INTERACTIONS OF DIETARY PROTEIN AND THE FLUORIDE ION ON THE RUMEN FERMENTATION AND OSTEO-SKELETAL CHANGES IN CROSSBRED CALVES

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SUMMARY: In order to assess the influence of dietary protein and the fluoride ion (F) levels on the rumen fermentation and osteo-skeletal changes, 30 crossbred calves (6–8 months; 104 kg body weight) reared on high, medium, and low levels of dietary protein were exposed to either 0 or 200 mg/kg supplemental F (source: NaF) for a period of 210 days. The rumen liquor studies carried out at the end of experimental feeding showed that the diets did not (p>0.05) influence the rumen fermentation as assessed by the total volatile fatty acids and the TCA-precipitable nitrogen concentration in the rumen liquor, although a significantly lower pH was noted in the F-supplemented animals than in the non-F-supplemented groups. The radiological studies conducted at 90 and 210 days of feeding revealed signs of sub-periosteal new bone formation and cortical thickening in the F-supplemented animals. It is concluded that F in the diet at varied levels of protein nutrition did not have any adverse impact on the rumen fermentation but the radiological findings showed typical signs of fluorosis and the protein levels did not significantly influence the susceptibility to fluorosis.

Keywords: Calves; Dietary fluoride; Dietary protein; Fluoride retention; Rumen fermentation.

INTRODUCTION

Toxic levels of the fluoride ion (F) in drinking water and in many foods cause F intoxication (fluorosis) which is prevalent worldwide and endemic in different parts of India.¹⁻⁴ Earlier investigations showed that poor nutritional status is known to affect skeletal fluorosis, especially when malnutrition and low protein, calcium, and vitamin C intake are present.⁵⁻⁷ Low nutritional levels of food or protein intake increases the amount of F retained in the body,⁶⁻⁸ and an elevated deposition of F in bone occurs in guinea pigs fed low protein diets.⁹ Conversely, an ameliorative effect of high protein diets on F retention in rats is accompanied by increased urinary excretion of F.¹⁰ Tooth wear has been shown to be beneficially influenced by protein supplementation in industry-fluorosed goats.¹¹ Similar results suggesting a reduction in F deposition in the bones of pigs fed high protein diets as compared to low protein diets have also been reported.¹² Various studies, conducted by Chinoy et al. in mice and Wang et al. in rats and rabbits, have indicated the importance of dietary protein on the susceptibility to the varied adverse impacts of fluorosis.^{6,7,13-16} Whether these beneficial effects of protein are because of an influence on rumen fermentation has not been studied. In our earlier report, we found that dietary protein levels affect the performance of

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crossbred calves.¹⁷ We also reported the metabolic effects on growth and nutrient utilization of short- and long-term exposure to F in calves fed graded protein levels.^{18,19} The present study was conducted to investigate the possible interaction of protein and F in calves with respect to rumen fermentation. The study also investigated the possible role of protein nutrition on the susceptibility to bovine fluorosis by monitoring osteo-skeletal changes in crossbred calves exposed to long-term high dietary F.

MATERIALS AND METHODS

Our animal feeding experiment was the same as in the work of Lohakare et al.^{18,19} Thirty crossbred male calves (*Bos taurus* × *Bos indicus*) aged 6–8 months and initially exposed to different planes of protein nutrition for 90 days, were divided into 6 groups in a 3×2 factorial design. The factors included 3 different levels of protein, viz., normal protein (100%; NP), low protein (75%; LP) and high protein (125%; HP) as per the Kearl recommendations,²⁰ besides 2 levels of supplemental F (source: NaF) at 0 or 200 mg F/kg diet. From weaning through the trial period the drinking water contained 0.01 mg F/L; the oat straw 10–15 mg F/kg. The animals were fed on the respective concentrate mixtures (CMs) in addition to a basal diet of wheat straw, offered *ad libitum*, for 210 days.

Rumen fermentation was assessed at the end of experimental feeding. It involved collection of rumen liquor from 4 representative animals in each group. The liquor was collected 3 hours post-feeding for two consecutive days by using a stomach tube. About 150 mL of rumen liquor, collected each time, was filtered through four layers of muslin cloth. About 60 mL of strained rumen liquor (SRL) was acidified with five drops of 20% H₂SO₄ and preserved in a freezer for further biochemical analysis. The pH of rumen liquor was estimated using a digital pH meter immediately after collection before the addition of any preservative. The concentration of total volatile fatty acids (TVFA) was determined as per the procedure of Barnett and Reid.²¹ Five mL of SRL was directly distilled by Kjeltec auto analyzer to obtain the NH₃-N concentration. The total N was determined by the Kjeldahl method using a Kjeltec auto analyzer. For determining microbial N, 5 mL of SRL was precipitated with an equal volume of 20% trichloroacetic acid (TCA) and left overnight under refrigeration. This was then centrifuged at 4,000 rpm for 10 min and the supernatant was quantitatively transferred and digested. The concentration of TCA soluble N was determined as described above for the total N, and the TCA- precipitable N was expressed as the difference between the total- and the TCA-soluble N. The non-protein N was derived by deducting the TCA-precipitable and the NH₃-N fractions from the total N content of SRL.

Lateral radiographs of the metacarpal and metatarsal bones were taken after 90 days of F exposure from three representative animals of each group. In addition to these three animals, two other animals from each group were again subjected to radiological examination after 210 days of F exposure. The radiographs were assessed for changes in the trabecular pattern of the bones, radiographic density, sub-periosteal new bone formation, cortical thickness, exostosis, and other

radiological changes related to the musculo-skeletal system in the metacarpal and metatarsal region for comparison among the treatments.

The data on rumen fermentation were analyzed in a 3×2 design in order to ascertain the effects of protein, F, and their interaction. Means were subjected to the 'test of significance' by Duncan's multiple range tests as described by Snedecor and Cochran²² using the SPSS 10.0 package. All notations of significance are based on a probability of p<0.05 or p<0.01 unless otherwise indicated.

RESULTS

The ruminal pH did not vary (p>0.05) when different protein levels were compared (Table 1).

Attributes	Fluoride ion (0 mg/kg)			Fluoride ion (200 mg/kg)			Sig⁵
	Normal protein (NP)	Low protein (LP)	High protein (HP)	Normal protein (NP)	Low protein (LP)	High protein (HP)	
рН	6.50 ±0.1	6.57 ±0.1	6.53 ±0.1	6.23 ±0.1	6.42 ±0.1	6.41 ±0.1	F
TVFA	96.9	96.1	98.1	89.4	92.9	98.6	Ρ
(mEq/L)	±1.8	±1.5	±2.6	±2.1	±2.3	±1.8	
Total N	104.6 ^{cd}	101.7 ^d	99.8 ^{de}	110.1 [°]	93.0 ^e	102.9 ^{cd}	P,
(mg/dL)	±3.3	±2.6	±1.9	±3.0	±1.6	±1.5	PxF
NH₃-N	13.0	10.5	11.7	11.0	10.5	11.0	NS
(mg/dL)	±0.9	±0.7	±0.4	±0.7	±0.3	±0.4	
TCA ppt. N	61.8	61.1	61.0	64.4	58.9	60.6	NS
(mg/dL)	±1.5	±1.7	±2.0	±2.1	±1.7	±2.1	
TCA-sol. N	42.8	40.6	38.8	45.7	34.1	42.3	NS
(mg/dL)	±2.5	±3.7	±0.9	±3.3	±2.9	±2.3	

Table 1. Effect of different levels of protein and F on pH and rumen biochemical parameters in calves^a

^aData are presented as mean \pm SE; ^bSignificant (p<0.05) effect of fluoride ion (F), protein (P), or their interaction (PxF); NS: nonsignificant; ^{cde} Different superscripts in the same row differ significantly;

A reduction (p<0.05) in ruminal pH, however, was found upon feeding of F when compared to non-F diets. Significantly higher (p<0.05) TVFA was evident upon feeding high protein diets and total N was reduced (p<0.05) on LP diets compared to their counterparts. There was no effect (p>0.05) of the protein or the F content in the diets on the ruminal N fractions including NH₃-N, TCA-precipitable, and TCA-soluble N (Table 1).

The lateral radiographs of the metacarpal and metatarsal bones taken at 90-days showed dense, smooth cortices that were thicker in the mid-diaphyseal region as compared to that at the proximal and distal thirds of the bones in the calves fed the NP diet without F (Figures 1a and 1c).



Figure 1. Lateral radiographs of metacarpal (a and b) and metatarsal (c and d) bones at 90 (a and c) and 210 (b and d) days post-feeding in calves fed a normal protein diet without F.

The medullary cavity had a smooth endosteal surface, was narrow in the center, and gradually widened towards the proximal and distal metaphyses. The trabecular pattern in the metaphyseal region was normal having a regular distribution in the bones of the calves fed the NP, LP, and HP diets without F supplementation (Figures 1a and 1c, 2a and 2c, and 3a and 3c, respectively). At 210-days, the metacarpal and metatarsal cortices appeared relatively more dense and thicker (Figures 1b and 1d, 2b and 2d, and 3b and 3d, respectively), showing no effect of protein on the bones. All the other changes remained similar to those seen at 90-days.

The radiographs of the metacarpal and metatarsal bones at 90-days in the Fsupplemented calves fed the NP diet did not show marked radiographic changes suggestive of fluorosis (Figures 4a and 4c). However, at 210-days, the signs of fluorosis became apparent in the bones of the calves fed the NP diet and F (Figures 4b and 4d); there was sub-periosteal new bone formation leading to thickening of the cortices and an uneven periosteal surface. The changes were more marked in the metacarpal compared to the metatarsal. However, the medullary cavity appeared uniform in size in most of the animals in this group.

At 90-days, the calves on the F-supplemented LP and HP diets showed mild radiographic changes with sub-periosteal bone formation and a relative increase in the cortical thickness suggestive of fluorosis, but the trabecular pattern was normal and uniform (Figures 5a and 5c; 6a and 6c, respectively). The contours of the metacarpal bone appeared uneven due to the sub-periosteal new bone formation.

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Figure 2. Lateral radiographs of metacarpal (a and b) and metatarsal (c and d) bones at 90 (a and c) and 210 (b and d) days post-feeding in calves fed a low protein diet without F.



Figure 3. Lateral radiographs of metacarpal (a and b) and metatarsal (c and d) bones at 90 (a and c) and 210 (b and d) days post-feeding in calves fed a high protein diet without F.

At 210-days, lesions became more marked in the calves on the F-supplemented LP and HP diets, (Figures 5b and 5d; 6b and 6d, respectively). There was extensive sub-periosteal bone formation leading to thickening of the cortex and uneven contours of the bone. The newly formed bone was less dense and unevenly

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distributed. The medullary cavity appeared relatively narrow compared to that at 90-days exposure. The intensity and degree of the changes varied from animal to animal. However, consistent changes demonstrating the characteristic signs of fluorosis in the long bones of both these groups were observed.



Figure 4. Lateral radiographs of metacarpal (a and b) and metatarsal (c and d) bones at 90 (a and c) and 210 (b and d) days post-feeding in calves fed a normal protein diet with F.



Figure 5. Lateral radiographs of metacarpal (a and b) and metatarsal (c and d) bones at 90 (a and c) and 210 (b and d) days post-feeding in calves fed a low protein diet with F.



Figure 6. Lateral radiographs of metacarpal (a and b) and metatarsal (c and d) bones at 90 (a and c) and 210 (b and d) days post-feeding in calves fed a high protein diet with F.

DISCUSSION

There was a reduction in the ruminal pH in the calves fed the F-supplemented diets showing the negative effects of F. The ruminal pH range obtained in the study, as a function of time and the various diets, was, however, considered to be favorable for microbial activity in the rumen without affecting the cellulolytic microorganisms.²³ The total N content in the SRL varied significantly (p<0.05) among the six dietary groups exhibiting an interaction between the protein and F levels; the addition of F reduced the total N concentration on low protein feeding. A higher ruminal N is generally considered beneficial. The trend present in the study thus highlights that the negative impact of F is more pronounced on a low protein diet. Highly significant (p<0.01) variations among the different protein levels were observed which showed that low protein fed animals had a decreased content of total N in the SRL, which was positively correlated with the protein intake. The NH₃-N in the SRL did not show any variations among the diets or F levels and the values fell within the normal range required for efficient microbial production. As far as we know, this is the first systematic study showing the effect of F on rumen fermentation in calves. An earlier study on the replacement of dicalcium phosphate in cattle diet by high F-rock phosphate reported no effect of F on the ruminal pH and NH₃-N.²⁴ The present study also found no detrimental effects of F on rumen fermentation which, in turn, is reflective of the effective adaptation of ruminal microbes to high dietary F.

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Previous studies have demonstrated that F influences the molecular structure of collagen, an important framework protein in bone,¹¹ and can disorder its gene expression.²⁵⁻²⁷ The radiographic changes suggestive of fluorosis were more marked at 210-days than at 90-days of F exposure. The greater and faster metabolic accumulation of F in bones severely affects the skeletal tissues showing the impact on bones of fluorosis.²⁸⁻³¹ The sub-periosteal new bone formation and the relative increase in cortical thickness leading to a narrowing of the medullary cavity were the prominent features of F toxicity recorded at 210-days, and it was more prominent in the F-supplemented HP-fed calves radiographed at this stage (Figures 6b and 6d). An earlier report that the ingestion of F with a protein- and calcium-deficient diet had adverse effects on the bone structure and metabolism, and that enriching the diet with protein or calcium could offset or reduce these deleterious effects⁶ contradicts our findings, especially regarding bone structure. Excessive F ingestion significantly decreases the protein content, resulting in adverse changes in the histology and composition of both hard and soft tissues.⁶, ³²⁻³⁴ The lateral radiography of metacarpal and metatarsal bones after 90- and 210-days of feeding showed normal radiographs in all the three non-F fed groups where the cortices were uniform and smooth but, however, relatively thicker and more dense in the metatarsal than in the metacarpal. The medullary cavity was narrow at the centre and uniformly lined by the endosteal surface of the cortex throughout the entire length of bone (Figures 1, 2, and 3).

After the ingestion and absorption of F through the gastrointestinal tract, storage by bony tissues is the primary mechanism for the removal of F from the body fluids, with the secondary mechanism of excretion being via the kidneys. F has an affinity for bone and also accumulates in other calcified tissues.²⁵ F induces cell injury in both osteoblasts and osteocytes, initiates a repair response, and increases the serum alkaline phosphatase production from these cell populations.³⁵⁻³⁸ The effect of F on the bones of young animals in the present study also showed this to occur. The continuous ingestion of F may cause skeletal diseases like osteosclerosis.³⁹ periosteal hyperostosis, osteoporosis, osteomalacia, and osteophytosis as reviewed by Wheeler and Feel.⁵ The present study also found the same signs of periosteal hyperostosis and osteophytosis. Osteoclasts are responsible for bone resorption, help maintain bone integrity, and play a critical role in the normal skeletal development. However, F inhibits their capacity for bone resorption, thus resulting in osteosclerosis.³⁹ Protein establishes the structural foundation and framework of bone in preparation for the deposition of minerals.⁷ A deficiency of protein can adversely affect bone growth and metabolism.

Overall, the perusal of the rumen fermentation revealed that the incorporation of F on a low protein diet affects nitrogen metabolism. However, the radiological studies showed that the protein levels in the diet do not impart a significant influence on the susceptibility to fluorosis in crossbred calves when fed on a diet containing 200 mg F/kg.

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