vitro formation of crystals. This phenomenon may inaccurately indicate the presence or extent of crystalluria in vivo. In the study reported here, failure to differentiate between in vivo and in vitro crystal formation in urine samples stored at refrigeration temperature for 24 hours would have resulted in the erroneous interpretation that 28% (11/39) of these cats and dogs had crystalluria. Because crystals in urine can be templates for further crystal formation in a process known as heterogeneous nucleation, we believe that crystals formed in vivo are a risk factor for stone formation.

In our study, the number of MAP and CaOx crystals formed in vitro increased with storage time. This observation supports the recommendation to analyze fresh urine samples to avoid time-dependent effects on in vitro development of crystals.

After collection, storage of urine samples at room temperature is not generally recommended. In our study, however, crystals formed in vitro less frequently in urine samples stored at room temperature than in samples that were refrigerated. In part, the explanation for this finding may be that crystal formation is likely to increase as the temperature of a urine specimen decreases. Temperature-dependent electrostatic attractions between water molecules and calculogenic ions play an important role in crystal formation. As temperature decreases, water molecules are less likely to flow and disrupt the attraction between ions that are capable of forming crystals. Our findings are consistent with this generalization. Compared with MAP and CaOx crystals formed in vitro in urine samples stored at room temperature, those formed in samples stored at refrigeration temperature were larger and more numerous.

Changes in SG and pH of stored urine specimens might also be expected to influence formation of crystals in vitro. In our study, however, storage of urine was not associated with significant changes in pH or SG. We interpret these results to indicate that under the conditions of our study, pH and SG were not factors in the in vitro development of crystals.

In a study of 10 healthy Beagles, results of urinalyses performed on urine specimens immediately after collection and after refrigeration for 24 hours were compared. Only MAP crystals were reported. Similarly, in vitro formation of only MAP crystals was reported in a study to evaluate the effect of refrigeration on urine samples from cats. Therefore, we were surprised to discover that CaOx crystals formed in vitro more commonly than MAP crystals in our study.

In the context of urine samples with SG ≥ 1.025, results of our study support the recommendation to analyze urine specimens within 60 minutes of collection to minimize temperature-dependent and time-dependent effects on in vitro development of crystals.

Ideally, the clinical importance of crystals in stored urine samples should be verified via analysis of another sample immediately after collection. At the time of initial collection of urine, it may be known that further urine sampling will not be possible. Under such circumstances, it may be advantageous to collect a volume of urine sufficient to provide aliquots for storage at both refrigeration and room temperatures.

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1 Gilson Pipetman 1 to 5 mL, Gilson Inc, Middleton, Wis.
2 Universal pipette tips 1 to 5 mL, Lab Safety Supply, Janesville, Wis.
3 Corning brand 13 mL graduated sterile plastic tubes with conical bottoms, Corning Inc, Corning, NY.
4 Sunbeam thermometer, Opitcelc Tiernan Group, Westford, Mass.
6 AO veterinary refractometer, American Optical, Buffalo, NY.
Corning pH meter 430, Corning Inc, Corning, NY.
Kova Glasstic slide 10 with grids, IDEXX Laboratories Inc, Westbrook, Me.
JMP in 4, SAS Institute Inc, Cary, NC.

References