Effect of Gossypol from Cottonseed Meal Diets on Some Clinico-biochemical Parameters and Humoral Immune Response of Crossbred Calves Fed Barley or Sorghum

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ABSTRACT : Effects of feeding of 9.95 mg free gossypol/kg live weight through cottonseed meal (CSM) were studied in 20 intact male calves fed barley or sorghum as source of cereal during the experimental duration of 210 days. Serum concentration of total protein, albumin, globulin and their ratio did not vary because of protein (gossypol) or cereal sources. Serum level of cholesterol and urea were lower (p<0.05) in sorghum than barley fed calves. Feeding of gossypol through CSM enhanced (p<0.05) serum cholesterol. An interaction between protein and period was observed with respect to serum concentrations of urea, creatinine and alanine transferase. The levels of serum creatinine and alanine transferase increased (p<0.05) following 120 days of experimental feeding in calves fed CSM diets compared to the control animals fed groundnut meal diets. No effect of feeding gossypol was, however, evident on the serum enzymes viz. alanine and aspartate transferases and alkaline phosphatase. Moreover, the source of cereal and protein did not appear to influence the metabolic profile of the calves. Humoral immune response, measured through antibody titre against *Brucella abortus* S99 innoculation, revealed a delayed and depressed seroreactivity indicative of immunocompromisation because of the phytotoxin gossypol. In conclusion, the feeding of gossypol at the designated levels, although had no deleterious clinico-biochemical manifestations, affected the humoral immune response of the calves. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 9 : 1291-1296)***

Key Words : Cottonseed Meal, Gossypol, Blood Profile, Immune Response, Calves

INTRODUCTION

In order to mitigate the perennial gap between the demand and supply of quality protein concentrates in many developing countries including India, efforts are being made continuously to look for alternate feeding resources. Cottonseed meal is one such feed resource produced as a byproduct of cotton oil and fibre industry. India ranks third in cottonseed production (FAO, 1997) with an annual production of 5,430 million tonnes. After extraction of oil, the resultant undecorticated cottonseed meal (CSM) could serve as a potential source of protein (25-30% CP) and fibre in the diet of ruminants. However, the extensive use of CSM is handicapped by its gossypol content because of its potential toxicity. Gossypol is a natural yellow polyphenolic pigment contained in all parts of cotton plants (*Gossypium spp.*) and other plants belonging to *Malvaceae* family, and has been the source of potential scientific interest for over a century (Horn et al., 1987). Several substances interrelated to gossypol have been characterized and found to be toxic, and all of these substances have been generically termed as gossypol (Pons, 1977).

Gossypol has been known to be toxic to non-ruminants since long (Clawson et al., 1961; Haschek et al., 1989; Suryavanshi et al., 1993), while ruminants are assumed to be not susceptible because of the binding of the gossypol to soluble proteins in the rumen (Reiser and Fu, 1962). However, studies by Lindsey et al. (1980), wherein they have found presence of gossypol in tissues of mature lactating cows, established that the capacity of ruminants to detoxify dietary gossypol is limited. Excessive ingestion of CS products that contain high free gossypol can, therefore, overwhelm the detoxifying capabilities of rumen, and cause gossypol toxicosis (Kerr, 1989; Morgan, 1989). Although the effects of gossypol have been studied with greater details in non-ruminants, the picture with regards to ruminants, especially growing ruminants remains somewhat unclear. Few studies reported depict rather a conflicting view. Risco et al. (1992) have suggested that diets of dairy calves containing up to 200 mg free gossypol (FG)/kg diet dry matter (DM) are safe; toxicity approaches at levels beyond 400 mg, and death losses at 800 mg or more/kg diet DM. On the contrary, Zelski et al. (1995) have recorded >40 per cent mortality in calves fed 100-220 mg FG/kg DM. Hence, there is a need for establishing the safe limits of gossypol in calves especially when fed as cottonseed meal involving a practical long-term feeding trial. The present investigation was, therefore, undertaken to ascertain the effect of long-term effects of feeding gossypol as cottonseed meal on the clinico-biochemical parameters and humoral immune response of growing dairy calves.

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Table 1. Serum concentrations of total protein, albumin, globulin, urea and creatinine in calves fed gossypol through cottonseed meal

	Experimental groups				ANOVA[1]
	B-GN	S-GN	B-CS	S-CS	
Total protein, g/l					
day 0	65.0±3.57	66.31±4.21	68.0±2.39	68.0±5.21	D
day 120	59.6±2.38	64.4±1.05	64.4±1.87	60.6±1.52	
day 210	69.1±2.03	69.7±2.12	71.8±2.50	70.4±1.53	
Albumin, g/l					
day 0	39.4±2.82	38.5±1.09	39.6±2.50	38.2±1.92	
day 120	37.4±1.83	39.0±1.26	39.1±1.57	37.2±2.93	
day 210	38.5±1.67	40.4±1.07	38.8±2.42	39.9±0.64	
Globulin, g/l					
day 0	25.5±1.88	27.9±3.21	28.3±1.97	29.73±3.38	D
day 120	22.2±0.82	25.4±0.89	25.3±0.44	23.4±1.88	
day 210	30.2±0.64	29.3±1.25	33.0±1.00	30.5±1.03	
Alb : Glob ratio					
day 0	1.57±0.15	1.42±0.11	1.43±0.16	1.32±0.09	D
day 120	1.69±0.07	1.55±0.09	1.55±0.05	1.62±0.16	
day 210	1.26±0.05	1.38±0.05	1.18±0.08	1.31±0.03	
Urea, mmol/l					
day 0	6.07±0.44	3.76±0.19	6.13±0.74	5.21±0.58	G, P×G, P×D
day 120	2.93±0.25	1.77±0.06	2.74±0.22	2.36±0.33	
day 210	5.26±0.48	3.86±0.46	3.60±0.79	3.30±0.09	
Creatinine, µmol/l					
day 0	147.6±15.19	127.5±22.28	102.3±16.47	106.5±14.59	
day 120	130.8±23.60	120.2±23.43	167.1±27.49	183.0±34.83	
day 210	145.4±13.93	145.4±13.93	148.3±13.77	114.7±12.57	

[1] Significant effects ($p<0.05$). Grain (G), protein (P), time (D) and their interactions. B: barley; S: sorghum; GN: groundnut meal; CS: cottonseed meal.

MATERIALS AND METHODS

Animals, experimental design and feeding

The experiment was conducted with 20 intact male calves (*Bos taurus*×*Bos indicus*) of about 6 months of age with a mean body weight of 93 kg. During an initial period of two weeks, calves were fed on a standard wheat straw-based diet for adaptation. The diet included provision of 2.0 kg concentrate mixture (maize 40, groundnut meal 30, wheat bran 27, mineral mixture 2, and common salt 1 part per 100 kg) along with *ad libitum* wheat straw. Then the calves were assigned evenly on body weight basis to one of the four groups (GN-B, GN-S, CS-B and CS-S) each consisting of 5 animals. Two groups were fed a concentrate supplement containing groundnut meal (GN) along with barley (B) and sorghum (S), i.e., GN-B and GN-S, and serve as controls. The other two groups were fed the same barley and sorghum as cereal but a part of the groundnut meal in each was replaced by cottonseed meal, i.e., CS-B and CS-S. The final concentration of cottonseed meal in the supplement was 25 per cent, equivalent to 50 per cent of the groundnut meal nitrogen. All the four concentrate mixtures were formulated to be iso-nitrogenous and iso-caloric, and per kg of dry matter contained 223-235 g of crude protein, 18-23 g of crude fat (ether extract) and 606-659 g of total carbohydrates, and 10.5-10.6 MJ metabolizable energy besides minerals and vitamins. The calves were fed as per requirements (NRC, 1989) and amount of concentrate fed was adjusted based on mean body weight measured fortnightly. Besides the respective concentrate supplements, the calves had access to *ad libitum* supply of wheat straw.

The calves were housed in a well-ventilated barn with facilities for individual feeding and watering. Concentrate mixtures were offered at 08:00 h daily and wheat straw at 14:00 h. Feed residues were measured daily for each calf separately. Health of the animals was regularly monitored throughout the experimental period.

Blood collection

Before the onset of the experiment (i.e., 0 day), and thereafter at 120 and 210 d after experimental feeding blood samples were taken by jugular venipuncture and serum was separated within half an hour by centrifugation and frozen at -20°C for later analysis of various biochemical constituents.

Immune response study

Humoral immune response study was carried out after 180 days of experimental feeding using sonicated antigen of *Brucella abortus* S99 as an immunogen. Accordingly, the calves under each dietary treatment were sensitized by subcutaneous administration of 2 mg protein of *B. abortus* S99 sonicated antigen mixed equally with Freund's incomplete adjuvant. Prior to sensitization, the calves were screened for brucellosis by conducting Rose Bengal Plate Test (RBPT) as described by Alton et al. (1975). The alteration in immune response was assayed by indirect enzyme-linked immunosorbent assay (ELISA) using blood serum collected from the sensitized calves at 0, 7, 14, 21 and 28 d post-sensitization as per the procedure of Nielsen et al. (1987).

Laboratory analyses

The total and free gossypol contents of CSM were estimated as per the procedure of Botsoglou and Kufidis (1990) and Botsoglou (1991), respectively. Serum concentrations of glucose, cholesterol, total protein, albumin, globulin, urea, creatinine, aspartate aminotranseferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined as described elsewhere (Pattanaik et al., 1999).

Statistical analyses

Values are expressed as mean±SE. The effects of protein, cereal, day of collection and interaction between these factors were evaluated by analysis of variance (Snedecor and Cochran, 1967). Significance was declared at $p<0.05$.

RESULTS

Data for serum total protein, albumin, globulin, urea and creatinine are presented in Table 1. Total protein was not affected by dietary treatment, but was lower ($p<0.01$) at d 120 than at the initial and final collections (62.2 vs. 66.8 and 70.2 g/l, respectively). While no effect of dietary treatment was evident on serum levels of albumin and globulin, the values for the latter varied significantly ($p<0.001$) within the three collection periods. A similar trend was reflected in the albumin: globulin ratio. Serum urea exhibited an interaction ($p<0.05$) between protein and cereal sources; it was lower for calves fed GN-S than GN-B and CS-S (3.13 vs. 4.76 and 3.62 mmol/l, respectively), which varied between themselves. Also, the value was higher ($p<0.001$) for calves fed barley (B) than sorghum (S) diets (4.45 vs. 3.38 mmol/l, respectively). Moreover, an interaction ($p<0.05$) between protein source and day was also evident; at d 0 and d 120 serum urea was not different but at d 210 calves fed GN had higher urea (4.56 vs. 3.45 mmol/l). Serum urea was higher ($p<0.001$) at d 0 followed by d 210 and d 120 showing significant period effect. Serum creatinine was not apparently affected by dietary treatment, but exhibited an interaction ($p<0.05$) between protein source and day; at d 0 and d 210 serum creatinine was similar but at d 120 it was higher in calves fed CS (175.03 vs. 125.5 μmol/l).

Data for serum glucose, cholesterol, ALP and the transaminases AST and ALT are presented in Table 2. Serum glucose was higher ($p<0.001$) at d 120 and d 210 than at d 0 (3.84 and 3.52 vs. 3.05 mmol/l, respectively). An interaction ($p<0.05$) between protein type, cereal source and

Table 2. Serum concentrations of glucose, cholesterol, AST, ALT and alkaline phosphatase in calves fed gossypol through cottonseed meal

	Experimental groups				ANOVA[1]
	B-GN	S-GN	B-CS	S-CS	
Glucose, mmol/l					
day 0	3.24±0.25	2.80±0.33	3.10±0.07	3.07±0.04	D, PxGxD
day 120	3.32±0.22	3.94±0.14	4.35±0.05	3.76±0.24	
day 210	3.27±0.13	4.06±0.30	3.43±0.37	3.33±0.35	
Cholesterol, mmol/l					
day 0	2.56±0.15	2.21±0.22	2.68±0.07	2.16±0.10	G, P, D, GxP
day 120	2.63±0.07	2.34±0.11	3.09±0.07	3.10±0.10	
day 210	2.67±0.09	2.40±0.11	3.26±0.05	3.30±0.04	
AST, U/l					
day 0	23.68±1.20	24.64±1.67	23.52±1.22	20.91±1.26	D
day 120	24.27±1.30	27.88±1.24	22.53±1.34	25.73±1.60	
day 210	21.36±1.93	22.03±0.97	20.90±0.63	21.09±1.35	
ALT, U/l					
day 0	30.36±1.89	30.48±3.40	28.87±3.61	26.93±2.57	PxD
day 120	24.46±1.15	29.24±3.12	33.68±2.17	32.41±2.29	
day 210	28.01±2.56	29.61±2.63	24.11±2.04	22.07±1.24	
ALP, U/l					
day 0	147.5±26.22	132.4±15.82	144.3±29.66	153.2±27.81	D
day 120	165.4±24.59	171.2±22.97	181.3±14.32	206.7±25.15	
day 210	200.1±14.84	189.3±13.25	175.1±13.15	198.3±33.40	

[1] Significant effects ($p<0.05$). Grain (G), protein (P), time (D) and their interactions. B: barley; S: sorghum; GN: groundnut meal; CS: cottonseed meal.

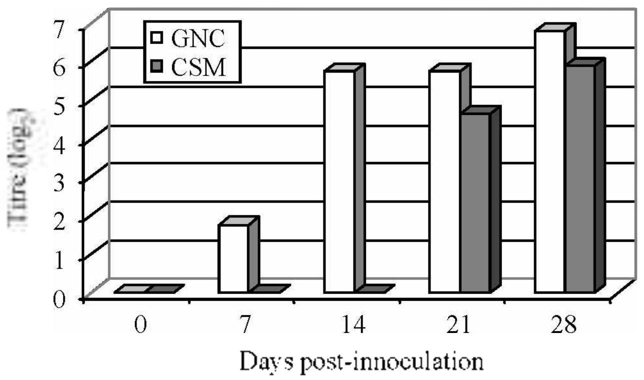

Figure 1. Effect of feeding (gossypol) cottonseed meal (CSM) or groundnut cake (GNC) on the mean titre (log_2) against *B. abortus* S99 antigen in calves.

day was observed; at d 0 and d 210 serum glucose was similar across diets. At d 120 the values were higher in calves fed B-CS diet (4.35 mmol/l) as compared to those fed S-CS (3.76 mmol/l) or B-GN (3.32 mmol/l) diets.

Serum levels of cholesterol varied significantly due to dietary treatments; animals on B diets had higher ($p<0.01$) level than those on S diets. Likewise, calves on CS diets had greater concentration of cholesterol as compared to GN diets fed calves. Moreover, the level of cholesterol in the serum increased ($p<0.01$) during the course of the experiment as indicated by significantly higher values at d 120 (2.79 mmol/l) and d 210 (2.91 mmol/l) compared to that of 0 day (2.40 mmol/l).

Concentration of AST was not influenced by source of protein and cereal. However, calves exhibited a lower ($p<0.001$) value at d 210 as compared to d 120 (21.34 vs. 25.10 U/l, respectively). On the contrary, no effect of type of protein and cereal was apparent on the serum levels of ALT. Moreover, serum ALT exhibited an interaction ($p<0.01$) between protein and day; the value was higher in calves fed CS at d 120 but subsequently becoming similar at d 210 in comparison to calves fed GN (33.05 vs. 26.85 and 23.09 vs. 28.81 U/l, respectively). The serum concentration of ALP did not show any variation attributable to protein or cereal sources. The values, however, were significantly ($p<0.01$) higher at d 120 and d 210 as compared to the d 0 value, showing a period effect.

The result of humoral immune response study is presented in Figure 1. As obvious, grain source had no influence on the immune responsiveness of the calves; hence the data were analyzed and depicted to reflect the effect of the protein sources. The pre-sensitization screening of the calves (through RBPT) revealed that all the experimental animals were negative for brucellosis. The antibody against *B. abortus* S99 was detected in calves fed GN diets as early as 7 d post-innoculation (PI) and showed a steady rise till 28 d PI. On the contrary, CS fed calves failed to respond by 14 d PI, and antibody was detected only after 21 d PI. The antibody titre (log_2) exhibited by CS fed calves was significantly lower on 14, 21 and 28 d PI as compared to GN fed calves.

DISCUSSION

The profile of blood serum proteins such as albumin, globulin, total protein and the ratio between albumin and globulin was not affected by dietary treatments. The variation observed with respect to days of collection appears to be related to that of the globulin concentration rather than albumin. However, these variations in serum proteins perhaps had no biological significance as these are well within the normal physiological ranges for bovines (Kaneko, 1980). Moreover, Murray et al. (1993) reported that, albumin concentration <3.8 g/dl and globulin concentrations >3.8 g/dl were signs of hepatic insufficiency. In non-ruminants gossypol consumption has been correlated with hypoproteinemia involving hypoalbuminemia (Jarquin et al., 1966). However, Lindsay et al. (1980) have reported a hypoproteinemia characterised by a disproportionate increase in globulin fraction in adult cows fed cottonseed meal. Zelski et al. (1995), on the other hand, have observed lower serum total protein accompanying a significant drop in albumin in young calves affected with gossypol toxicosis, and attributed this to a severely impaired liver function.

Blood urea concentration in ruminants is closely regulated by the rate of protein breakdown and ammonia utilization for bacterial protein synthesis in the rumen. An increase in the level of blood urea may reflect an accelerated rate of protein catabolism rather than decreased urinary excretion (Kaneko, 1989), while a reduction was observed in severe hepatic insufficiency and renal affections (Kaysen et al., 1985). In the present study, the values observed were within the reported normal range (Kaneko, 1980), indicating no impact of CS gossypol feeding. Similar to the present findings, feeding of cottonseed at 21 (Keery et al., 1991) and 30 per cent (Nikokyris et al., 1991) levels to lambs did not affected the blood urea levels.

Serum creatinine levels in the present study are similar irrespective of protein type or cereal sources. Increased creatinine levels are generally seen in degenerative muscular diseases (Prasse, 1986). Similar to the present findings, Barraza et al. (1991) have also found no variation in creatinine levels of Holstein calves fed 50 per cent whole cottonseed in their diets. Also, no impact of gossypol feeding was evident when it was fed either as cottonseed meal to lambs (Nagalakshmi et al., 2001) or as whole cottonseed to heifers (Colin-Negrate et al., 1996). Serum creatinine level was higher in CS fed calves on d 120; but it became comparable to the GN fed group towards the end of the study. A similar trend in serum creatinine level was also recorded by Warren et al. (1988); they have observed a significant increase in serum creatinine value in wethers after 127 days of whole cottonseed feeding and attributed this to muscle damage because of gossypol.

No impact of dietary treatments was evident on serum glucose similar to the observations of Colin-Negrate et al. (1996) in heifers and Nagalakshmi et al. (2001) in lambs. Feeding of CS, on the other hand, significantly increased the serum cholesterol as have been reported by Williams et al. (1989). The significant increase in cholesterol with advancement of feeding period was again in agreement with the observations of Colin-Negrate et al. (1996), who have found increased blood cholesterol level in growing heifers upon long term feeding of whole cottonseed. Serum cholesterol besides being influenced by thyroid activity also varies with a variety of factors such as the nature of the diet and hepatic function among others (Kaneko, 1980).

The feeding of cottonseed exerted no influence on the activity of serum ALP. ALP is a membrane bound enzyme used for diagnosis of bone and liver disorders. Because of its association with osteoblastic acivity, bone contains more ALP activity than any other tissue in growing animals. But once growth ceases, liver becomes the primary source of ALP. Its activity increases in response to primary and secondary hepato-cellular disorders including gossypol toxicity, which induces degenerative changes in the liver. According to Murray et al. (1993), high levels of ALP might be because of a direct response to liver and bone injury as a result of gossypol toxicity. However, the present findings of similar serum ALP activity in calves fed CS and GN diets indicates absence of any toxicity due to gossypol. Velasquez-Pereira et al. (1998) have also observed no impact of CS feeding (equivalent to 12.9 mg free gossypol/kg body weight) on serum ALP activity.

Young calves with gossypol intoxication have exhibited high serum levels of AST (Zelski et al., 1995). Acute hepatic disorders causing membrane damage or cell necrosis usually results in an appreciable increase in plasma AST activity (Kaneko, 1980). AST, although not used as an organ specific enzyme, nonetheless could be useful in conjunction with other enzymes as an index of hepatic and muscular cell damage (Kaneko, 1980). In the present study, feeding of gossypol at the designated levels did not induce any changes in serum AST activity. Similarly, Nagalakshmi et al. (2001) have also found no impact of feeding 40 per cent cottonseed meal in the diet of lambs on serum AST activity. Feeding of CS resulted in an increase in ALT activity at d 120 in comparison to calves fed GN. However, subsequently by d 210, the values declined to become comparable to that of the GN fed calves. Colin-Negrate et al. (1996) have also recorded a linear increase in serum ALT activity in heifers with increasing levels of whole cottonseed meal inclusion in their diets.

The humoral immune response of the calves as assessed through seroreactivity against *B. abortus* S99 was indicative of the fact that feeding of CS diets delayed the onset of immune response, which was not apparent till 21 d PI. Moreover, the seroreactivity of the CS fed calves was poor as evident from the significantly lower antibody titre (log_2) in comparison to the GN fed calves. Very little research has been done to assess the impact of gossypol on the immunocompetence of farm animals. Rogozhin et al. (1986) have observed a depression in immunological reactivity and destruction of immunity in vaccinated pigs upon long term feeding of cottonseed meal. More recently, Nagalakshmi et al. (2001) have reported that incorporation of 40 per cent cottonseed meal in the concentrate supplement significantly affected both the cell-mediated and humoral immune response of the lambs. These findings further confirmed the negative impact of CS feeding on the immunocompetence of farm livestock.

Based on the average daily consumption of dry matter through concentrate and wheat straw (data not given), the average intake of total and free gossypol (FG) in the present study works out to be 2.70 and 1.40 g/d, respectively, equivalent to 798 mg TG and 416 mg FG/kg feed dry matter. Brahman bulls of about 6 months of age when fed on 1.8 g FG/bull/day for 196 days, tended to have lower weight gain compared to the control, with no apparent clinical signs of gossypol toxicity (Chase et al., 1994). In a previous long duration study in heifers for 431 days, Colin-Negrate et al. (1996) have concluded that although feeding of total gossypol (TG) at 7.8 mg/kg BW was without any significant adverse effects, any further increase beyond this level needs further validation. In the present study, feeding of TG at 19.11 mg/kg BW (equivalent to 9.95 mg FG/kg BW) resulted in no apparent clinical and biochemical manifestation of toxicity. Accompanying the non-existent detrimental physiological changes, there was also lack of any physical symptoms, apparently suggestive of absence of gossypol toxicity. However, the humoral immune response of the calves was severely compromised, indicating possibly the presence of a sub-clinical toxic condition. It is, therefore, quite pertinent to conclude that blood biochemistry may not be of much usefulness for monitoring sub-clinical gossypol toxicosis in calves. Similar was the view of Risco et al. (1992), who stated that the changes in enzyme activities might be indicative of liver failure only during terminal stages of the disease in calves.

IMPLICATIONS

From the results, it is evident that long-term feeding of moderate level of cottonseed meal, at 25 per cent of concentrate and supplement supplying about 19 mg total gossypol/kg BW, did not apparently induce any toxic symptoms in calves even up to 210 days. Although the animals exhibited alterations in some blood biochemical parameters, none of the metabolites was beyond the normal physiologic range so as to signify drastic impact of gossypol poisoning. The humoral immunity of the calves, on the contrary, was significantly affected: this needs further research in the context of the clinical wellbeing of cottonseed (meal) fed ruminants.

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