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Effect of Dietary Cottonseed Meal on the Occurrence of Urolithiasis in Chinese Merino Sheep from Xinjiang Region (China): A Pilot Study

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Abstract: The objective of this 90 days study was to establish possible association of addition of cottonseed meal with occurrence of urolithiasis in sheep. Total 18 chinese merino male sheep were allotted randomly to three dietary treatments which were the control (C, corn-soybean meal), corn-cottonseed meal A (CA, 461.5 g day^{-1} cottonseed meal) and corn-cottonseed meal B (CB, 661.5 g day^{-1} cottonseed meal), respectively. Total 5 sheep with urolithiasis (urinary tract blockage) were observed in CB group. Blood and urine samples were obtained from three treatments every 30 days intervals. Cottonseed meal diets significantly increased the levels of blood Magnesium (Mg) ($p<0.05$), potassium (K) ($p<0.05$), Phosphorus (P) ($p<0.05$). The higher levels of blood magnesium (Mg) ($p<0.05$), potassium (K) ($p<0.05$) were observed in sheep with urolithiasis (4 bladder stones and one kidney stone) while the level of blood Phosphorus (P) ($p<0.05$) was low in comparison with that of sheep given cottonseed meal. There were some prismatic crystals with granules similar to Potassium Magnesium Phosphate ($KMgPO_4.6H_2O$) and ammonium magnesium phosphate ($NH_4MgPO_4.6H_2O$) in bladder and urine sediments. In this study, high intake of cottonseed meal with increase of Mg, K and P may contribute to urolithiasis under the condition of this study, high level cottonseed meal is a good dietary model for inducing urolithiasis in sheep.

Key words: Sheep, urolithiasis, magnesium, potassium, cottonseed meal, intake

INTRODUCTION

Urolith is the serious disease in domestic animals and human (Bartges, 1998; Gutierrez *et al*., 2002; Heilberg and Schor, 2006; Picavet *et al*., 2007; Macbeth, 2008). It causes many economic losses in weathers and steers. Over the years, there has been increasing interest in the pathogenesis of urinary stone formation. Most of the calculi are composed of minerals. The minerals tend to crystallize when they are at a high level then aggregate and develop into calculi in urine. Many animal researchers had reported that high amount of concentrate feeding and dietary mineral imbalance especially high amount of Mg, K, P intake, contributed to the formation of struvite (Wang *et al*., 1997). In addition, low Ca/P ratio had also been given more attention in the aetiology and prevention of urinary stone formation.

Xinjiang is abundant in cotton, cottonseed byproduct are facilitated to become a kind of high protein feed for ruminants while cottonseed meal (an important byproduct of cottonseed) contains imbalance minerals. We found sheep fed diet containing high level cottonseed meal had high occurrence of urolithiasis in the previous investigation of urolithiasis in Xinjiang. Urolithiasis is the major cause of death for sheep in the Xinjiang region. Wang *et al*. (1993) reported that struvite was the main component of urinary calculi in water buffalo fed a diet containing high level cottonseed meal and found crystals of some bladder stones and urinary sediments which were rich in potassium seemed to be chemically different from struvite. Wang *et al*. (1997) found the newly prismatic crystals which were rich in Magnesium (Mg), Potassium (K) and similar to struvite in crystals structure were identified as potassium magnesium phosphate ($KMgPO_4.6H_2O$) in some bladder stones and urinary sediments. However, very few studies describe the changes of blood and urinary minerals caused by feeding cottonseed meal to sheep. Why did lots of sheep die of urolithiasis in the Xinjiang region? The reason for

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inducing urolithiasis might be related to the diet containing high cottonseed meal to sheep. Therefore, some studies to find the factors causing urinary calculi are very necessary now. Accurate analysis of changes of urine and blood are essential for understanding the precise etiology of urolithiasis and establishing valid medical management. On the basis of the diet of the previous observed sheep with urolithiasis, we fed sheep a traditional diet containing high level of by product of cottonseed (cottonseed meal) in the present experiment. The diet provided a high level of protein, low level of energy and minerals imbalance. The experiment reported herein was conducted to determine the influence of cottonseed meal as dietary protein feed on the changes of blood and urinary minerals and formation of urinary calculi in sheep.

MATERIALS AND METHODS

Animals and diets: The experimental protocol was approved by state and local laws and regulations. Eighteen, 5 months old, Chinese Merino male sheep (23.0±2.0 kg initial body weight) were randomly and allocated randomly to three groups. During a 15 days pre-experimental period, the sheep were fed corn-soybean meal-hay basal diet. We devised, the experimental diets by making modifications according to sheep nutrition requirements (NRC, 1985) and the diet of the sheep with urolithiasis from the previous investigation of urolithiasis in xinjiang region. Sheep were assigned to three dietary treatments (corn-soybean meal (C) (Control), corn-cottonseed meal A (CA, 461.5 g days^{-1} cottonseed meal), corn-cottonseed meal B (CB, 661.5 g days^{-1} cottonseed meal)) for a 90 days study. The diet ingredients were shown in Table 1. The diets were made available in a self-feeder thrice a day (at 8:00, 13:00, 20:00). Salt and water were offered *ad libitum*. The hardness of water accorded with the standards (200 ppm) recommended by WHO.

Table 1: Ingredients and composition of pre-testing and the experimental dit* (DM basis)

	Groups**		
Ingredients (g day^{-1})	C	CA	CB
Corn	210.00	210.00	210.00
Soybean meal	261.50	-	-
Cottonseed meal***	-	461.50	661.50
Wheat bran	64.00	64.00	64.00
Limestone	10.50	10.50	10.50
Salt	4.00	4.00	4.00
Silage corn	750.00	750.00	750.00
Hay	100.00	100.00	100.00
Alfalfa meal	300.00	-	-
Total	1500.00	1500.00	1500.00
Composition (g kg^{-1} DM)**			
Crude protein	130.11	138.13	187.03
Calcium	6.45	3.57	3.85
Phosphorous	3.23	2.04	4.33
Ca:P ratio	1.95	1.75	0.89
Magnesium	2.30	2.78	3.60
Potassium	8.90	11.95	13.90

*The experimental diets devised according to making modifications according to sheep nutrition requirements (NRC, 1985) and the diet of the previous sheep with urolithiasis investigated in Xinjiang region. **C: Corn-soybean meal diet; CA: Corn-cottonseed meal A diet (461.5 g day^{-1} cottonseed meal in a diet); CB: Corn-cottonseed meal B diet (661.5 g day^{-1} cottonseed meal in a diet). ***Cottonseed meal contain 0.81% P, 1.465% K, 0.612% Mg and 0.21% Ca on the basis of DM. ****Measured value

Clinical observation, blood and urine samples preparation and analysis: Blood samples were obtained by jugular venepuncture into heparinised blood tubes on days 0, 30, 60 and 90 and centrifuged at 3000 rpm for 10 min to obtain serum and stored frozen until analysis. Sheep of each group were placed in steer metabolism cages for a period of 24 h for urine collections on days 0, 30, 60 and 90 of this experiment. In anuretic sheep urine was obtained by bladder puncture with 20-gauge needle during days 1-2 after the occurrence of dysuria (days 78-85 of the experiment) at the same time blood samples were collected. Urine pH value was determined by pH meter (Model IQ 140 pH Meter, IQ Scientific Instruments Inc., Carlsbad, CA) and then centrifuged at 1000 rpm for 10 min to obtain the supernatant and sedimentary crystals. The supernatant of samples were transferred to a freezer at -20°C as soon as possible to keep them stable. The crystals were washed twice with deionized water and air-dried for further analysis. Samples of serum were analyzed for P, Mg, Ca by Beckman CX5PRO fully-automatic biochemical analyzer (biochemical analysis reagent, Beckman Coulter, Inc., Fullerton, CA, USA) using commercial kits (ZhongSheng BeiKong bio-technology and science Inc.) and level of K was analyzed by using flame photometry (6400-A, Shanghai). During the experiment all experimental sheep were clinically examined on a daily basis focusing especially on signs of urolithiasis. Affected sheep were necropsied 2-5 days after the onset of dysuria were sacrificed by exsanguination at end of the trail. The sheep were anesthetized with thiopental sodium (15 mg kg^{-1}) prior to bleeding out. Total 5 sheep developing urinary tract blockage (named as urolithiasis sheep) were sacrificed when death appeared imminent in group CB. The urethra, bladder and kidney of sheep were carefully examined and the calculi samples were obtained from the sheep with urolithiasis for further analysis.

Calculi: Total 5 urinary tract blockage sheep (4 bladder stones and one kidney stone) were observed in group CB fed high level cottonseed meal. Sheep with urolithiasis were slaughtered when death appeared imminent. About 4 bladder stones and one kidney stone samples were collected for further analysis.

Statistical analysis: Values were expressed as mean±SD of six animals. Statistical analysis of the resulting data was performed using SPSS version 15.0 and the data were subjected to one-way analysis of variance to compare variables among treatments. Significant differences ($p<0.05$) were reanalyzed by Turkey's multiple-range test.

RESULTS

Blood and urine: In this experiment, the change of serum mineral levels is shown for the same group on days 0, 30, 60 and 90 and urolithiasis sheep group (Table 2). Serum magnesium and potassium levels were raised in urolithiasis sheep (urinary tract blockage observed in group CB) compared with the others whereas serum phosphorus declined. From day 30 to the termination of experiment, serum magnesium, potassium and phosphorus levels were significantly increased ($p<0.05$) in groups CA and CB. Magnesium and potassium levels in urolithiasis sheep were higher ($p<0.05$) than that in others at all time. It is clear that serum magnesium and potassium levels were affected by increase of cottonseed meal.

The effect of cottonseed meal in diet on serum mineral elements is shown in Table 3. In the group CB,

Table 2: The dynamic changes of blood mineral elements of Chinese Merino sheep fed dietary cottonseed meal *, **(n = 6)

Element (mmol L^{-1})	Day	Group*** C	CA	CB
Ca	0	2.49±0.10^{ab}	2.49±0.24^{a}	2.39±0.18^{a}
	30	2.36±0.19^{a}	2.26±0.24^{a}	2.43±0.11^{a}
	60	2.58±0.17^{b}	2.47±0.25^{a}	2.28±0.23^{a}
	90	2.66±0.07^{b}	2.48±0.02^{a}	ND
	Urolithiasis	2.55±0.14^{ab}	2.55±0.14^{a}	2.55±0.14^{a}
P	0	2.18±0.15^{a}	2.24±0.07^{b}	2.22±0.15^{b}
	30	2.24±0.11^{a}	3.04±0.16^{a}	3.47±0.49^{a}
	60	2.31±0.08^{a}	3.62±0.72^{a}	3.78±0.66^{a}
	90	2.27±0.18^{a}	3.34±0.07^{a}	ND
	Urolithiasis	2.32±0.50^{a}	2.32±0.50^{b}	2.32±0.50^{b}
K	0	5.18±0.51^{b}	5.52±0.33^{b}	5.43±0.15^{c}
	30	5.35±0.19^{b}	6.33±0.39^{b}	6.80±0.29^{b}
	60	5.40±0.29^{b}	5.93±0.30^{b}	6.58±0.76^{b}
	90	5.43±0.26^{b}	6.15±0.06^{b}	ND
	Urolithiasis	8.25±1.17^{a}	8.25±1.17^{a}	8.25±1.17^{a}
Mg	0	1.11±0.07^{b}	1.14±0.05^{b}	1.10±0.05^{c}
	30	1.09±0.04^{b}	1.19±0.13^{b}	1.38±0.10^{b}
	60	1.07±0.07^{b}	1.22±0.24^{b}	1.32±0.15^{b}
	90	1.04±0.08^{b}	1.12±0.05^{b}	ND
	Urolithiasis	2.19±0.56^{a}	2.19±0.56^{a}	2.19±0.56^{a}

Urolithiasis sheep: About 5 sheep with urinary tract blockage were observed in group CB; when death appeared imminent, their blood samples were collected. ND: there was only one sheep in group CB on 90th day. *Means within a column with different letters (a-c) differ significantly ($p<0.05$). **Values were expressed as mean±SD of six animals. ***C: corn-soybean meal diet; CA: corn-cottonseed meal A diet (461.5 g $days^{-1}$ cottonseed meal in a diet); CB: corn-cottonseed meal B diet (661.5 g $days^{-1}$ cottonseed meal in a diet)

serum magnesium, potassium and phosphorus were raised whereas there were no significant differences for serum calcium from the groups C and CA on days 30, 60. There were significantly high in serum magnesium and potassium ($p<0.05$) in urolithiasis sheep but significant low for phosphorus ($p<0.05$) in comparison with others. The high level of cottonseed meal (661.5 g $days^{-1}$ cottonseed meal in the diet) caused significant increase in both serum magnesium and phosphorus ($p<0.05$). Serum potassium was slightly elevated when the level of cottonseed meal was increased from 461.5-661.5 g $days^{-1}$ The high occurrence of urolithiasis (5/6) in group CB was unexpected. Cottonseed meal might be a critical factor in the course of development of urolithiasis.

Urine PH from different groups are shown in Table 4. A slight increase in pH (avg, 0.25 units) did promote

Table 3: Effect of dietary cottonseed meal on blood mineral elements of Chinese Merino sheep at different stage*, ** (n = 6)

Element (mmol L^{-1})	Group*** (sheep)	Day 0	30	60	90
Ca	C	2.49±0.10^{a}	2.36±0.19^{ab}	2.58±0.17^{a}	2.66±0.07^{a}
	CA	2.49±0.24^{a}	2.26±0.24^{b}	2.47±0.25^{ab}	2.48±0.02^{a}
	CB	2.39±0.18^{a}	2.43±0.11^{ab}	2.28±0.23^{b}	ND
	Urolithiasis	2.55±0.14^{a}	2.55±0.14^{a}	2.55±0.14^{a}	2.55±0.14^{a}
P	C	2.18±0.15^{a}	2.24±0.11^{b}	2.31±0.08^{b}	2.27±0.18^{b}
	CA	2.24±0.07^{a}	3.04±0.16^{a}	3.62±0.72^{a}	3.34±0.07^{a}
	CB	2.22±0.15^{a}	3.47±0.49^{a}	3.78±0.66^{a}	ND
	Urolithiasis	2.32±0.50^{a}	2.32±0.50^{b}	2.32±0.50^{b}	2.32±0.50^{b}
K	C	5.18±0.51^{b}	5.35±0.19^{c}	5.40±0.29^{c}	5.43±0.26^{b}
	CA	5.52±0.33^{b}	6.33±0.39^{b}	5.93±0.30^{bc}	6.15±0.06^{b}
	CB	5.43±0.15^{b}	6.80±0.29^{b}	6.58±0.76^{b}	ND
	Urolithiasis	8.25±1.17^{a}	8.25±1.17^{a}	8.25±1.17^{a}	8.25±1.17^{a}
Mg	C	1.11±0.07^{b}	1.09±0.04^{c}	1.07±0.07^{c}	1.04±0.08^{b}
	CA	1.14±0.05^{b}	1.19±0.13^{c}	1.22±0.24^{c}	1.12±0.05^{b}
	CB	1.10±0.05^{b}	1.38±0.10^{b}	1.32±0.15^{b}	ND
	Urolithiasis	2.19±0.56^{a}	2.19±0.56^{a}	2.19±0.56^{a}	2.19±0.56^{a}

Urolithiasis sheep: Total 5 sheep with urinary tract blockage were observed in group CB; when death appeared imminent, their blood samples were collected. ND: there was only one sheep in group CB on 90th day. *Means within a column with different letters (a-c) differ significantly ($p<0.05$). **Values were expressed as mean±SD of six animals. ***C: Corn-soybean meal diet; CA: Corn-cottonseed meal A diet (461.5 g day^{-1} cottonseed meal in a diet); CB: Corn-cottonseed meal B diet (661.5 g day^{-1} cottonseed meal in a diet)

Table 4: Effect of dietary cottonseed meal on urine pH of Chinese Merino sheep*, ** (n = 6)

Groups***	Days 0	30	60	90
C	7.38±0.13^{b}	7.63±0.24^{a}	7.25±0.14^{b}	7.25±0.14^{b}
CA	8.00±0.20^{ac}	7.63±0.13^{a}	7.25±0.14^{b}	7.75±0.14^{a}
CB	8.38±0.13^{a}	8.00±0.20^{a}	7.50±0.00^{bc}	ND
Urolithiasis sheep	7.75±0.14^{bc}	7.75±0.14^{a}	7.75±0.14^{ac}	7.75±0.14^{a}

Urolithiasis sheep: Total 5 sheep with urinary tract blockage were observed in group CB; when death appeared imminent, their blood samples were collected. ND: there was only one sheep in group CB on 90th day. * Means within a column with different letters (a-c) differ significantly ($p<0.05$). **Values were expressed as mean±SD of six animals. ***C: Corn-soybean meal diet; CA: Corn-cottonseed meal A diet (461.5 g day^{-1} cottonseed meal in a diet); CB: Corn-cottonseed meal B diet (661.5 g day^{-1} cottonseed meal in a diet) urinary calculi in group CB compared with C (the control) howerver, no significant differences were observed between urolithiasis sheep and others. Urine PH had not remarkable difference with increasing cottonseed meal in the diet.

Calculi and urinary sediments: Visual observation was made up of the external appearance, size, hardness and colour of calculi. The calculi samples appeared finely granular, irregular mass and balls (bladder stones, Fig. 1) or prismatic crystals and irregular block (kidney stone, Fig. 2). The urinary sediments were prismatic crystals with granules (Fig. 3). The calculi samples were analysed by Pan *et al*. (2010), the result indicated that potassium magnesium phosphate ($MgKPO_4$) and potassium magnesium phosphate hexahydrate ($MgKPO_4.6H_2O$) were major components in the bladder stones while less magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4.6H_2O$) however, the kidney stone were rich in magnesium and pyrophosphate were identified as magnesium pyrophosphate ($Mg_2P_2O_7$).

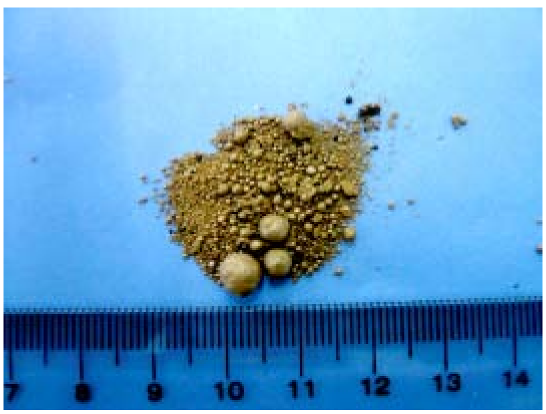

Fig. 1: Bladder stones from four sheep with urinary tract blockage showing the size and surface character similar to potassium magnesium phosphate ($KmgPO_4.6H_2O$) and ammonium magnesium phosphat ($NH_4MgPO_4.6H_2O$) in crystals structure

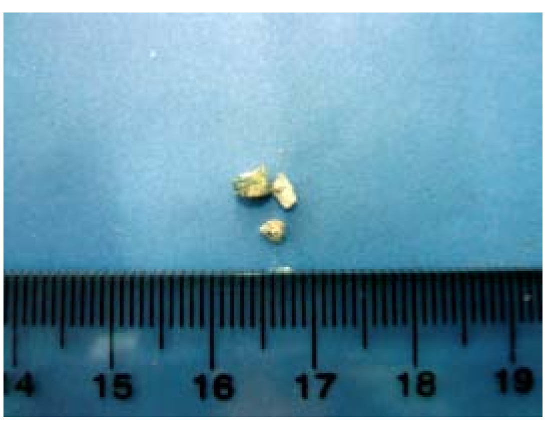

Fig. 2: Kidney stone from one sheep in the kidney showing the size and surface character (promatic crystals)

Urolithiasis might be related to the following factor: the pH value of the urine, the minerals of the urine such as Calcium (Ca), Magnesium (Mg), Potassium (K), Phosphorus (P). Here in, we used an imbalance experimental diet (high levels of CSM) to provide excess P, K and Mg (the ratio of Ca:P is 0.89, 0.36% Mg, 1.39% potassium in the diet) compared with the nutritional requirements of sheep (NRC, 1985). There was higher incidence of urolithiasis (one sheep with kidney stone and 4 sheep with bladder stones from 6 sheep, 5/6) in high levels of CSM group while uroliths were not observed in the control and medium groups.

Some previous studies indicated that serum Ca level positively correlated with both calcium intake and urinary Ca excretion whilst urinary Ca excretion have been correlated with the risk of urolithiasis (Ljunghall, 1977; Resnik *et al*., 1968; Welshman and McGeown, 1975). While we found there were no significant difference in serum Ca in this study. This result probably resulted from low Ca:P ratio in diet. This would support the observation that sheep fed the diet CB developed bladder stones composed primarily of potassium magnesium phosphate ($MgKPO_4$), potassium magnesium phosphate hexahydrate ($MgKPO_4.6H_2O$) and ammonium magnesium phosphate ($MgNH_4PO_4.6H_2O$) and magnesium pyrophosphate ($Mg_2P_2O_7$) in the kidney stone (Pan *et al*., 2010). Sheep normally excrete alkaline urine containing low P while sheep fed a cottonseed meal diet with a low Ca:P ratio and high level P in this study therefore, the urinary P was increased. Most veterinary scientists have attributed the high urinary excretion of P in these circumstances to the absolute deficiency of dietary Ca and therefore to the stimulation of parathyroid hormone secretion by low serum Ca concentration which in turn decreases renal tubular reabsorption of P.

Serum magnesium level was elevated significantly in CB group in this study, it might be attributed to addition of high level cottonseed meal containing the high level of magnesium. The result of serum Mg is in agreement with the previous study (Petersson *et al*., 1988) reported blood and urine Mg levels were increased with the increase of dietary Mg. Gentry *et al*. (1978) showed that when high level magnesium was fed, magnesium concentration in calves urine increased much more than that in blood. Kallfelz *et al*. (1987) reported calves with urinary tract obstructions were observed that only level of serum Mg was increased. Wang *et al*. (1993) also reported this observation for water buffalo fed with a cottonseed meal diet.

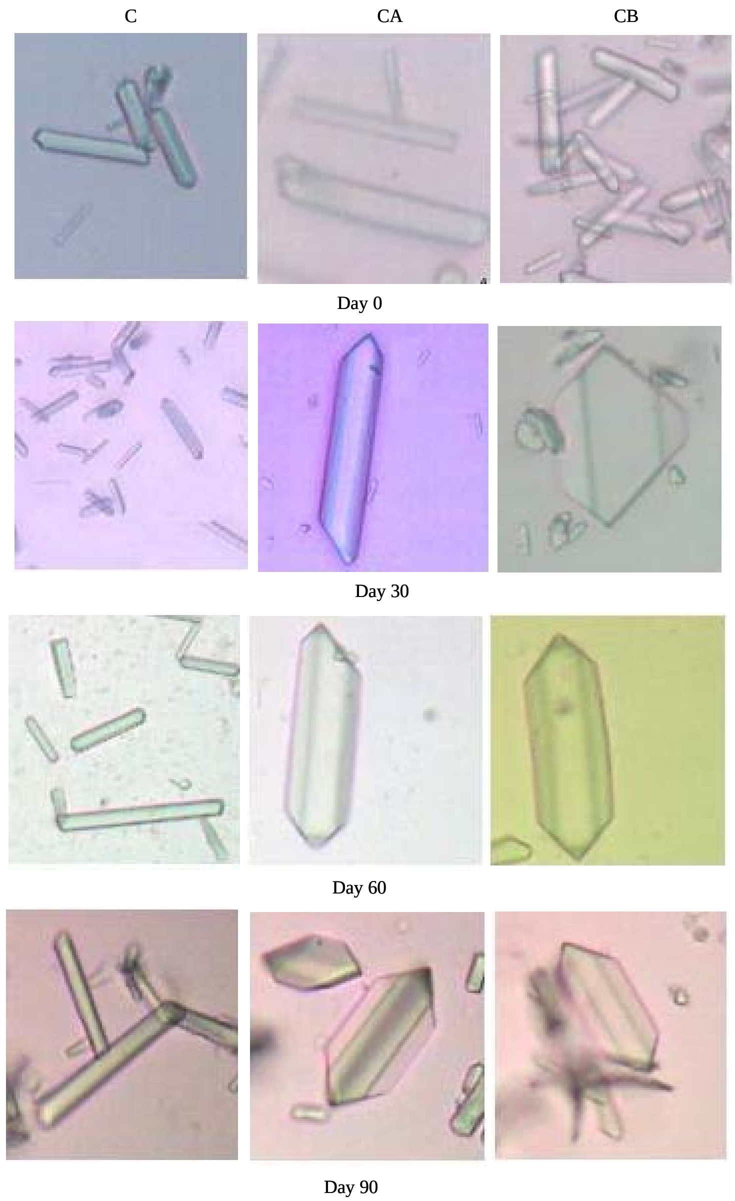

Fig. 3: Micrographs (10×40) of urinary sediments of Chinese merino sheep by microscope. Micrographs (10×40) of urinary sediments of sheep fed with corn-spybean meal died (C), corn-cottonseed meal diet A (CA), corn-cottonseed meal diet B (CB) on days 0, 30, 60, 90, respectively

Under normal conditions, K is excreted via faeces, skin, sweat gland, urine and milk. Urine is a main path of excretion in sheep, approximately 95% potassium via urine. Moreover, potassium would be excreted more when sheep were fed with high levels of potassium in the diet. The nutritional requirement of potassium in sheep is 0.50-0.80% (NRC, 1985). The dietary ingredients in the experiment mainly consisted of CSM containing 1.47% potassium. Potassium in the diet (1.39%) remarkably surpassed nutrient requirement of sheep and the result shew that serum potassium was increased in the groups containing cottonseed meal, leading to elevated concentrations of potassium in urine.

In sheep with urolithiasis, serum Mg and K levels were observed significant increase as previous studies (George *et al*., 2007; Packett *et al*., 1968). Cottonseed meal is also rich in protein (43% DM) resulting in increase of urea which is easily decomposed to ammonium ions in urine, especially in the presence of urease-producing bacteria. Under the condition of alkaline urine (Table 4) when concurrently there are high levels of magnesium, phosphate and ammonium ions in the urine, the optimal conditions are provided for precipitation of the highly insoluble ammonium magnesium phosphate (struvite) ($NH_4MgPO_4 .6H_2O$) (Hedelin, 2002). Since, the radii of potassium and ammonium ions are similar, the crystal structures of $KmgPO_4.6H_2O$ and $NH_4MgPO_4.6H_2O$ are almost completely similar, they both belong to the orthogonal crystal system and they have similar solubility products. Either $KMgPO_4.6H_2O$ or $NH_4MgPO_4.6H_2O$ can be produced in the urinary tract depending on the concentration of potassium ion and ammonium ion. This might be the mechanism for the formation of potassium magnesium phosphate ($KMgPO_4.6H_2O$) similar to that of struvite calculi, as suggested by McIntosh (1978).

CONCLUSION

Addition of high level cottonseed meal increased remarkably the levels of blood magnesium, potassium, phosphorus. Total four bladder stones and one kidney stone were collected in CB group as well as urinary sediments from different groups were similar to struvite and potassium magnesium phosphate ($KMgPO_4.6H_2O$). It is clear that blood phosphorus, magnesium and potassium levels were affected by addition of cottonseed meal. The addition of cottonseed meal might be an very important factor for occurrence of calculi in this region.

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