Low performance of vitamin C compared to ammonium chloride as an urinary acidifier in feedlot lambs

Baixo desempenho da vitamina C comparado ao cloreto de amônio como acidificante urinário em cordeiros confinados

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Abstract
Obstructive urolithiasis is highly prevalent disease in feedlot sheep. Urinary acidification is effective for disease prevention. Forty-five healthy 3-4 month-old male Santa Inês crossbred feedlot lambs were distributed into three groups of 15 animals each. Ammonium chloride (G A) at 400 mg/kg/day/animal, vitamin C (G C) at 4 mg/kg/day/animal, and a combination of the two (G AC) were administered orally for 21 d. Blood and urine samples were taken 7 d before beginning treatment (M0), immediately before (M1), and weekly for 21 d (M2, M3, and M4) for renal function tests, levels of Ca, P, and Mg in serum and urine, urinalysis, and fractional excretion (FE) analysis in these minerals. In groups G A and GAC, pH decreased in M2 and remained acidic throughout the experiment. A significant decrease in serum P and a urinary increase in Ca and Mg occurred in GA. The FE of Ca increased during treatments, but there was no interference with Mg. The FE of P was significantly lower in GA. Ammonium chloride was an effective urinary acidifier in sheep, but vitamin C administered orally did not provide stable results. Thus, based on our results, vitamin C supplementation may not effective for urinary acidification to prevent obstructive urolithiasis.

Keywords: pH urinary, fractional excretion, small ruminants, urinalysis, urolithiasis.

Resumo
A urolitíase obstrutiva é uma enfermidade de alta incidência em ovinos confinados. A acidificação urinária é um dos métodos mais eficazes para a prevenção da doença. Utilizaram-se 45 cordeiros clinicamente sadios, machos, mestiços Santa Inês,
com três a quatro meses de idade, em confinamento, distribuídos em três grupos de 15 animais cada. Foi administrado 400mg/kg/dia/animal de cloreto de amônio (G_A), 4mg/kg/dia/animal de vitamina C (G_C) e associação dos dois produtos (G_AC), durante 21 dias, ambos por via oral. As colheitas de sangue e urina foram realizadas sete dias antes do início do tratamento (M0), imediatamente antes (M1) e depois, semanalmente, até 21 dias após (M2, M3 e M4) para realização de exames de função renal (ureia e creatinina), dosagem de Ca, P e Mg no soro e na urina, urinálise e cálculo de EF desses minerais. Nos grupos G_A e G_AC, houve diminuição do pH no M2, permanecendo ácido até o final do experimento. Houve diminuição significativa do P sérico no G_A, além de aumento urinário nos teores de Ca e Mg nesse grupo. A EF de Ca aumentou após o início dos tratamentos, porém não houve interferência para Mg. A EF de P foi significativamente menor somente no G_AC. O cloreto de amônio se mostrou eficaz como acidificante urinário em ovinos, porém a vitamina C, por via oral, apresentou oscilação e não atingiu estabilidade. Portanto, a suplementação com vitamina C não foi eficaz para acidificação urinária e, por isso, não deve ser utilizada na prevenção de urolitíase obstrutiva.

Palavras-chave: pH urinário; excreção fracionada; pequenos ruminantes; urinálise; urolitíase.

Introduction

Obstructive urolithiasis is a high-incidence disease that occurs during the rearing of sheep, especially in confined males\(^{(1,2)}\). After the appearance of clinical signs, there is little chance of reversal of the condition, and if surgical treatment is necessary, a vast majority of animals become unfit for reproduction\(^{(3)}\). Thus, prevention of the disease is the best strategy, and consequently, it is necessary to understand the chemical composition of the uroliths and correct factors potentially related to their formation\(^{(4-6)}\).

Urine acidification is one of the most efficient and inexpensive methods for preventing urolithiasis. It can be performed by the administration of an anionic diet\(^{(7,8)}\) and the use of substances that induce a decrease in urinary pH. Ammonium chloride can be used to prevent struvite and calcium phosphate uroliths, which are preferably formed at an alkaline pH\(^{(9)}\). It can be used in the total diet, at a proportion of 0.5% to 1.0% or 2.0% of the concentrate\(^{(10,11)}\), as well as in individual doses of 5 to 10 g/animal/day\(^{(12)}\). Mavangira et al.\(^{(9)}\) obtained a urinary pH less than 6.5 in goats with a dose of 450 mg/kg/PV of ammonium chloride/day, or 2.25% of the dry matter (DM) intake. Ferreira et al.\(^{(13)}\) described ammonium chloride efficacy in sheep at a dose of 400 mg/kg of body weight (BW), which maintained the pH below 6.1.

Uroliths are formed from predisposing factors, such as intensive management of animals, excessively high-protein diet, or those with a high content of phosphorus, magnesium, or calcium, as well as the ingestion of plants with a large amount of oxalate or silica\(^{(14)}\). However, the disease is more frequently present in confinement,
where the feed is made up of grains. This type of food, in general, has a high content of phosphorus and magnesium, but low content of calcium. Thus, the ratio of Ca and P ranges from 1:4 to 1:6, whereas the ideal ratio should be 1:1 to 2:1. A Ca:P imbalance results in high excretion of phosphorus in the urine, which is an important factor in the genesis of uroliths. The urine of ruminants is alkaline, which makes phosphorus insoluble, precipitating it, and forming crystals with calcium and magnesium. The determination of the biochemical composition of urine is recommended to detect the underlying mechanisms of specific types of uroliths. Higher levels of serum and urinary phosphorus may occur in animals that have stones than that in healthy animals. The measurement of urinary ion concentrations of Ca, P, and Mg can provide data regarding the mineral balance by quantifying the excretion of these elements. However, the simple measurement of the concentration of urinary electrolytes cannot be interpreted correctly without considering the urinary volume produced. For this, the values of electrolytes in serum and urine, in addition to serum and urinary creatinine, must be obtained for the calculation of fractional excretion (FE) because the variation in water absorption and excretion hinders interpretation caused by the significant diversity in the concentration of solutes in the urine.

There is a correlation between urinary creatinine and specific urine density in cattle, indicating that creatinine is almost completely passively filtered by the glomeruli and that the secreted or reabsorbed amounts are insignificant. Therefore, creatinine is used in the calculation of fractional excretion (FE) in cattle and sheep. Thus, the objectives of the present study were to evaluate the effectiveness of the administration of ammonium chloride, vitamin C, and their combination in urinary acidification in confined sheep. Additionally, the differences in the urinalysis results and the serum levels of urea, creatinine, Ca, P, and Mg, as well as the urinary concentrations and EF of these electrolytes, were determined between the treated and untreated groups throughout the experimental period.

**Material and Methods**

A total of 45 healthy, non-castrated male, crossbred Santa Inês sheep, aged 3–4 months with an average weight of 22.6 ± 5.4 kg, were randomly divided into three groups. The animals were numbered, randomly selected, and distributed into nine collective confinement 12 m²-masonry pens, with five lambs each (2.4 m²/animal), arranged in the same location and under the same conditions of temperature, air humidity, and light. The feed consisted of 70% crushed Coast Cross grass hay (cultivar *Cynodon dactylon*) and 30% feed for finishing lambs with 85% DM, 18% crude protein (PB), and 75% neutral detergent fiber (NDT), according to the recommendations of the NRC, for an average daily weight gain of 300 g. The Ca:P ratio was 1.9:1. Water and mineral salt (Ovinofós with Monensina®, Tortuga Companhia Zootécnica Agrária, Mairinque-SP, Brazil) were available *ad libitum*. This ration was supplied twice a day (at 7:00 am and 5:00 pm) in a mash, consisting of corn bran, soybean meal, wheat bran, and calcitic limestone, along with crushed hay to allow mixing and homogenization with ammonium chloride.
Before the experimental study, all animals were dewormed with moxidectin (Cydectin® 1% injectable, Fort Dodge, Campinas-SP, Brazil), vaccinated against clostridiosis (Sintoxan Polivalente®, Merial, Campinas-SP, Brazil) and allowed an adaptation period of at least 21 d. Subsequently, they received the treatments for another 21 consecutive days. During this period, the animals continued to receive the same diet as during the adaptation period and specific treatments were provided for each group. The total confinement time (adaptation and experimental period), of 42 d, was established in this experiment to mimic the conditions in a lamb finishing field, with weaning at 80 to 90 d of life (20 to 22 kg), followed by ingestion of diet for early weight gain (weight gain of 250 to 300 g/day) for 2 months, and reared till 120 to 130 d to achieve an average weight of 35 to 40 kg.

The three experimental groups received three different treatments: group A (G_A) – 400 mg/kg/BW of ammonium chloride/animal/day; AC group (G_AC) – 4 mg/kg/BW of vitamin C and 400 mg/kg/BW of ammonium chloride/animal/day, and group C (G_C) – 4 mg/kg/BW of vitamin C/animal/day.

Vitamin C was administered orally using an automatic dosing syringe (Hauptner Brasil, São Paulo-SP), and ammonium chloride was added daily to the total diet. To avoid the interference of light in the degradation of ascorbic acid, care was taken in carrying out this work to protect vitamin C by wrapping the vial with aluminum foil and administering it immediately to the animal after dilution. After adapting to conditions for 21 d, urine and blood samples were collected from the animals in the three groups.

Samples were collected at 6:00 am using the standard method, before feeding, and were defined as: M0 - 7 d before the start of treatment; M1 - immediately before treatment; M1a - 1 d after treatment; M1b - 2 d; M1c - 3 d; M1d - 4 d; M1e - 5 d; M1f - 6 d; M2 - 7 d; M2a - 8 d; M2b - 9 d; M2c - 10 d; M2d - 11 d; M2e - 12 d; M2f - 13 d; M3 - 14 d, and M4 - 21 d. Blood samples were collected for biochemical tests and urine for urinalysis weekly, at five times: M0, M1, M2, M3, and M4.

The sheep were manually held in a quadrupedal position, using a halter for blood and urine collection. The latter was performed by natural, spontaneous urination or by induction after brief asphyxia for approximately 15 s.

Urinalysis was performed immediately after the collection of urine in sterile 70 mL flasks (J. Prolab. Indústria e Comércio de Produtos para Laboratório Ltda. São José dos Pinhais-PR). The urine samples were sent to the Clinical Pathology Service of the Department of Veterinary Clinic of the Scholl of Veterinary Medicine and Animal Science (FMVZ), UNESP, Botucatu Campus. During the physical examination, aspect (clear or cloudy) and density were evaluated (Atago® T2 refractometer, NE Clinical, Atago Brasil Ltda. Ribeirão Preto-SP, Brazil). The chemical examination was performed using reagent strips (Combur10 Test®, Roche Diagnóstica Brasil Ltda. São Paulo-SP, Brazil), to evaluate proteins (mg/dL), glucose (mg/dL), acetone, urobilinogen, bilirubin, occult blood, and bile salts. The pH was evaluated using a portable pH meter (pH100 PHTEK® Labmais Comércio de Equipamentos Ltda. Curitiba-PR, Brazil), which was calibrated every day and after analyzing sample from every five animals in a solution of pH 4.0 and pH 7.0. The peagameter electrode was completely immersed inside the urine sample.
until stabilization and was only placed in the next sample after being washed in distilled water and dried on absorbent paper.

To examine the urinary sediment, 5 mL of urine was centrifuged (Excelsa II®, Fanen, São Paulo-SP, Brazil) in conical tubes at 400 g for 5 min. After centrifuging and discarding the supernatant, 0.5 mL of urine was used to perform the sediment examination, which included identification of different types of cells from urinary tract (renal, pelvis, bladder, and urethral cells), prostate cells, and other structures, such as red blood cells, leukocytes, cylinders, bacteria, sperm, mucus, and crystals.

The adopted quantitative criteria were: rare (<1 cells/field); a cross (+) (1 to 3 cells/field); two crosses (++) (3 to 5 cells/field); three crosses (+++) (> 5 cells/field) and full field (countless cell numbers/field). All of these observations were made using standard optical microscopy, with 400-fold magnification.

A 10 mL sample of blood was collected in a vacuum tube without anticoagulant (BD Vacutainer®, BD Medical, Curitiba-PR, Brazil), by puncturing the jugular vein of each animal at different times (M0, M1, M2, M3, and M4). After the clot retraction, the collected samples were centrifuged (Centrífuga Combate Celm® - Cia. Equipadora de Laboratórios Modernos, Barueri-SP, Brazil) at 2000 g for 5 min to obtain serum, and frozen at lower than 20°C in 2 mL aliquots in tubes (Eppendorf do Brasil Ltda. São Paulo-SP, Brazil).

All biochemical tests were performed simultaneously at the Clinical Pathology Service of the Department of Veterinary Clinic of FMVZ, UNESP, Botucatu Campus, using commercial reagents (Katal® Biotecnológica Ind. Com. Ltda. Belo Horizonte-MG, Brazil) and spectrophotometer readings were obtained (SB-190 Celm® Apparatus - Company. Modern Laboratories Equipments, Barueri-SP, Brazil).

Methods used for serum measurements included enzymes for the colorimetric determination of urea concentration (modified Berthelot), creatinine (Jaffe), Ca (cresolphthalein complexone), P (ammonium molybdate), and Mg (sulfonated Magon). Concentrations of urine calcium and phosphorus were obtained after acidification of the samples, according to the technique described by Fleming et al. (25).

The FE electrolyte calculations were performed after their measurement in serum and urine, as well as the determination of serum and urinary creatinine. Thus, it was possible to compare the electrolyte clearance with that of endogenous creatinine and determine its renal excretion by using the equation below: FE (%) = [(EU/ES) x (CRS/ CRU)] x 100. Where, EU was urinary electrolytes, CRU was urinary creatinine, ES was serum electrolytes, and CRS was serum creatinine.

The data were analyzed using the IBM SPSS Statistics Software, v.21, with a 95% significance level (p<0.05). Because of the non-normal distribution of quantitative variables, the Kruskal-Wallis non-parametric test was used among the three experimental groups (G_A, G_AC, G_C) to identify differences among groups within the time points (M), and when there was a statistically significant difference, and pairwise tests were conducted using Dunn's post-hoc test. The Kruskal-Wallis test was also performed to assess the difference among the five-time points (M0, M1, M2, M3, and M4), within
each experimental group. When there was a statistically significant difference, it was verified by the Friedman test. Dunn's post-hoc tests identified pairwise differences. For the appearance of urine, a Chi-square test was used.

This study was submitted and approved by the Ethics Committee on the Use of Animals of School of Veterinary Medicine of São Paulo State University, Botucatu, under protocol 38/2007.

Results and Discussion

Urinary pH

The pH remained alkaline (7.0 to 7.75) before the start of treatment (M0 and M1). In the GA and GAC groups, there was a decrease in pH 1 d after the administration of ammonium chloride (p<0.05) (M1b), urine remained acidic until the end of the experiment (M4). The pH values did not decrease linearly from the baseline in Gc group, and rather oscillated between alkaline and acidic pH throughout the experimental period, differing from the GA and GAC groups in M3 and M4 (Figure 1).

![Figure 1. Medians of urinary pH of feedlot lambs submitted to supplementation with ammonium chloride (GA), ammonium chloride and vitamin C (GAC), and vitamin C (GC), at different times. Arrows indicate statistical difference among the groups (p<0.05).
Source: The authors.](image)

At the beginning of the experiment (M0 and M1), the mean urinary pH values were 7.05 ± 1.10, which was within the reference values for the sheep (7.0 to 8.0) (24). After
the initiation of treatments with acidifiers, the urinary pH of the groups that received ammonium chloride (G_A) and ammonium chloride associated with vitamin C (G_AC) were significantly lower (p<0.05) than those of animals that received only vitamin C (G_C), from time M1b to M4.

At 2 d after the beginning of the treatment (M1b), the G_A group showed acidification of the urine below 5.3, and pH values were maintained below 5.9 for the 21 d of treatment (M4). The G_AC group exhibited a pH decrease to 5.5 2 d after the start of treatment (M1b) and maintained values below 6.0 until M4 at the end of the experiment. This was similar to the results obtained in the G_AC group, most likely because both groups received ammonium chloride.

The G_C group could not effectively stabilize and maintain the urinary acidification, as shown by medians of pH below 6.0 only in M2 and M2c. The animals in this group exhibited fluctuations from alkaline (pH 7.08 to 8.4) to acidic (pH 5.9 to 6.9) urine during the experimental period. The time points at which the G_C group showed acidic urinary pH values (M2 and M2c) could be related to the diet composed of high levels of protein and carbohydrate, which could cause transient metabolic acidosis, leading to renal compensation for H⁺ excretion. This was also described by Ferreira and collaborators (13) in confined lambs not supplemented with a urinary acidifier and fed a high-grain content diet, which resulted in an acidic urinary pH, but did not cause metabolic acidosis. In sheep with urolithiasis due to a calculogenic diet (Ca:P 1:2), the occurrence of compensated metabolic alkalosis due to the elevated levels of bicarbonate and CO₂ pressure has been reported (26).

According to McEvoy (27), ammonium chloride has also been used as an adjunct in the treatment of urinary tract infections when low urinary pH is desired. However, the literature on human medicine cites the occurrence of concomitant systemic acidosis, stating that acidosis can be prevented by the administration of other acidifying agents, such as ascorbic acid. In veterinary medicine, repeated doses of 75 g of ammonium chloride have been used for therapeutic purposes in cattle without any adverse effects. In addition to daily doses of 31 to 47 g for calves or 8 g for sheep have been used without toxic effects (28). Ferreira et al. (13) described a dose of 400 mg/kg/day of ammonium chloride to compensate for hyperchloremic metabolic acidosis in confined lambs. This was confirmed by the reduced values of bicarbonate, excess of bases and strong ions difference (SID), high chloride values, and normal venous blood pH. Therefore, these authors concluded that ammonium chloride, despite causing a decrease in the alkaline reserves in the body, did not interfere with the development of the animals, and could be used as a preventive agent of obstructive urolithiasis in sheep.

The large fluctuation in the pH for group G_C, observed at M2 and M2c when compared with other groups, proved the inefficiency of oral vitamin C as a urinary acidifier and in the maintenance of acidic pH in sheep, corroborating the findings of other researchers who tested the dose of 1 g/animal/day (29,30). Additionally, this fact was supported by the comparison with the effectiveness of ammonium chloride for urinary acidification in sheep in this study. The use of vitamin C for urinary acidification was recommended in doses of 3 to 4 mg/kg/day (31), which was the dose used in this experiment; however,
the oral route of the administration does not seem to be the best choice in ruminants because of the difficulty in administration\(^\text{12}\) and the possibility of ruminal degradation\(^\text{32}\). Further, it is economically unfeasible and almost impossible to daily administer vitamin C systemically\(^\text{12}\).

**Urinalysis**

A cloudy aspect was detected in 22 samples from the G\(_A\) group (22/75), in 23 samples from the G\(_{AC}\) group (23/75), and in 34 samples from the G\(_C\) group (34/75), but there was no statistical difference between the groups according to the Chi-square test at any of the five analyzed time points. In M0, the cloudy appearance in the samples of the G\(_{AC}\) (7/15) and G\(_C\) (9/15) groups was coincident with the appearance of crystals in the urine\(^\text{33,34}\), a fact also observed in 8/15 samples at M4 in group G\(_C\).

The G\(_A\) group presented two animals with triple phosphate crystals, one in M1 (rare) and another in M3 (+), and one of amorphous urate in M3 (+++). In the G\(_{AC}\) group, there was only one sheep with amorphous urate in M3 (+++). In the G\(_C\) group, seven animals showed triple phosphate crystals, one in M2 (+), three in M3 (+++), and two in M4 (+), as well as one animal with amorphous urate in M4 (+++). Although the difference between the groups was not significant, it was noted that the animals in the G\(_C\) presented crystalluria even after 15 d of treatment (M2), which persisted up to 21 d (M4) after the administration of vitamin C. Thus, it was noted that acidic urine, regardless of the four (4/150) samples in animals that received ammonium chloride (G\(_A\) and G\(_{AC}\)), prevented the formation of these crystals. Crystals produced in urine are eliminated periodically and are only of diagnostic value if in large quantities or associated with clinical signs of urolithiasis\(^\text{15,24,32}\). Since this disease was absent in this study cohort, the presence of crystals could be related to the diet rich in grains.

Hyaline cylinders were absent in most animals, except for four samples in G\(_A\) (4/75), three in G\(_{AC}\) (3/75), and two in G\(_C\) (2/75) groups. The formation of cylinders is favored by acidic pH and was observed in the time points M2, M3, and M4 in the different groups, when the animals were already under treatment for urinary acidification\(^\text{35}\). According to Garcia-Navarro\(^\text{24}\), the hyaline cylinders are formed exclusively by protein and may be present in small numbers in physiological proteinuria\(^\text{36}\). The possible explanation for this fact is that during renal filtration most proteins are retained because of their high molecular weight; however, they are not completely excluded from the filtrate. Despite this, no clinically significant proteinuria and/or glycosuria was observed among the treated animals.

The values for ketone, urobilinogen, bilirubin, occult blood, and bile salts were within the normal range. As for red blood cells and leukocytes, and other components of the urinary sediment, such as mucus and bacteria, no clinically relevant differences were observed in different groups at various time points. The commonly observed different cell types in the urine sample were from the urethral, bladder, renal cells, followed by the pelvis cells and finally the prostate cells. Some rare cells were observed (one to three/field) at all time points, which was similar in the three experimental groups. Despite being present in the vast majority of samples, their presence is considered normal\(^\text{24,33,36}\).
The density of the samples remained between 1,017 to 1,039 at all time points and in all treatment groups, and was within the normal values for the species (range: 1,015–1,045)\(^{36}\), with no significant difference among groups (Table 1). However, there was a statistically significant difference during the different time points in \(G_{AC}\) group, when the median of urinary density values were lower after a week of supplementation with both acidifiers and remained so until the final measurement. According to Garcia-Navarro\(^{24}\), density measures the concentration of total solids in the urine. From this, we identified that the \(G_{AC}\) group had the least crystalluria among the groups, with only one animal exhibiting amorphous urate at M3.

### Table 1. Urine density of feedlot lambs submitted to supplementation with ammonium chloride (\(G_A\)), ammonium chloride and vitamin C (\(G_{AC}\)), and vitamin C (\(G_C\)), at different times.

| Times | \(G_A\) (n=15) | \(G_{AC}\) (n=15) | \(G_C\) (n=15) |
|-------|----------------|-----------------|----------------|
|       | \(X \pm s\)  | \(md\)           | \(X \pm s\)     | \(Md\)                 |
| M0    | 1027.87 ± 15.83 | 1026.0          | 1038.90 ± 12.83  | 1040.0\(^a\)          |
| M1    | 1030.40 ± 10.32 | 1030.0          | 1033.90 ± 8.42   | 1038.0\(^b\)          |
| M2    | 1028.00 ± 8.94  | 1024.93 ± 11.03 | 1024.0\(^b\)     | 1029.47 ± 8.40         |
| M3    | 1023.33 ± 13.56 | 1024.0          | 1018.40 ± 8.72   | 1018.0\(^b\)          |
| M4    | 1031.60 ± 6.68  | 1032.0          | 1027.73 ± 7.52   | 1026.0\(^ab\)         |

\(^{ab}\) different letters in the column differ statistically among the time points within the group (Dunn’s post-hoc test).

Source: The authors.

### Serum urea and creatinine

Serum values for urea (17.12 to 42.8 mg/dL) and creatinine (1.2 to 1.9 mg/dL) (Table 2) were close to the reference values for sheep\(^{37}\) in all the groups.

At M0 and M1, the animals did not receive treatment with ascorbic acid and ammonium chloride. Therefore, the values obtained could be considered the baseline for the three groups. At M2, M3, and M4, there was a statistically insignificant decrease in urea concentration in the three groups. This decrease in urea can be explained by the action of an acidifying salt, such as ammonium chloride, which produces a diuretic effect, in addition to compensating metabolic acidosis\(^{13}\). With an increased urinary flow, there is a decrease in tubular reabsorption of urea. Consequently, the serum level of urea is lower than that observed with low urinary velocity\(^{38-40}\), although it had normal values in the groups. This explains the lower urinary density obtained after supplementation with acidifiers, especially that of \(G_{AC}\).

Creatinine remained below the normal range at all times and in all groups, so it can be inferred that the acidifying substances administered in this study did not cause damage to the renal tubule cell walls; therefore, creatinine was effectively excreted from the
blood circulation\(^{38}\). Creatinine is a more effective marker of kidney damage than urea, because in healthy animals it is not reabsorbed by the renal tubule cell wall, and is not influenced by diet. Although creatinine was not elevated, not more than 50\% of the nephrons were impaired in this study. Therefore, the combined analysis of urea and the clinical status of the animals indicated that 21 d of treatment did not cause damage to the renal cells\(^{39,40}\).

### Table 2. Urea (mg/dL) and creatinine (mg/dL) concentrations of feedlot lambs submitted to supplementation with ammonium chloride (G\(_A\)), ammonium chloride and vitamin C (G\(_{AC}\)), and vitamin C (G\(_C\)), at different times.

| Times | Variable | G\(_A\) (n=15) | G\(_{AC}\) (n=15) | G\(_C\) (n=15) |
|-------|----------|----------------|------------------|-----------------|
|       |          | \(\bar{X} \pm s\) | \(md\) | \(\bar{X} \pm s\) | \(md\) | \(\bar{X} \pm s\) | \(Md\) |
| M0    | Urea     | 45.73 ± 4.49 | 45.00\(^a\) | 41.93 ± 9.28 | 38.30\(^a\) | 43.42 ± 6.90 | 43.70\(^a\) |
|       | Creatinine | 0.80 ± 0.08 | 0.80\(^a\) | 0.80 ± 0.11 | 0.80 | 0.84 ± 0.15 | 0.80 |
| M1    | Urea     | 42.51 ± 4.74 | 42.50\(^a\) | 40.56 ± 12.79 | 38.90\(^a\) | 46.36 ± 6.39 | 47.20\(^a\) |
|       | Creatinine | 0.74 ± 0.12 | 0.70\(^a\) | 0.72 ± 0.11 | 0.70 | 0.76 ± 0.16 | 0.80 |
| M2    | Urea     | 29.69 ± 10.72 | 29.00\(^b\) | 32.61 ± 11.04 | 27.80\(^ab\) | 29.12 ± 13.87 | 26.60\(^b\) |
|       | Creatinine | 0.77 ± 0.12 | 0.80\(^a\) | 0.73 ± 0.12 | 0.70 | 0.82 ± 0.19 | 0.80 |
| M3    | Urea     | 32.81 ± 4.08 | 33.50\(^b\) | 34.73 ± 4.14 | 33.60\(^ab\) | 35.54 ± 5.07 | 36.40\(^ab\) |
|       | Creatinine | 0.71 ± 0.11 | 0.70\(^a\) | 0.69 ± 0.12 | 0.70 | 0.79 ± 0.13 | 0.80 |
| M4    | Urea     | 23.22 ± 7.10 | 22.20\(^c\) | 29.95 ± 8.94 | 30.70\(^b\) | 29.07 ± 10.34 | 26.20\(^b\) |
|       | Creatinine | 0.67 ± 0.08 | 0.70\(^ab\) | 0.69 ± 0.12 | 0.70\(^AB\) | 0.76 ± 0.08 | 0.80\(^A\) |

Mean (\(\bar{X}\)), standard deviations (s) and medians (md).

\(^{a,b}\) Medians different letters in the column differ statistically among the time points within the group (Dunn’s post-hoc test).

\(^{A,B}\) Medians followed by different capital letters on the line differ statistically among groups within the time points (Dunn’s post-hoc test).

Source: The authors.

### Serum, urinary, and EF measurements of Ca, P, and Mg

There was no statistical difference between the groups or times in relation to serum calcium (Ca) (Table 4), and the mean serum values were below the reference values\(^{37,41}\) for sheep species, similar as that described by Maciel et al. \(^{29}\) in Santa Inês lambs fed a calculogenic diet.
The median serum Ca of animals at M0 was 8.91 mg/dL, 8.59 mg/dL, and 8.53 mg/dL for GA, GAC, and GC, respectively, and they remained close to these values until the end of the experiment. Larsen et al. (42) observed that with a sudden change in feeding to a tender pasture, a reduction in Ca reabsorption could be expected. This may explain the lower values observed during the adaptation period. Its maintenance, over time, could be attributed to the homeostatic mechanism of calcium, which is maintained by the body to improve the efficiency of the absorption of this mineral and increase bone resorption (41). There was variation in the median value of serum calcium in GA, (8.91 mg/dL to 9.24 mg/dL), which is corroborated by the scientific studies which reported that an increase in the acidity of the intestinal tract due to the ingestion of ammonium chloride increased the absorption of calcium, which could be used to prevent puerperal hypocalcemia in cows consuming an anionic diet (7,11).

The mean values for serum P (Table 4) were above the reference values for sheep (5.0 to 7.3 mg/dL) proposed by Kaneko et al. (37). This was justified by the diet rich in grains fed to the animals throughout the experiment. Phosphorus-rich diets increase

| Table 3. Serum (mg/dL), urinary (mg/dL) and FE (%) dosages of Ca of feedlot lambs submitted to supplementation with ammonium chloride (GA), ammonium chloride and vitamin C (GAC), and vitamin C (GC), at different times. |
|---|---|---|---|
| Times | Variable | GA (n=15) | GAC (n=15) | GC (n=15) |
| | | x ± s | md | x ± s | md | x ± s | md |
| M0 | Serum | 8.70 ± 0.74 | 8.91 | 8.59 ± 0.92 | 8.59 | 9.02 ± 1.42 | 8.53 |
| | Urine | 29.70 ± 35.43 | 11.97 | 25.66 ± 33.05 | 8.64 | 30.52 ± 34.91 | 14.04a |
| | FE | 3.45 ± 3.56 | 2.23a | 2.50 ± 3.27 | 1.74a | 4.44 ± 7.67 | 1.99a |
| M1 | Serum | 8.90 ± 0.59 | 8.86 | 9.16 ± 0.98 | 9.23 | 8.88 ± 0.68 | 9.05 |
| | Urine | 84.80 ± 55.70 | 106.62ab | 106.85 ± 13.07 | 103.95ab | 115.79 ± 19.54 | 113.40ab |
| | FE | 6.42 ± 4.91 | 5.98ab | 10.33 ± 7.17 | 6.79ab | 14.60 ± 9.70 | 11.21ab |
| M2 | Serum | 8.88 ± 0.63 | 8.88 | 8.87 ± 0.36 | 8.71 | 9.38 ± 1.87 | 8.62 |
| | Urine | 153.09 ± 29.24 | 151.20c | 150.82 ± 23.96 | 143.64c | 125.44 ± 13.67 | 134.19c |
| | FE | 15.10 ± 7.66 | 14.06bc | 17.97 ± 12.10 | 14.92bc | 12.34 ± 4.95 | 13.32c |
| M3 | Serum | 9.04 ± 0.35 | 9.06 | 8.99 ± 0.44 | 8.97 | 8.85 ± 0.57 | 8.71 |
| | Urine | 161.53 ± 43.84 | 156.87c | 137.47 ± 22.10 | 134.19c | 138.85 ± 10.40 | 139.86c |
| | FE | 25.91 ± 15.86 | 18.77c | 26.95 ± 19.49 | 23.03c | 33.60 ± 55.71 | 19.91b |
| M4 | Serum | 9.20 ± 0.25 | 9.24 | 8.94 ± 0.59 | 8.88 | 9.06 ± 0.90 | 8.97 |
| | Urine | 179.55 ± 19.68 | 171.99bc | 159.52 ± 14.98 | 154.98bc | 135.83 ± 10.17 | 134.19bc |
| | FE | 13.96 ± 4.83 | 12.41bd | 14.39 ± 4.46 | 14.19b | 10.17 ± 5.63 | 8.84a |

Means (x±s), standard deviations (s) and medians (md).
a,b,c Medians different letters in the column differ statistically among the time points within the group (Dunn’s post-hoc test).
A,B,C Medians followed by different capital letters on the line differ statistically among groups within the time points (Dunn’s post-hoc test).
Source: The authors.
serum phosphate, and consequently, increase urinary phosphorus excretion, favoring calculogenesis. Although the concentration of P in the diet of small ruminants is very important to prevent damage from hypophosphatemia, it should be noted that the low Ca:P ratio in the diet also results in hyperphosphatemia, which contributes to the formation of stones\(^{(43)}\). Another important form of P excretion in ruminants is through saliva; therefore, the ingestion of low-quality fibers or in small amounts reduces the production of saliva and can increase the excretion of phosphates by the kidneys\(^{(1)}\).

**Table 4.** Serum (mg/dL), urinary (mg/dL) and FE (%) dosages of P of feedlot lambs submitted to supplementation with ammonium chloride (G\(_A\)), ammonium chloride and vitamin C (G\(_{AC}\)), and vitamin C (G\(_C\)), at different times.

| Times | Variable | \(G_A\) (n=15) | \(G_{AC}\) (n=15) | \(G_C\) (n=15) |
|-------|----------|----------------|-----------------|----------------|
|       |          | \(\bar{X} \pm s\) | \(md\) | \(\bar{X} \pm s\) | \(Md\) | \(\bar{X} \pm s\) | \(md\) |
| M0    | Serum    | 7.65 ± 2.10   | 6.86\(^a\)  | 7.18 ± 1.12   | 7.21\(^b\)  | 7.32 ± 1.31   | 7.39\(^b\) |
|       | Urine    | 33.65 ± 34.88 | 29.40\(^ab\) | 6.19 ± 7.13   | 2.40\(^b\)  | 17.51 ± 42.38 | 1.80\(^b\) |
|       | FE       | 7.47 ± 8.93   | 4.50\(^b\)  | 1.02 ± 1.48   | 0.47        | 3.05 ± 6.32   | 0.26        |
| M1    | Serum    | 19.85 ± 6.16  | 22.17\(^b\) | 19.77 ± 5.83  | 21.47\(^b\) | 20.57 ± 5.93  | 22.35\(^a\) |
|       | Urine    | 2.47 ± 4.84   | 1.00\(^b\)  | 9.65 ± 31.08  | 1.20        | 7.20 ± 9.64   | 1.80        |
|       | FE       | 0.12 ± 0.26   | 0.04\(^b\)  | 0.46 ± 1.44   | 0.05        | 0.43 ± 0.73   | 0.11        |
| M2    | Serum    | 21.38 ± 2.58  | 21.83\(^b\) | 10.71 ± 2.54  | 11.44\(^b\) | 16.60 ± 1.66  | 16.74\(^b\) |
|       | Urine    | 12.20 ± 23.69 | 3.40\(^b\)  | 25.92 ± 54.47 | 2.40        | 10.40 ± 16.63 | 1.60        |
|       | FE       | 0.56 ± 1.32   | 0.13\(^b\)  | 5.11 ± 1.44   | 0.24        | 0.59 ± 0.98   | 0.07        |
| M3    | Serum    | 15.56 ± 1.76  | 16.11\(^b\) | 17.30 ± 2.88  | 17.17\(^ab\)| 10.89 ± 6.23  | 7.63\(^b\)  |
|       | Urine    | 15.35 ± 42.51 | 2.60\(^ab\) | 2.65 ± 4.25   | 1.20\(^b\)  | 42.57 ± 58.49 | 6.20\(^b\)  |
|       | FE       | 1.03 ± 2.36   | 0.20\(^ab\) | 0.21 ± 0.30   | 0.09\(^b\)  | 4.20 ± 6.76   | 1.36\(^b\)  |
| M4    | Serum    | 14.79 ± 1.24  | 14.62\(^b\) | 18.71 ± 1.68  | 19.08\(^b\) | 14.88 ± 1.79  | 15.05\(^b\) |
|       | Urine    | 18.24 ± 1.45  | 14.30\(^b\) | 35.73 ± 48.78 | 4.60        | 12.48 ± 25.19 | 2.00        |
|       | FE       | 0.87 ± 1.45   | 0.14\(^b\)  | 1.84 ± 2.65   | 0.16        | 0.54 ± 1.21   | 0.08        |

Means (\(\bar{X}\)), standard deviations (s) and medians (md).

\(^{ab}\) Medians different letters in the column differ statistically among the time points within the group (Dunn’s post-hoc test).

\(^{A,B}\) Medians followed by different capital letters on the line differ statistically among groups within the time points (Dunn’s post-hoc test).

Source: The authors.

The group of animals that received ammonium chloride (G\(_A\)) initially had an average phosphate of 19.85 ± 6.16 mg/dL in M1. At 14 d (M3), the values dropped to 15.56 ± 1.76 mg/dL, and then to 14.79 ± 1.24 mg/dL in M4. The administration of ammonium chloride was satisfactory in preventing urolithiasis by phosphate urolith by urinary acidification, which makes P soluble; therefore, it hinders its precipitation and the formation of crystals with Ca and Mg\(^{(41)}\).

The animals that received vitamin C supplementation showed an average serum P value
of 20.57 ± 5.93 mg/dL, and after 14 d (M3) it dropped significantly to 10.89 ± 6.23 mg/dL; however, with 21 d of supplementation (M4), the values increased to 14.88 ± 1.79 mg/dL. The same trend was observed for the animals in the G_{AC} group, which started with a mean phosphorus of 19.77 ± 5.83 mg/dL, which at M1 was 10.71 ± 2.54 mg/dL; however, at 21 d experimentation, the mean value was similar to that at M0: 18.71 ± 1.68 mg/dL. Although the initial reduction in phosphorus was significant, vitamin C was not sufficient in acidifying urinary pH and was not effective in reducing the serum P level, even when used in combination with ammonium chloride. Maciel and collaborators\(^{(29)}\) also observed an increase in serum P during the ingestion of an unbalanced diet in lambs supplemented with vitamin C.

The mean serum magnesium values (Table 5) were within the reference values (2.2 to 2.8 mg/dL) established by Kaneko \textit{et al.}\(^{(37)}\) and Radostitis \textit{et al.}\(^{(41)}\), and varied from 2.27 to 2.63 mg/dL, although other authors have observed serum Mg concentrations of up to 3.33 mg/dL in confined lambs\(^{(29)}\).

Throughout the time points, the serum Mg values did not show statistical differences; however, in the G_{C}, an increase was noted when the average value in M0 (2.19 mg/dL) was compared to that in M4 (2.61 mg/dL). It was noted that vitamin C intake increased serum Mg concentration during confinement, which could lead to renal Mg retention and increased P excretion, which in turn increases the ion concentration in the urine and favors urolithiasis\(^{(20)}\). The analysis among the groups showed that, at two time points (M2 and M4), the G_{C} value was similar to that of the G_{A}, and both had medians greater than that of the G_{AC} group. This group, which received both products, exhibited the lowest mean Mg when compared to others, except at M1; however, all values were within the reference values for electrolyte in sheep\(^{(37,41)}\).

There are no normal standards for the concentration of these electrolytes in urine, but other authors have studied the influence of different diets on the occurrence of urolithiasis in goats\(^{(5)}\) and sheep\(^{(29)}\) and described different values corresponding to the amount of the minerals in each dietary ingredient.

There was a statistical difference in urinary Ca concentration between the groups (Table 3) at M1 and M4, when the highest median values were observed in the G_{C} and G_{A} groups, respectively. However, the highest results for urinary Ca occurred in sheep supplemented with ammonium chloride (G_{A} and G_{AC}) in M2, M3, and M4.

Over time, the urinary Ca concentration in the three groups increased from M0 to M2, then remained stable until M4. There was a statistical difference across the time points in the three groups, with the lowest median values at M0 and the highest at M4. This was not observed by Maciel \textit{et al.}\(^{(29)}\), who described a drastic reduction in urinary Ca excretion in Santa Inês lambs during confinement.
Takagi and Block (43) attested that acidogenic diets increased urinary Ca excretion and decreased retention. Braithwaite (44) mentioned that the urinary excretion of Ca was controlled by a renal mechanism, which is affected by pH; thus, acidosis acts directly on the renal tubular cells, causing decreased renal tubular reabsorption of Ca and resulting in lower levels of serum Ca, as previously reported.

Regarding the dosage of urinary P (Table 4), there was a difference at M0, when the GA group had a higher median than that of the others; however, at that time, the animals were adapting to the diet and the environment, and the administration of acidifiers had not begun. Diets rich in phosphorus cause an increase in serum phosphate, and consequently, an increase in its urinary excretion, which favors calculogenesis. However, in healthy ruminants, the excretion of phosphorus is also conducted via feces, whereas in the case of an increase in the serum concentration of this electrolyte, the excretion becomes urinary (45).

The medians of urinary P values exhibited a statistical difference across the time points.
only in G\textsubscript{A}. After 7 d of administration of the acidifiers, a decrease in the urinary P levels was already noticeable. In G\textsubscript{A}, the median ranged from 29.40 mg/dL to 1 mg/dL and remained low until the end of the experiment, illustrating the beneficial effect of the acidifier in preventing hyperphosphaturia.

The concentrations of urinary Mg (Table 5) exhibited a statistical difference across the time points in the three groups and exhibited wide variation; however, the highest median values were observed at M4 in all groups, with the highest value in G\textsubscript{AC}. Similarly, Maciel \textit{et al.}\textsuperscript{(29)} reported a progressive increase in urinary Mg excretion in sheep fed an unbalanced diet.

As previously mentioned, the role of Mg in lithiasis is still debatable. Asplin \textit{et al.}\textsuperscript{(46)} highlighted that Mg is considered an inhibitor of crystallization, nucleation, and growth of calcium oxalate uroliths. Therefore, greater excretion of Mg may indicate greater secretion of this electrolyte by the renal tubules, which could lessen the predisposition to urinary stones\textsuperscript{(29)}. Changes in Mg metabolism are determining factors in the development of urolithiasis, although abnormal phosphorus metabolism is also necessary\textsuperscript{(29)}. These authors described high levels of Mg, P, and low Ca levels as a result of an unbalanced diet, which increased the possibility of urolith formation because of renal Mg retention and increased P excretion, thereby increasing urinary concentration of P.

In general, the medians EF values of the three electrolytes were similar to those observed in the urinary biochemical analysis, with higher calcium excretion (Table 3) and low P excretion (Table 4); however, there was little influence on fractionated Mg excretion (Table 5). The fractional excretion (EF) of urinary electrolytes was determined. According to Caple \textit{et al.}\textsuperscript{(17)}, variation in water absorption and excretion make it difficult to interpret the values of electrolytes in the urine.

Despite the distinct efficacy in urinary acidification between the three treatments, there was no significant difference in FE, indicating that the interventions used did not cause any changes among animals supplemented at different time points. The G\textsubscript{C} exhibited higher results of FE of Ca than G\textsubscript{A}, whereas the G\textsubscript{AC} was similar for both. At M3, the CG exhibited higher FE values of P than did the G\textsubscript{AC} group, and that of the G\textsubscript{A} group was similar to the groups supplemented with vitamin C.

Across the time points, the FE of Ca exhibited a statistical difference, but it behaved similarly in the three groups, with a progressive increase in values from M0 to M3, and a decrease in M4 demonstrating that the treatments provided greater Ca excretion.

Regarding the FE of P, there was a difference only in G\textsubscript{A}, which exhibited a decrease in value from M0 to M1, with lower values continuing until the end of the experiment. Thus, it was demonstrated that the use of ammonium chloride in the diet decreased the excretion of phosphorus, which agrees with the preventive effect of urolithiasis\textsuperscript{(9,10,13)}.

Regarding the FE values of Mg, the median values at all times points and for all groups were similar, illustrating that this was not a good parameter to evaluate treatments.

Under the conditions of the present study, ammonium chloride caused the most rapid decrease in the urinary pH of the lambs and kept it acidic throughout the study period.
Ammonium chloride combined with vitamin C ($G_{AC}$) showed similar effects regarding urine pH as observed in the group treated with only ammonium chloride ($G_A$). Due to the fluctuation in the urinary pH values observed in the group supplemented with ascorbic acid ($G_C$), this was not an efficient treatment for maintaining urinary acidification.

The treatments did not interfere with the parameters evaluated in the urinalysis, nor with the values of urea and creatinine. There was a significant decrease in serum P in $G_A$, as well as a urinary increase in calcium and magnesium levels in this group. Ca FE increased after treatments started, but there was no interference with Mg. FE of P was significantly lower only in $G_A$.

**Conclusions**

In conclusion, the administration of oral vitamin C is not effective in the acidification of urine; therefore, it could be inferred that it may ineffective as a preventive method for obstructive urolithiasis in sheep. However, ammonium chloride was successful in urinary acidification 24 h after its administration; therefore, it can be used to prevent this disease.

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**Conflict of interest**

The authors declare no conflict of interest.

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