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# Urine storage under refrigeration preserves the sample in chemical, cellularity and bacteriuria analysis of ACS

## *Armazenamento da urina, sob refrigeração, preserva a amostra nas análises químicas, celularidade e bacteriúria no EAS*

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### ABSTRACT

**Introduction:** The analysis of urine abnormal constituents and sediment (ACS) comprises tests of great diagnostic and prognostic value in clinical practice. When the analysis of ACS cannot be performed within two hours after collection, the sample must be preserved in order to avoid pre-analytical interferences. Refrigeration is the most applied technique due to its cost effectiveness. Moreover, it presents fewer inconveniences when compared to chemical preservation. However, changes in ACS may also occur in samples under refrigeration. **Objective:** To analyze the influence of refrigeration at 2 to 8°C on the storage of urine samples within 24 hours. **Material and method:** A total of 80 urine samples were selected from patients admitted at Universidade Federal de Juiz de Fora (UFJF) university hospital, which were tested for ACS at room temperature and stored under refrigeration for 6, 12 and 24 hours. **Results:** The results showed that refrigeration proved to be effective when compared to samples kept at room temperature, inasmuch as the physical, chemical, microbial and cellularity features were preserved. Nevertheless, crystalluria was present after a 6- hour storage period. **Conclusion:** The tests revealed that cooling preserved cellularity and chemical characteristics of urine samples for up to 12 hours. Nonetheless, the precipitation of crystals was evident in this storage method. Thus, the possible consequences of storing urine samples for ACS test under these conditions should be included in the analysis report.

**Key words:** urinalysis; urine storage; crystalluria; pyuria; hematuria Introduction.

### INTRODUCTION

The routine urine test or urinalysis is a vital laboratory tool in clinical practice. One of the most important urine analysis test is the screening of abnormal constituents and sediment (ACS). The ACS test comprises physical (organoleptic features, volume and density), chemical and microscopic analysis of the urinary sediment<sup>(2, 5, 7)</sup>. This is an inexpensive and commonly available test, which provides a considerable amount of useful information on the diagnosis of metabolic and genitourinary tract disorders<sup>(1,3)</sup>.

Several systemic clinical conditions that may affect renal function and / or change the composition of urine, namely hydration status, progression and diagnosis of diabetes, hepatopathy, hemolytic anemia or poisoning, may have higher chances of being diagnosed and/or monitored by ACS analysis<sup>(2, 4-6)</sup>. Furthermore, the ACS assessment is vital in detecting renal and urogenital system diseases such as glomerulonephritis, nephrotic syndrome, cystitis, pyelonephritis, renal failure, lithiasis and even cancer<sup>(2, 6)</sup>.

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In clinical pathology laboratories, all analysis procedures, including urine tests, are divided into pre-analytical, analytical and post-analytical phases<sup>(8, 17)</sup>. The preanalytical phase is the step in which fundamental procedures are required in order to guarantee the quality and service performance. It involves the collection, handling, processing, delivery of the sample to analyzers and storage when it is not processed on arrival at the laboratory. It also accounts for 46% to 70% of laboratory errors, which may lead to inaccurate results, thus hindering clinical diagnosis and patient's healthcare<sup>(8, 9, 11, 18)</sup>.

Therefore, urine collection (pre-analytical phase) should follow basic procedures in order to obtain a sample that reflects physicochemical, cellular and microbiological changes<sup>(2, 7)</sup>. Ideally, the sample should be collected after cleaning the genitourinary tract through spontaneous and preferably long midstream urine after nocturnal concentration (first morning urination). The dispatch and sample analysis should occur within two hours after collection if kept at room temperature<sup>(7, 12)</sup>.

According to the Brazilian Association of Technical Standards (*Associação Brasileira de Normas Técnicas*- ABNT)<sup>(2)</sup>, when the analysis cannot be performed within this period, preservatives should be used in order to conserve the characteristics of the urine and prevent inaccurate analysis results. The main factors directly or indirectly linked to the patient's condition that are able to change the results of AES analysis are the following: medications, vitamins, physical exercises, diet and poor sample storage<sup>(2, 5)</sup>.

Many situations do not allow the analysis within two hours after micturition, therefore the preservation of urine sample becomes a commonly applied pre-analytical step in order to avoid the interferences previously mentioned. There are several preservatives that offer advantages and disadvantages: boric acid, chloroform, phenol, formalin, toluene, thymol and refrigeration at 2 to 8°C, which is the most widely applied method<sup>(2, 7, 13)</sup> due to its cost-effectiveness and fewer drawbacks in comparison with chemical preservatives<sup>(2, 12, 13)</sup>.

The use of refrigeration may lead to changes in ACS that include density, precipitation of crystals and even cellular alterations<sup>(2, 13, 15)</sup>. Moreover, the length period of cooling efficiency is controversial, insofar as some authors claim to be 12 hours<sup>(2)</sup>, while others recommend up to 24 hours<sup>(12)</sup>.

## OBJECTIVES

The present study aimed at verifying the influence of refrigeration at 2 to 8°C on urine storage for 24 hours before ACS

analysis. The refrigerated samples were compared with those kept at room temperature for the performance of physical, chemical and microscopic ACS analysis.

## MATERIAL AND METHOD

### Patient and samples

The urine samples were obtained from adult patients aged from 18 to 60 years from both genders, who were hospitalized at *Universidade Federal de Juiz de Fora* (UFJF) - university hospital and who had ACS test requests.

80 urine samples containing 80 ml each were from clean-catch midstream one-time urine collection after nocturnal concentration. They were stored in containers standardized by the World Health Organization (WHO) and immediately dispatched to the urinalysis sector of the Clinical Analysis Laboratory from UFJF University Hospital and analyzed at arrival.

After the performance of ACS test, the samples were divided and stored at different temperatures (refrigeration at 2 to 8°C and at room temperature). Additionally, they were assessed at different periods (6, 12 and 24 hours).

All research procedures were approved by the Research and Ethics Committee of UFJF - *Hospital Universitário - Centro de Atenção à Saúde* (number: 0085.0.420.000-10). The laboratory has external quality control according to the standards of the Brazilian Society of Clinical Pathology (*Sociedade Brasileira de Patologia Clínica*).

### Storage

After performance of ACS test at immediate arrival, the remaining urine volume (70 ml) was split into two vials that were stored for 24 hours following different procedures: one was stored in the refrigerator and the other was kept at room temperature. The refrigerator temperature was adjusted between 2 to 8°C with time check by maximum and minimum thermometer throughout the experiment. ACS test was performed again.

### ACS

Once the material was collected, the aliquot of 10 ml of urine was applied for analysis. Physical examination was carried out by observing color, density, appearance and odor. The density was assessed with the use of a refractometer, which was subjected to calibration with distilled water after each analysis.

The chemical analysis was performed with the aid of reagent strips (UriquestPlus®) in order to detect the presence of leukocytes, nitrite, protein, blood, bilirubin, urobilinogen, ketone body, glucose as well as pH control. After homogenization, the reagent areas were immersed in fresh urine and immediately removed to avoid dissolving reagent from the strip. While the strip was withdrawn from the urine, the excess was drained to prevent the mixing of chemicals. The specific time of reaction for each analyzed parameter was observed and, subsequently, it was carefully compared with the product label.

Once the chemical analysis was conducted, microscopy was performed using a common optical microscope, followed by centrifugation of 10 ml of urine at 1,500 rpm for 5 minutes. Afterwards, the supernatant was removed and the sediment was resuspended and transferred to a slide. After that, we proceeded to the microscopic analysis at  $100 \times 400$  and  $\times$ . The sediment was assessed in order to verify the presence of elements such as crystals, mucus, renal cast, cells, bacteria and yeasts among others. The aforementioned procedures were adopted in all sample analyzes and performed after 6, 12 and 24 hours. It is particularly worth mentioning that the refrigerated samples reached room temperature before being handled in each of these steps.

### Pyuria and hematuria assessed in ACS

Twenty urine samples that showed more than five pyocytes and / or red blood cells / microscopic field were selected to assess pyuria and hematuria. Accordingly, 10 ml of urine were centrifuged at 1500 rpm for 5 minutes. Without shaking and without resuspending the sediment, the upper 9 ml were removed

and the remaining 1 ml was homogenized and placed in Newbauer chamber. Afterwards, pyocytes and red blood cells were counted as described in the literature<sup>(17)</sup>. The result was released with the number of red blood cells or pyocytes/ml. Both ACS test and the quantification of pyocytes and red blood cells were carried out by three trained analysts, who adopted the same criteria.

### Statistic analysis

Statistical procedures were employed using the software Graph Pad Prism, version 5.0, by analysis of variance (ANOVA), followed by Tukey's test, with significance level of 5%. Data were expressed as mean  $\pm$  standard error of the mean (SEM).

## RESULTS

Initially, the physical analyzes (color, appearance and density) of 80 urine samples were carried out. **Figure 1** shows that the urine samples that were at room temperature and the refrigerated ones showed virtually no difference in terms of color. Nevertheless, after 24 hours at room temperature, four samples changed their hue from yellow to dark yellow (3) and red (1).

Regarding aspect (**Figure 2**), 38 out of 80 urine samples, which had been kept at room temperature, were clear at the time of collection and 31 remained with the same aspect throughout the period of analysis. The remaining 7 samples became cloudy in analyzes performed after 24 hours. A completely different result was observed in the samples stored under refrigeration. In this method

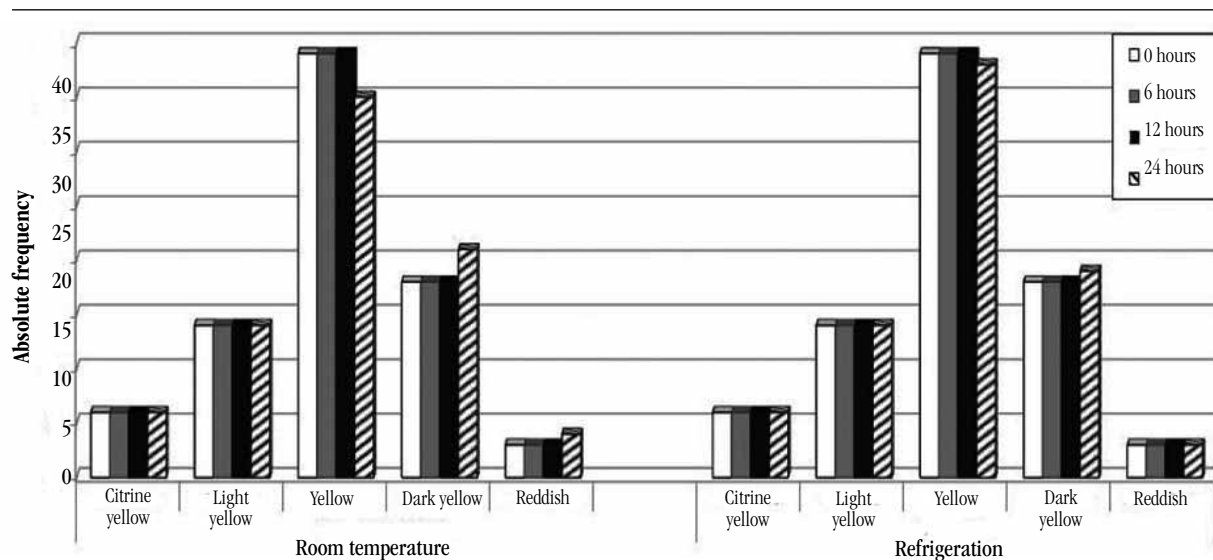


FIGURE 1 – Color of urine samples kept at room temperature and refrigerated storage at 2 to 8°C for up to 24 hours (n = 80)

of storage, it was observed that after a six- hour refrigeration period some samples became cloudy or slightly cloudy despite the fact that most of them remained clear. In analyzes performed 12 and 24 hours after collection, there was an inversion of the profile with the predominance of cloudy and slightly cloudy features. Therefore, at time zero, 38 samples were limpid and only 11 samples presented this characteristic by the end of the 24- hour refrigeration period.

As to the density analysis, it was observed that all samples, refrigerated or not, yielded results within 1.005 and 1.035. 9 out of

80 samples kept at room temperature demonstrated a maximum increase of 0.010 units. Regarding the samples that were kept under refrigeration, only 3 showed an increase in density by 0.005.

In the chemical assessment, it was observed that 45 out of 80 selected urine samples showed no chemical change detected by the reagent strip when analyzed at room temperature at the time of collection. Even after refrigeration, these 45 samples did not present any chemical change, corroborating the data presented during this study. Thus, refrigeration did not exert any effect on chemical analysis, which could lead to positivity.

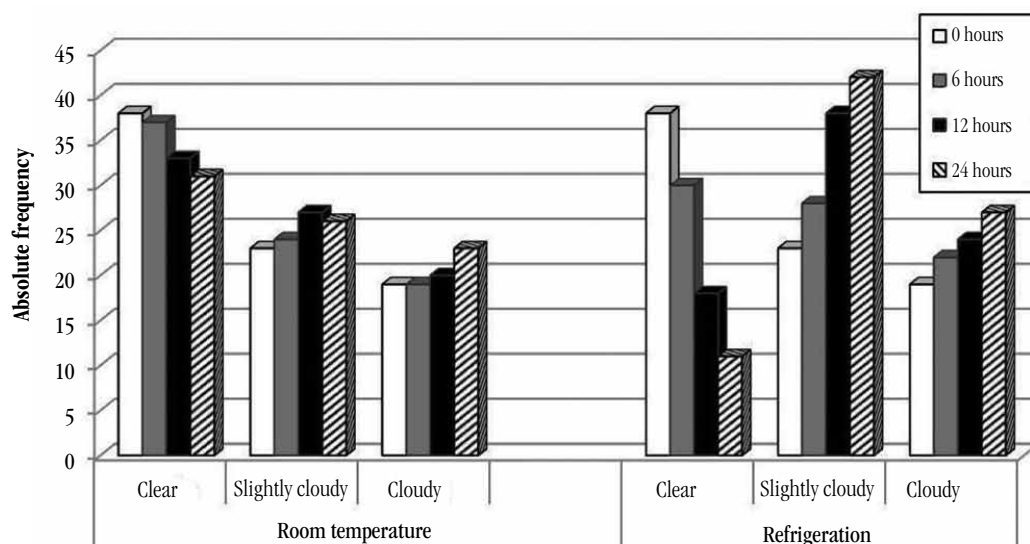


FIGURE 2 – Features of urine samples kept at room temperature and refrigerated storage at 2 to 8°C for up to 24 hours (n = 80)

In the remaining 35 samples, one or more abnormal elements were detected on the reactive strips immediately after collection (**Table 1**). In these samples with chemical changes, which had been kept under refrigeration and analyzed after 6, 12 and 24-hour periods, there was no qualitative or semi-quantitative change in the screening in contrast with the analysis upon arrival at the laboratory.

Regarding the samples stored at room temperature, **Table 2** shows changes in some chemical parameters in contrast with the results obtained from those immediately analyzed. In the analysis after 24 hours, the three samples began to show a positive nitrite reaction, two were negative for leukocytes and one was negative for glucose.

As to the pH of the 80 samples kept under refrigeration and assessed by the reactive strips, there was pH change from 6.0 to 7.0 in only one sample. Conversely, in samples kept at room

temperature (**Table 3**), there was change in 9 of them, mainly 24 hours after collection.

After the physical and chemical analyzes, ACS screening of formed elements in the refrigerated urine samples revealed no differences in the results of epithelial cells, pyocytes count, erythrocyte and bacteria in relation to samples assessed at the time of collection.

However, a rise in urinary sediment crystals proportional to the cooling period was observed during the qualitative and semi-quantitative analysis, especially calcium oxalate, urate and amorphous phosphate crystals (**Table 4**). Initially, only 7 out of 80 samples presented crystals. By the end of the 24-hour cooling period, 36 urine samples showed precipitated crystals and several with more than one type of crystal. When kept at room temperature for 24 hours, the number of samples with crystals at the time of collection changed from 7 to 10 with precipitation of crystals.

**TABLE 1** – Absolute number of positive findings from non-refrigerated urine samples in chemical analysis by reactive strips (collection = time zero)

Chemical analysis	Leucocytes	Nitrites	Proteins	Blood	Bilirubin	Urobilinogen	Glucose	Ketone bodies
Number of positive samples	12	6	7	9	2	4	4	3

**TABLE 2** – Absolute number of positive findings from non-refrigerated urine samples (time zero) kept at room temperature (6, 12 and 24-hours after collection) in chemical analysis by reactive strips

Storage time	Leucocytes	Nitrites	Proteins	Blood	Bilirubin	Urobilinogen	Glucose	Ketone bodies
0 hours	16	6	7	9	2	4	4	3
6 hours	16	6	7	9	2	4	4	3
12 hours	14	7	7	9	2	4	4	3
24 hours	12*	9	7	9	2	4	3*	3

**TABLE 3** – Reactive strip results related to pH in urine samples with changes and kept at room temperature

Sample	0 hours	6 hours	12 hours	24 hours
1	pH 6.5	pH 6.5	pH 6.5	pH 7.0*
2	pH 6.0	pH 6.0	pH 6.0	pH 6.5*
3	pH 6.0	pH 6.0	pH 6.0	pH 8.0*
4	pH 6.0	pH 6.0	pH 6.0	pH 8.0*
5	pH 5.0	pH 5.0	pH 6.0*	pH 7.0*
6	pH 7.0	pH 7.0	pH 7.0	pH 8.0*
7	pH 5.0	pH 6.0*	pH 6.0*	pH 7.0*
8	pH 6.0	pH 6.0	pH 6.0*	pH 7.0*
9	pH 6.0	pH 6.5*	pH 7.0*	pH 8.0*

**TABLE 4** – Absolute number of crystal findings in non-refrigerated urine samples (time zero) and after refrigeration (6, 12 and 24 hours after collection)

Crystals	Classification	Time after sample collection			
		0 hours	6 hours	12 hours	24 hours
Calcium oxalate	Rare	2	4	7	8
	Some	2	2	5	4
	Numerous	1	2	4	6
Amorphous urate	Rare	0	3	2	2
	Some	2	0	2	4
	Numerous	0	3	7	9
Uric acid	Rare	2	1	1	2
	Some	0	1	0	1
	Numerous	0	1	2	4
Amorphous phosphate	Rare	1	1	1	2
	Some	2	3	3	1
	Numerous	0	2	5	11
Ammonium magnesium phosphate	Rare	0	0	0	1
	Some	0	0	0	1
	Numerous	0	0	0	0

Regarding the urine stored at room temperature, the analysis of pyuria by microscopy revealed that the number of cells decreased during the 12-hour period. Initially, 6 samples presented 5 to 10 pyocytes/field and only 3 remained with the same count by the end of the 24 hour-period. 6 out of 8 urine samples that showed 10 to 20 pyocytes/field remained with the same count after 12 hours and this total dropped to 5 samples by the end of the 24 hour-period.

As to hematuria, 10 samples showed values higher than 4 erythrocytes/field, which is established as the normal value in the clinical laboratory from the University Hospital. This was corroborated by the urine strip results that showed 9 positive samples. When the samples were kept at room temperature and analyzed after 6 hours, a significant decline was observed in hematuria, insofar as only 2 out of 5 samples containing 5-10 red erythrocytes/field remained with the same number. In other urine samples with results ranging from higher than 10/field up to numerous erythrocytes/field, there was also a considerably lower number after the first six hours. This reduction was not observed in urine samples kept under refrigeration even after 24 hours.

In the samples stored at room temperature, 10 samples showed significant bacteriuria after 12 hours, accruing to 14 samples after 24 hours, whereas the 8 urine samples that initially presented bacteria did not undergo any changes.

The results of the quantitative analysis of pyocytes revealed that there was no statistically significant changes ( $p > 0.05$ )

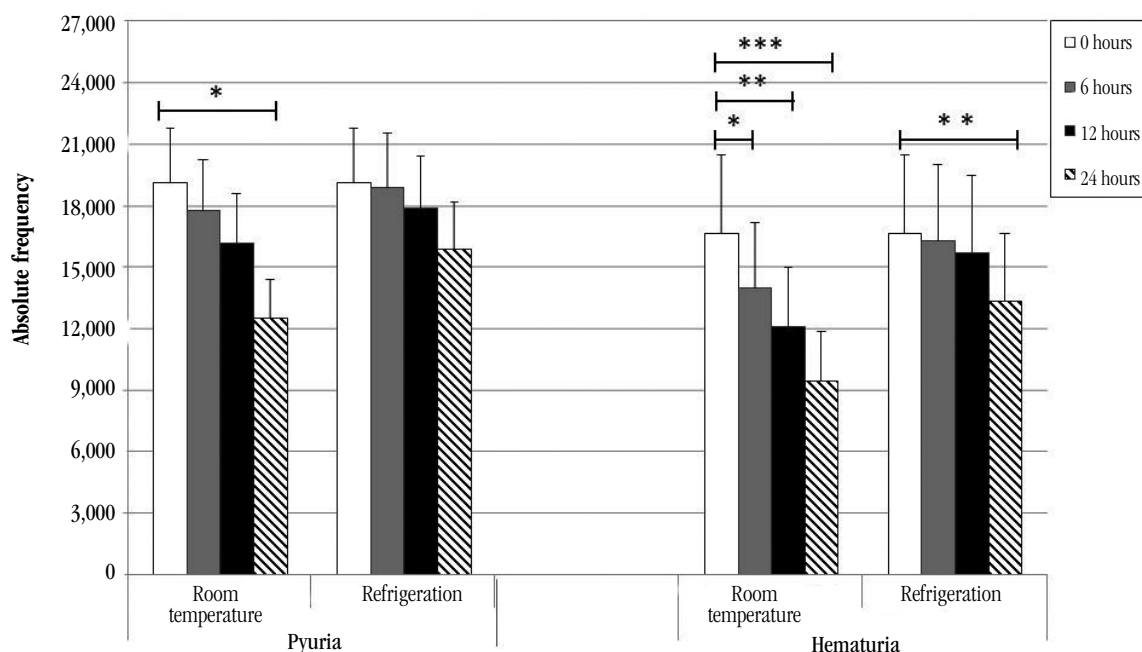
in samples kept under refrigeration, even after 24 hours in comparison with the counts obtained in the urine samples analyzed at the time of collection. When kept at room temperature, the analyzed urine samples only had a significant reduction ( $p < 0.05$ ) in the pyocytes count 24 hours after collection (**Figure 3**).

We also performed a quantitative analysis of red blood cells (**Figure 4**) kept under refrigeration and there was substantial reduction ( $p < 0.01$ ) only after 24 hours. In samples that were stored for 24 hours without refrigeration, the results were quite different, insofar as after six hours there was a decline in red blood cell count ( $p < 0.05$ ) and this reduction was more significant after 24 hours ( $p < 0.001$ ).

## DISCUSSION

Urinalysis is one of the most requested tests in clinical medicine. Therefore, collection of material and analysis should be appropriate so that accurate results may be yielded, hence assisting in the diagnosis, treatment and/or patient monitoring<sup>(6, 10)</sup>.

The preanalytical phase is of paramount importance in all laboratory tests, including urine examinations. In ACS analysis, some guidelines should be followed as to cleaning of external genitalia, suitable collection of material and immediate referral to the laboratory<sup>(2, 14, 17)</sup>.



**FIGURE 3** – Effect of urine storage at room temperature or under refrigeration for up 24 hours on quantified pyuria and hematuria

0 hours was the control group ( $n = 20$ ); stored for 6 hours ( $n = 20$ ); stored for 12 hours ( $n = 20$ ); stored for 24 hours ( $n = 20$ ). Data are presented as mean  $\pm$  SD.

\*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ , always compared with the control group.

The urine sample submitted to the laboratory must be analyzed within two hours after collection at most. In case the delivery and/or analysis are not performed immediately, the sample must undergo some method of conservation, either physical (cooling) or chemical (chloroform, thymol, boric acid, among others)<sup>(2,16)</sup>. Refrigeration is the most applied preservation method in clinical laboratories and it is able to prevent bacterial decomposition. According to Strasinger & Di Lorenzo<sup>(17)</sup>, refrigeration does not interfere with chemical testing, preserves cellularity and prevents bacterial growth. This result was confirmed herein, inasmuch as both patients with negative values for the chemical tests ( $n = 45$ ) and those with detected positive reactions ( $n = 35$ ) did not present any changes in the chemical examination after storage under refrigeration. Nevertheless, the literature describes that cooling may result in increased density assessed by urine densitometer<sup>(16,17)</sup>. This data was corroborated by the experiment, which also varied in relation to the density of cooled samples, insofar as six of them indicated increase in this parameter.

Conversely, the samples kept at room temperature underwent changes in the chemical tests, namely an increase in the presence of nitrite and a decrease in the samples with leukocytes and glucose (Table 2). The rise in nitrite correlates with the bacterial metabolism in the urine sediment, which is equally observed in the material maintained at room temperature during sedimentoscopy. The study by Santos *et al.*<sup>(15)</sup> also underscores the bacterial growth in urine when the sample is not analyzed immediately or performed without the use of conservation methods.

As noted by Silva *et al.*<sup>(16)</sup>, some samples maintained at room temperature for a prolonged period and without preservatives demonstrated an increase in pH (Table 3), turbidity (Figure 2), and disintegration of red blood cells and casts.

In contrast, cooling showed no significant changes in the number of urine samples with bacteria, leukocytes and erythrocytes, proving to be a great method for such analyzes.

Furthermore, the study showed precipitation of crystals in most cooled samples, which can hamper the microscopic examination of the sediment, as described in the literature<sup>(16,17)</sup>.

In the present experiment, the amorphous urate crystal was frequently present in the samples after cooling. Initially, only two samples showed it. After a six-hour refrigeration period, it was present in six samples. By the end of the experiment, it was present in fifteen of them (Table 4). However, according to Machado *et al.*<sup>(11)</sup>, the presence of these crystals usually has no clinical relevance, although it may interfere with the microscopic observation of the formed elements when in large quantities.

As to the presence of uric acid crystals in the urine, there was a considerable augmentation in cooled samples from 12 hours of storage onward (Table 4). This crystal can be detected in patients with leukemia, Lesch-Nyhan syndrome and in some patients with gouty arthritis<sup>(17)</sup>.

The oxalate and calcium phosphate crystals were among the most commonly detected herein in both room temperature and mostly cooled samples (Table 4). These crystals are usually present in the urine from patients with chemical poisoning, use of high doses of ascorbic acid and also ingestion of oxalic acid rich foods<sup>(6,11,14)</sup>. Although little importance is given to the role of oxalate and calcium phosphate crystals in the diagnosis of kidney stones<sup>(17)</sup>, this fact may lead to misinterpretation as to the risk of developing renal lithiasis, insofar as the oxalate calcium may account for up to 80 % of the cases of nephrolithiasis worldwide<sup>(6,12)</sup>.

As stated previously, the cooled samples were returned to room temperature so that they could be examined, as recommended in the literature<sup>(16)</sup>. According to Aguilar-Vallejo *et al.*<sup>(1)</sup>, the crystals present in the samples due to refrigeration tended to redissolve in urine after being returned to room temperature. The data gathered herein did not corroborate previous literature. In this study, the crystals remained present when the samples were returned to room temperature at all time intervals.

## Analysis of quantitative pyuria and hematuria

Quantitative data regarding pyuria and hematuria also support the findings of Silva *et al.*<sup>(16)</sup>. In the quantitative analysis of erythrocytes, when the samples were not cooled, there was a significant decrease after six hours from the initial analysis ( $p < 0.05$ ) whereas in leukocytes kept under the same conditions, there was a significant reduction only 24 hours after collection ( $p < 0.05$ ) (Figure 3).

By submitting these samples to refrigerated storage, the erythrocytes were preserved for up to 12 hours after collection, showing a significant reduction in hematuria only 24 hours after the initial analysis ( $p < 0.01$ ). Regarding pyuria, the reduction was not statistically significant in any of the analyzed periods (Figure 3).

In this investigation, it was demonstrated that the chemical tests, the cellularity (pyocytes) and bacteriuria of samples stored under refrigeration did not change (except erythrocytes within the 24-hour storage period), confirming the importance of this method in urine preservation. However, the precipitation of crystals was evident as pre-analytical variation at 2 to 8°C.

Considering the yielded results, if the refrigeration of urine samples is required, it is imperative that the conditions under which the sample was stored and the possible consequences of the ACS screening process should be included in the analysis report.

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## RESUMO

**Introdução:** A pesquisa de elementos anormais e sedimentoscopia na urina (EAS) compreende testes de grande valor diagnóstico e prognóstico na prática clínica. Quando a análise do EAS não puder ser realizada dentro de duas horas após a coleta da amostra, esta deve ser conservada para que interferências pré-analíticas sejam evitadas. A refrigeração é a técnica mais utilizada devido ao custo-benefício e por apresentar menos inconvenientes quando comparada com conservantes químicos. No entanto, alterações no EAS também podem ocorrer na amostra sob refrigeração. **Objetivo:** Analisar a influência da refrigeração entre 2 a 8°C no armazenamento do EAS por um período de até 24 horas. **Material e método:** Foram selecionadas 80 amostras de urina de pacientes internados no hospital da Universidade Federal de Juiz de Fora (UFJF) testadas para EAS, à temperatura ambiente, e armazenadas sob refrigeração em 6, 12 e 24 horas. **Resultados:** Os resultados mostraram que a refrigeração foi eficaz quando comparada com amostras mantidas à temperatura ambiente, já que as características físicas, químicas, da celularidade e da microbiota da urina foram preservadas. No entanto, a cristalúria se fez presente desde as 6 horas de armazenamento. **Conclusão:** Os testes demonstraram que a refrigeração preservou as características químicas e a celularidade da urina por até 12 horas. No entanto, precipitações de cristais mostraram-se evidentes neste método de armazenamento. Dessa forma, a sugestão de se relatar no laudo as possíveis consequências dessa forma de armazenamento de urina para o EAS pode ser importante..

**Unitermos:** uroanálise; armazenamento da urina; cristalúria; piúria; bematúria.

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