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Crystallurical profile in sheep after ammonium chloride supplementation

[*Perfil cristalúrico em ovinos suplementados com cloreto de amônio*]

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ABSTRACT

Although urinary crystals are habitual components, urolithiasis formation is always preceded by these concretions. We aimed to identify the change in the crystalline profile in sheep supplemented with ammonium chloride. Twenty-five male sheep aged three months, feedlot and randomly distributed into three groups were used: Control Group (CG) n = 5 did not receive Ammonium Chloride; G200 Group (n=10) (200mg/kg) of Ammonium Chloride for 56 consecutive days; G500 Group (n=10) (500mg/kg) of Ammonium Chloride for 56 consecutive days, administered daily orally. Sampling times and clinical evaluation were performed at seven days, with M0 (immediately before Ammonium Chloride), M1 (seven days after) until M8, totaling 70 days of feedlot. Urine samples were performed to identify the presence, type, and quantity of crystals. There was an increase in crystalluria in all groups in relation to time due to dietary influence, mainly in the CG, which presented more crystals of amorphous calcium phosphate and calcium oxalate. In addition, the G500 Group presented a higher presence of urate/uric acid crystals after urinary acidification, which are closely related to urinary pH.

Keywords: urinalysis, urinary acidification, crystals, urine, urolithiasis

RESUMO

Apesar de cristais urinários serem componentes habituais, a formação de urolitíase é sempre precedida dessas concreções. O presente estudo objetivou identificar a mudança do perfil cristalúrico em ovinos suplementados com cloreto de amônio. Foram utilizados 25 ovinos, machos, com idade de três meses, confinados e distribuídos aleatoriamente em três grupos: grupo controle (GC) (n=5) não recebeu cloreto de amônio; grupo G200 (n=10) (200mg/kg) recebeu cloreto de amônio por 56 dias consecutivos; grupo G500 (n=10) (500mg/kg) recebeu cloreto de amônio por 56 dias consecutivos, administrados diariamente por via oral. Os momentos (M) de colheita de amostras e avaliação clínica foram realizados com intervalo de sete dias, sendo M0 (imediatamente antes da administração do cloreto de amônio), M1 (sete dias após) até M8, totalizando 70 dias de confinamento. As amostras de urina foram analisadas para se identificar a presença, o tipo e a quantidade de cristais. Houve aumento da cristalúria em todos os grupos em relação ao tempo por influência dietética, principalmente no GC, que apresentou mais cristais de fosfato de cálcio amorfo e oxalato de cálcio. Além disso, o grupo G500 apresentou maior presença de cristais de urato/ácido úrico após acidificação urinária, estando esses intimamente relacionados ao pH urinário.

Palavras-chave: urinálise, acidificação urinária, cristais, urina, urolitíase

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INTRODUCTION

Thirty years ago, it was impossible to imagine sheep farming as an organized and profitable business in Brazil. At the time, production of small ruminants was seen as a secondary activity (Aquino Neto *et al*., 2007). The intensification of production, combined with commercialization of animals of high genetic value, led to profound changes in the feeding management of sheep, triggering an increase in occurrence of nutritional and metabolic diseases, and among the main ones, obstructive urolithiasis stands out (Guimarães *et al*., 2012). Considered as the most important disease of urinary tract of ruminants, it particularly affects males, and causes serious economic losses related to premature exit of animals from breeding, treatment costs, death of affected animals and condemnation of carcass in emergency slaughter (Guimarães *et al*., 2012).

The pathological process characterized by presence of stones or concretions in the urinary system is called urolithiasis. Disease becomes clinically important in ruminants when stones cause urinary tract obstruction, which normally occurs in the urethra and rupture of urethra or bladder will occur in 2-3 days if the obstruction is not relieved (Constable *et al*., 2016). Uroliths occur in animals of either sex, but obstruction rarely occurs in females due to short, unflexed, and larger-diameter urethra (Van Metre and Divers, 2006). Sigmoid flexure, ischial curvature, and urethral process of sheep and goats are the most common sites for uroliths to lodge and cause obstruction (Van Metre and Divers, 2006). Sheep and goats castrated at an early age are more susceptible, as they have less development of urethra, which is of smaller caliber than urethra of non-castrated animals (Constable *et al*., 2016). All sheep breeds are susceptible (Van Metre and Divers, 2006), however, Texel sheep are particularly efficient in absorption and excretion of minerals, when compared to Suffolk and Blackface breeds, being more predisposed to urolithiasis due to high concentration of minerals in urine (Guimarães *et al*., 2012). First signs of urolithiasis are anorexia and meteorism, abdominal pain, muscle weakness, apathy, anuria, or dysuria with passing a few drops of bloody urine (hematuria) after straining to urinate, pain on palpation of inguinal region, tachycardia, tachypnea, and blood vessels engorged scleral nerves (Van Metre and Divers, 2006). Urethral obstruction is not a result of stone formation but is predisposed by anatomical factors (Ewoldt *et al*., 2008). After the appearance of clinical signs, reversal of condition is difficult, and surgical treatment may be necessary when, in most cases, animals become unfit for reproduction (Ewoldt *et al*., 2008). Such economic limitations, due to prolonged clinical therapy and difficult surgical access, often lead to the animal being discarded (Ewoldt *et al*., 2008). This implies an economic loss to the producer, because in addition to the animal, genetic material of high zootechnical value is also lost (Aquino Neto *et al*., 2007).

Crystals are frequent components of the urinary tract and stone formation is invariably preceded by these substances, which can lead to urinary obstructions and consequent need for invasive treatments, with risk of renal loss and death (Frochot and Daudon, 2016). In this sense, prevention of crystallogenesis becomes important to maintain the reproductive integrity of the animal (Van Metre and Divers, 2006; Ewoldt *et al*., 2008). The use of ammonium chloride (AC) in sheep aims to promote urinary acidification and, therefore, reduce the incidence of urolithiasis, since these animals develop uroliths because of alkaline pH, which is influenced by diet, geographic location, water restriction, temperature, among others (Videla and Van Amstel, 2016). Urinalysis is an inexpensive and accessible test and should be performed by a qualified professional under ideal conditions, preferably using a microscope with polarized light (Daudon and Frochot, 2015). It is hypothesized that urinary acidification in sheep supplemented with AC can lead to changes in the crystalluric profile, mainly regarding the presence, type and number of crystals. Thus, the study aimed to identify the change in the crystalluric profile in sheep supplemented with ammonium chloride.

MATERIALS AND METHODS

The project was approved by the Ethics Committee on Animal Experimentation at the School of Veterinary Medicine at UNESP in Araçatuba, FOA process no. 2015-00635–CEUA. Twenty-five male, non-castrated, Dorper, Santa Inês and Morada Nova sheep

were used, aged approximately three months, feedlot, randomly divided into three groups: CG (n=5) control, G200 (n=10) 200mg/kg AC/animal/day and G500 (n=10) 500mg/kg AC/animal/day over 56 days. Experimental groups (G200, G500 and CG) were evaluated for incidence of crystals throughout the experiment according to the protocol below, divided into the following moments (M), namely:

M0 - Before the beginning of AC ingestion (14 days of confinement)
M1 - 7 days of AC ingestion in groups G200 and G500 (21 days of confinement)
M2 - 14 days of AC ingestion in groups G200 and G500 (28 days of confinement)
M3 - 21 days of AC ingestion in groups G200 and G500 (35 days of confinement)
M4 - 28 days of AC ingestion in groups G200 and G500 (42 days of confinement)
M5 - 35 days of AC ingestion in groups G200 and G500 (49 days of confinement)
M6 - 42 days of AC ingestion in groups G200 and G500 (56 days of confinement)
M7 - 49 days of AC ingestion in groups G200 and G500 (63 days of confinement)
M8 - 56 days of AC ingestion in groups G200 and G500 (70 days of confinement)

Animals and experimentation environment - Model developed by Ferreira *et al*. (2014), being modified in the following aspects, such as: individual administration of AC; highest dose administered; not supplying mineral salt diet, thus avoiding alteration or influence on measured urinary pH; and, finally, longer evaluation time (56 days). All animals were dewormed (Monepantel 2,5mg/kg, oral) and vaccinated against clostridiosis (Glanvac 1mL, SC). Previously, they were adapted to the environment in which the work would take place (northwest region in State of São Paulo) for about 14 days, whether in relative humidity conditions, temperature, and luminosity, for a total period of 70 days of confinement. The area was 730 square meters, corresponding to 29.2 square meters per animal, with daily cleaning. On Tuesdays, animals were weighed to establish the amount of AC that would be administered in a week, as well as physical examination of all animals, such as heart rate (HR), respiratory rate (RR), rectal temperature (RT) and ruminal movements (RM), in order to assess the health status of the animals, according to the recommendations by Feitosa (2020). On Wednesdays, urine and blood samples were collected from all animals, and samples were kept and stored in a refrigerated environment, with subsequent (and immediate) delivery to laboratory for analysis. On Thursdays, ultrasound evaluation of all animals was performed. AC was administered orally daily, based on weight and group studied, and diluted in 20mL of distilled water. The CG animals received only 20mL of distilled water.

Feed was provided daily in the morning at a ratio of 0.5m trough/animal. Mineral salt was not provided. Water was made available in two troughs, ad libitum. Commercial feed (LB Total diet for sheep) was used and sampled according to guidelines from the analysis company (Arasolo - www.arasolo.com.br), which was submitted to chemical analysis, being characterized by the following DM levels: 16.19% of crude protein, 3.27% of ether extract, 15.67% of crude fiber and 5.87% of minerals. Mineral analysis of the feed was performed. Calcium and phosphorus dosages were 12,900 ppm and 2,400 ppm of DM, respectively, which resulted in a Ca:P ratio of 5:1. The average daily feed consumption was 3% of BW, with an average experimental period of 1.15kg of total feed/day/animal. Clinical, laboratory (urine), and ultrasound assessments were performed weekly, between 7-9 am.

Collection of urine samples - Samples were collected with natural or forced urination, interrupting breathing with occlusion of nostrils for 10 to 20 seconds, as described by Garcia-Navarro (2005). The samples were placed in sterile flasks, stored in refrigeration at 4 to 8^{o}C for up to 2 hours after collection, and always processed by the same researcher. Volume (mL), color, odor, appearance, density, pH, proteins, glucose, acetone, urobilinogen, occult blood, and bile salts were evaluated using a reagent strip. The urine (5mL) was centrifuged at 7000 revolutions per minute for five minutes, discarding supernatant, leaving 0.5 mL of urine for sediment analysis, including identification of urinary tract cells, red blood cells and leukocytes, in addition to casts, crystals, bacteria and mucus, through optical microscopy with polarized light, with a magnification of 400 times. The urinary crystals found were classified according to their quantity as: rare

(less than 10 crystals per field), frequent (10 to 30 crystals per field) and numerous (above 30 crystals per field) and identified according to their birefringence and format.

Ultrasound Examination - Portable ultrasound device DP-2200 Vet Mindray was used, with a convex transducer and a frequency of 5.0 MHz, on a veterinary stretcher, after manual restraint, in contralateral decubitus position to evaluated kidney, assuming a dorsal position when evaluating bladder and with limbs abducted pelvises (Stockham and Scott, 2008). If there was suspicion of urethral obstruction, a new evaluation was performed. Conductive gel was used in a transducer for better evaluation and images were recorded and analyzed in real time and rigorously afterwards.

Necropsy of animals – The three animals sampled in each group were necropsied after the study period, aiming to verify if there were alterations in the urinary system. The protocol used was: 0.2mg/kg of xylazine, intravenously, for sedation. After lying down 10mg/kg of thiopental (5% solution) was administered intravenously. Then, 1mL/kg of potassium chloride (19.1% KCl amp, 2.5mEq/mL) was injected intravenously.

Statistical Analysis - Urinalysis data were analyzed by the Action 2.7 (Portal Action – Estaticamp) software, with a significance level of 5% ($p<0.05$). Continuous variables were analyzed using the Kruskal-Wallis test to identify differences between groups within same time of collection (M) and, when there was a statistically significant difference, verified using Dunn's post-hoc test. Medians were evaluated by Friedman test in eight moments, namely: Urinalysis: volume, density and pH. Categorical variables were evaluated using chi-square test: Urinalysis: color, appearance, protein, blood, bacteria, crystals, casts, mucus, red blood cells and leukocytes. Anova showed no statistical differences between groups ($p>0.05$).

RESULTS AND DISCUSSION

The data in the present study were collected from an experiment carried out previously (Navarro, 2016) and recently published (Navarro *et al*., 2021). The animals remained throughout the experiment without changes on physical examination (FC, FR, MV and TR), except for one animal that died in the G500, at M6. This lamb presented ataxia, reduced rumen mobility and apathy. Vitamin B1 and B12 were administered, associated with dexamethasone, due to the hypothesis of polyencephalomalacia, not responding to therapy. It underwent necropsy (N313-16) with a report of acute pneumonia, subacute enteritis and mild to moderate multifocal tubular necrosis. Average daily weight gain remained within usual range in all experimental groups, without interference even in the group that received a dose of 500mg/Kg per day/animal, corroborating previous studies (Ferreira *et al*., 2014). All urinary parameters analyzed remained within normal limits in all groups, with small variations and without statistical significance, except for urinary pH (Table 1), urinary crystal count and types.

Table 1. Means (*m*), standard deviations (*s*) and medians (*Md*) of urine pH of sheep during moments (M) in the experimental groups (CG, G200 and G500)

	CG (n=5)		G200 (n=10)		G500 (n=10)	
	m ± *s*	*Md*	*m* ± *s*	*Md*	*m* ± *s*	*Md*
M0	9.0±0.00	9.0^{Aa}	8.7±0.95	9.0^{Aa}	8.6±1.26	9.0^{Aa}
M1	8.5±1.11	9.0^{Aa}	8.1±1.00	8.0^{Aa}	7.0±1.70	6.8^{Bb}
M2	9.0±0.00	9.0^{Aa}	9.0±0.00	9.0^{Aa}	8.5±1.30	9.0^{Aa}
M3	9.0±0.00	9.0^{Aa}	6.3±1.70	5.5^{Bb}	6.3±1.49	6.0^{Bb}
M4	9.0±0.00	9.0^{Aa}	8.7±0.90	9.0^{Aa}	8.6±1.30	9.0^{Aa}
M5	9.0±0.00	9.0^{Aa}	9.0±0.00	9.0^{Aa}	8.3±1.49	9.0^{Aa}
M6	8.1±0.41	8.0^{Aa}	8.2±0.35	8.5^{Aa}	6.6±1.05	6.2^{Bb}
M7	8.1±0.22	8.0^{Aa}	7.4±0.95	7.7^{Ba}	6.2±1.06	6.0^{Bb}
M8	8.7±0.62	9.0^{Aa}	8.5±0.54	9.0^{Aa}	6.3±1.80	5.0^{Bb}

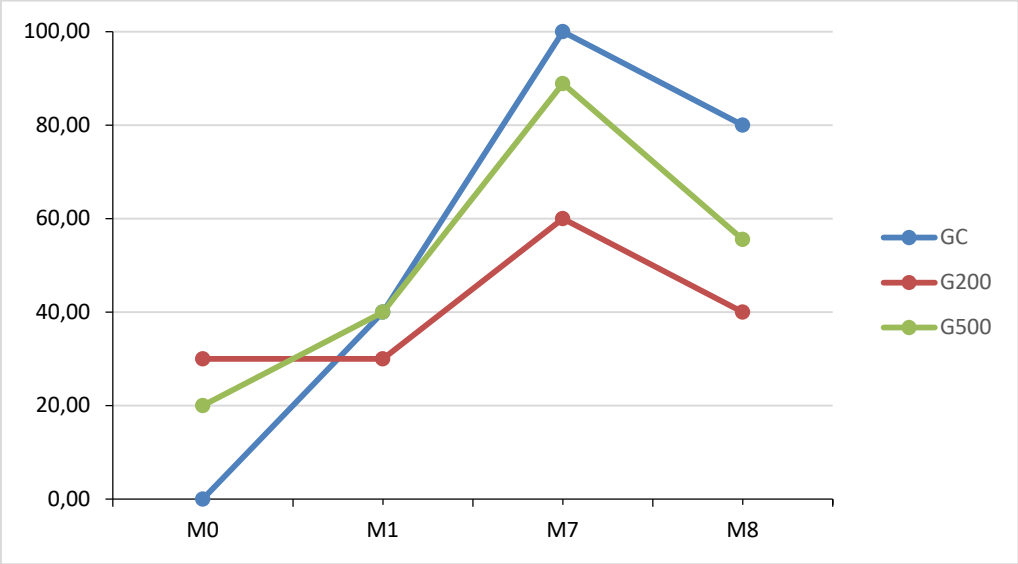
[ab] Different lowercase letters indicate difference between groups at each moment.
[AB] Different capital letters indicate difference between moments in each group.

Urinary pH of CG showed little variation throughout the study, contrary to what happened with G200 and G500. At beginning of *the* experiment and without administration of CA, alkaline pH of urine was observed in all groups, which is usual in sheep (6-8,5) (Garcia-Navarro, 2005). In M1 of G500 there was urinary acidification and during M3, G200 and G500 presented urinary acidification, but without maintenance, whose reasons require further investigation. There was permanent urinary acidification in G500 from M6 onwards, with a similar behavior until the end of the experiment, corroborating the findings of Mavangira *et al*. (2010), who found urinary acidification for more than 24 hours with the use of this dose.

An increase in number of urinary crystals was observed in all groups throughout the study (Figure 1), especially in the Control Group, which started the experiment without the presence of crystals, having evolved to up to 100% of affected animals (M7) and ending the study with 80% of them with crystals (Figure 2), mainly of Amorphous Calcium Phosphate (80% in M8) and of calcium oxalate (20% in M8). The presence and type of crystals, as well as interference of the urinary medium (acid or alkaline), can determine the formation of lithiasis (Garcia-Navarro, 2005). In this aspect, diet, mainly through the intake of proteins and salt, can influence the type of crystals. This increase in the number of crystals likely occurred due to the imbalance found in the bromatological analysis of the diet provided, since it presented a Ca:P - 5:1 ratio, far beyond recommended, which could lead to calciuria and, consequently, to the formation of crystals dependent on levels of calcium, as in the case of animals in CG, having presented calcium crystals in all animals at end of the study.

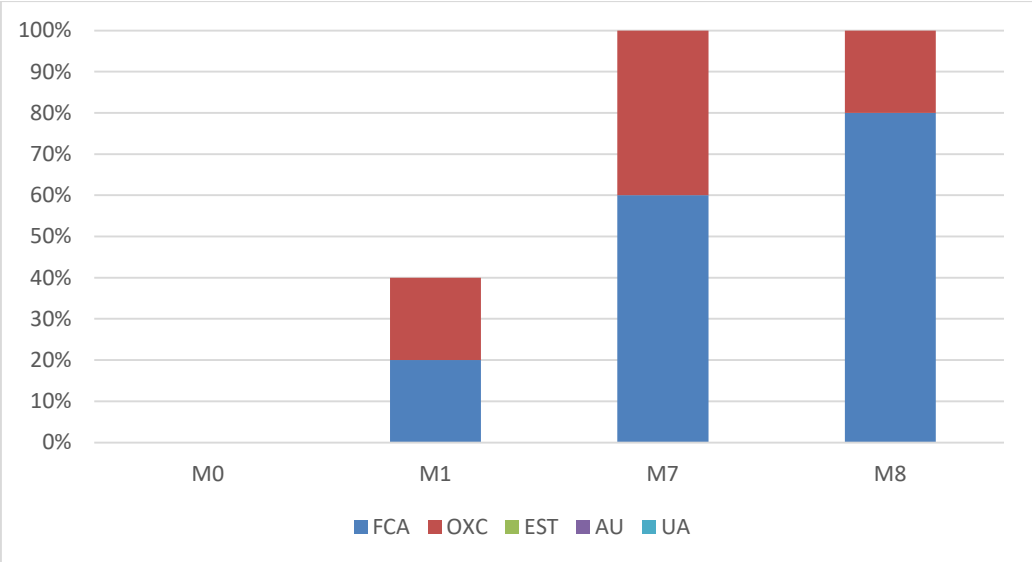
When using diets rich in grains and phosphorus, there may be an increase in struvite uroliths. Jones *et al.* (2015) developed an experimental model in goats proving this statement, using a ratio of 1:1.5 Ca:P. Santarosa *et al*. (2016) also found a predominance of struvite crystals in urine samples in the control group when compared to the group that received AC. Despite Ca:P ratio being favorable to the induction of uroliths of this type (1:1.33), this occurred despite animals having already started the study with acidic pH. This type of crystal was seen in 7.5% (21/280) of urine samples from animals in AC group and in 25.7% (36/140) in control group, suggesting a protective effect of AC. Mavangira *et al*. (2010) also observed a greater number of crystals (struvite and calcium oxalate) in animals that did not receive AC (27% X 8%), demonstrating a protective effect on crystallogenesis in supplemented animals. Furthermore, when analyzing number of crystals in samples, only the control group had numerous crystals in urine. However, authors did not evaluate pre-treatment urine samples, making their interpretation difficult. Riet-Correa *et al.* (2008) suggested the use of AC as a way to acidify urine, in addition to preventing precipitation of phosphates, although there should be a ratio around Ca:P 2:1. Additionally, sodium chloride can be used in a proportion of up to 4% of ration, as a way of stimulating diuresis, although the use of mineral salt should be avoided due to high concentration of phosphorus and magnesium (Riet-Correa *et al.*, 2008). Diet acidification increases production of diluted urine and decreases the concentration of precursors in the same, mainly to prevent struvite and calcium phosphate stones (Ewoldt *et al.*, 2008). Stratton-Phelps and House (2004) and Jones *et al.* (2009) have adopted dietary modification strategies, using an anionic diet as a form of urinary acidification, especially considering frequency of struvite stones. However, this strategy predisposes to an increase in calciuria and, consequently, a greater risk of calcium stones (Stratton-Phelps and House, 2004; Mavangira *et al*., 2010). Latter obtained a urinary pH lower than 6.5 in goats with a dose of 450mg/kg of AC/day and observed greater urinary calcium excretion in these animals, which suggests that this may also have occurred in the present study due to association of AC to diet with increased Ca:P ratio, but appearance of calcium crystals in G500 was not observed, probably because of urinary acidification. In a recent study, Jones *et al*. (2017) analyzed chemical composition of uroliths from goats and sheep with obstructive urolithiasis through optical crystallography and infrared spectroscopy and found a high association of calcium with struvite stones (13/36). The presence of calcium in stones that were initially expected to be just struvite, most reported composition in urinary obstruction of

small ruminants consuming a high-grain diet, may promote changes in therapeutic decision-making in such cases, especially in terms of changing urinary pH (Constable *et al*., 2016). Thus, it is suggested that acidification be monitored with repeat urinalysis and for a determined period to reduce the chances of complications.



Data didn't show significant differences between the groups (p >0.05).

Figure 1. Percentage of urine samples with crystals in sheep during moments (M) in the experimental groups, control group (CG), group receiving 200mg/kg (G200) and group with administration of 500mg/kg (G500).

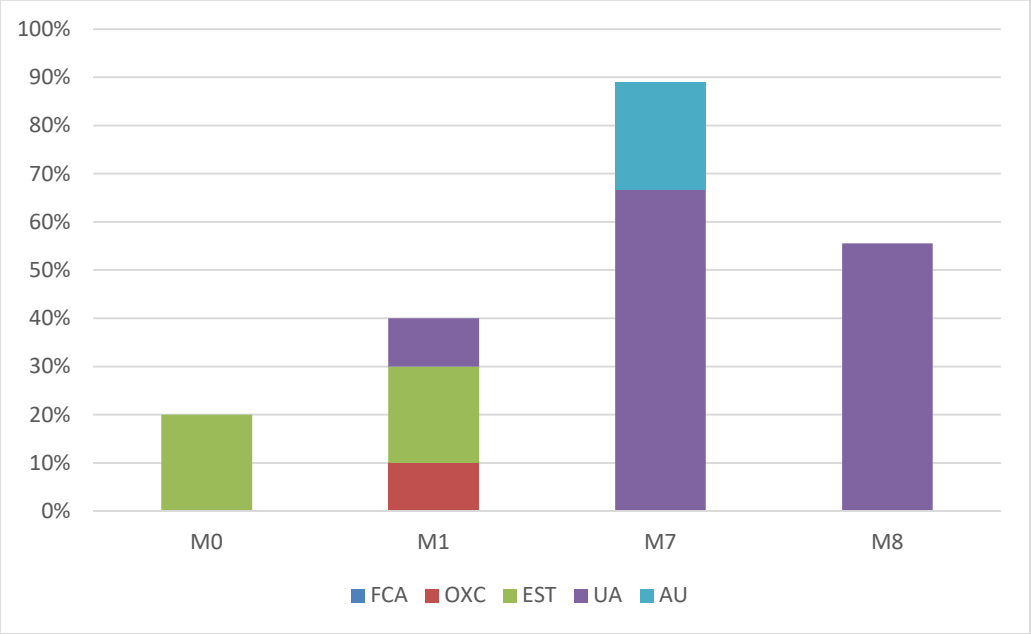


Legend: FCA – Amorphous Calcium Phosphate, OXC- Calcium Oxalate, EST-Estruvite, AU - Uric Acid, UA-Amorphous Urate

Data didn't show significant differences between the groups (p >0.05).

Figure 2. Percentage and types of crystals in the urinary samples of sheep during moments (M) in the control group (CG)

At the beginning of this study, before administration of AC, 20% of animals in G500 had struvite crystals (Figure 3), which are common in alkaline media, having disappeared at the end of study, when crystals of uric acid and amorphous urate were found in abundance in this group after urinary acidification. These crystals are extremely dependent on acidic pH and are therefore rare in sheep. If, on the one hand, this strategy contributed to prophylaxis of most common crystals in sheep (struvite, phosphate) (Mavangira *et al.*, 2010; Ferreira *et al.*, 2014), it may incur the development of crystals based on urate/uric acid and, potentially, if stones develop leading to urinary obstruction. According to this study, it is strongly suggested that from induction of acidic pH, there is a routine monitoring of urine through urinalysis (mainly of sheep in confinement, with a diet rich in grains and a history of stone formation) so that acidification is stopped immediately if potential damage is identified, acting in a prophylactic manner. In addition, it is suggested that urinary acidification should be performed for a short period, reducing the chance of complications from this therapy. In any case, additional long-term studies are needed to confirm this hypothesis. In the present study there was no formation of uroliths in kidneys and urinary tract, evaluated by ultrasonography or even by necropsy by sampling animals from all groups. Different findings by Ferreira *et al*. (2014) who observed the formation of stones in three groups (23/100 of the cases), with the highest percentage in the control group, demonstrating greater protection with the use of AC in the prevention of urolithiasis. It should be noted that urinary acidification was obtained from beginning of the experiment on all groups, probably caused by a diet rich in grains, with little roughage and use of mineral salt ad libitum. In addition, chemical analysis of stones found at necropsy showed a large amount of calcium in samples, despite a large amount of struvite crystals being identified. This fact infers the possibility of mixed lithiasis, corroborating findings by Jones *et al*. (2017).



Legend: FCA – Amorphous Calcium Phosphate, OXC- Calcium Oxalate, EST-Estruvite, AU - Uric Acid, UA- Amorphous Urate
Data didn't show significant differences between the groups (p >0.05).

Figure 3. Percentage and types of crystals in the urinary samples of sheep during moments (M) in the G500 group.

Prevention of uroliths and their complications is extremely important. For this purpose, urinalysis and assessment of presence of crystals, as well as their type and quantity, should be used. However, the presence of crystals alone is not a conclusive diagnosis for the presence of lithiasis, as some patients without stones have crystals in their urine (Robert *et al*., 1998), although these crystals are reported to be smaller and less aggregated when compared to those found in urine samples from repeat stone formers. A human study evaluated 188 patients with multiple urine samples and showed that having 50% or more urine samples with crystals was predictive of stone recurrence with a sensitivity of 88% and a specificity of 84% (Daudon *et al*., 2005). These authors proposed a "crystaluria index" defined as the ratio of the number of urine samples containing crystals to total number of samples examined in a given patient. A crystalluria index > 0.50 may be indicative of persistent lithogenic activity and risk of recurrence of lithiasis. Even because, in stone-forming patients, the disappearance of crystalluria indicates that lithogenic activity is under control, especially in calcium or uric acid uroliths (Daudon and Frochot, 2015). Under these conditions, a decrease in number of crystals present in urine samples is often enough to reduce the lithogenic process. Presence of crystals in urine shows that the balance between solutes and promoters on the one hand and crystallization inhibitors on the other, has tipped towards precipitation. Such as, crystalluria may provide evidence of urine's propensity for lithiasis formation. Daudon *et al*. (2005) studied in human presence of crystalluria in a routine urine sample in asymptomatic nephrolithiasis patients with those who had already had at least one renal crisis and showed a higher incidence of crystals in the latter, suggesting that serial search for crystals should be a useful tool in these cases. Furthermore, investigation of crystalluria is an inexpensive and valuable tool for detection and monitoring of inherited and acquired diseases associated with urolith formation or impairment of acute or chronic kidney function due to crystal precipitation. In this way, research on new methods (such as the automation of crystal counting), standardization of notification criteria and how to apply test results for diagnosis and treatment of patient will be important for dissemination of crystalluric determination in treatment of lithiasis (Willians *et al*., 2021). Thus, there was confirmation of hypothesis that under the conditions of the present study in those animals supplemented (G500) after urinary acidification regarding change in type and quantity of crystals. Therefore, we strongly recommend follow-up through serial urinalysis in those animals submitted to urinary acidification so that the crystallurical profile change is identified and can rotate preventively to avoid its potential complications. As limiting factors, we can mention three different breeds of sheep studied, urinary acidification isolated in G200 and G500 in M3, without such an obvious explanation and obtaining acidification only after 42 days of AC ingestion.

In conclusion, an increase in incidence of urinary crystals was observed in relation to time in all groups studied, especially in the CG. Meanwhile Group 200 (G200) did not show permanent urinary acidification and urinary acidification obtained by ammonium chloride supplementation in G500 promoted greater formation of crystals, due to acidic pH, such as uric acid and urate.

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