EFFECTS OF GOSSYPOL ON REPRODUCTION AND HEMATOLOGY IN SHEEP

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ABSTRACT

Twenty-nine sheep were used to determine the effects of gossypol on estrous cycle length, corpus luteum function in cycling sheep, embryonic development in pregnant sheep, and blood fragility. The sheep were randomly assigned to a control diet containing no cottonseed products (T0 = 0 g gossypol/days), a diet containing cottonseed meal and cottonseed hulls (T1 = 0.96 g gossypol/d), and diet containing whole cottonseed and cottonseed hulls (T2 = 2.42 g gossypol/ days). Weekly blood sample were obtained to monitor serum concentration of progesterone and to analyze of total hemoglobin and percentage of met-hemoglobin. After about 60 days, the sheep were synchronized using SMB implants and superovulated using FSH-P. Five days after estrous detection, embryos were surgically flushed from the uterus. There were no differences ($P>0.05$) among dietary treatment groups in interval to onset of estrus after synchronization, number of ovulation after superovulation, and number of embryos recovered. The diets had no effects ($P>0.05$) on the length of the first or second cycle and progesterone concentration when the sheep were on the diets for 42 - 58 days, indicating that normal luteal function was occurring during the estrous cycle. The only significant difference observed on this study were the total hemoglobin ($P <0.005$) and percentage of met-hemoglobin ($P < 0.001$) which increased in a linear fashion as the amount of free gossypol fed per day increased. Therefore, diets containing cottonseed products, in which the daily free gossypol intake did not exceed 2.42 g, had no effects on reproduction.

(Key Words: Gossypol, Cottonseed, Estrous Cycle, Reproduction, Hemoglobin, Methemoglobin).

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PENGARUH GOSSYPOL TERHADAP REPRODUKSI DAN HEMATOLOGY PADA DOMBA

INTISARI

Dua puluh sembilan domba digunakan untuk mengetahui pengaruh gossypol terhadap lama siklus estrus, perkembangan embrio dan kerusakan darah. Domba-domba tersebut dibagi dalam tiga kelompok perlakuan pakan yaitu pakan tanpa produk tanaman kapas (T0 = 0 g gossypol/hari), pakan mengandung bahan biji kapas dengan kulitnya (T1 = 0,96 g gossypol/hari), dan pakan mengandung biji kapas ditambah kulit bijinya (T2 = 2,42 g gossypol/hari). Pengambilan sampel darah mingguan dilakukan untuk mengukur serum progesteron, hemoglobin dan persentase serum methemoglobin. Setelah 60 hari dalam perlakuan, domba disinkronisasi estrus dengan menggunakan syncro-mate B dan disuperovulasi dengan menggunakan FSH-P. Lima hari setelah estrus terditeksi, embrio dikeluarkan dengan jalan operasi. Hasilnya menunjukkan tidak ada perbedaan terhadap munculnya estrus pertama atau kedua setelah sinkronisasi, jumlah ovulasi setelah superovulasi, dan jumlah embrio yang didapat (P> 0,05). Perlakuan juga menunjukkan tidak ada perbedaan pada lama sikus estrus pertama dan kedua dan terhadap konsentrasi progesteron selama domba dalam perlakuan 42 - 58 hari menunjukkan berfungsi normalnya korpus luteum selama siklus estrus. Beberapa parameter yang berbeda adalah total serum hemoglobin (P<0,005) dan persentasi serum methemoglobin (P<0,001) yang meningkat seiring dengan peningkatan kandungan gossypolnya Ternyata bahwa penggunaan gossypol sampai sejumlah 2,42 g dalam pakan domba tidak mempengaruhi reproduksinya.

(Kata Kunci: Gossypol, Biji Kapas, Siklus Estrus, Reproduksi, Progesteron, Hemoglobin, Methemoglobin).

Introduction

Gossypol is yellowish phenolic compound occurring naturally in certain species of cotton plants of family *Malvaciae*, mostly in the seed and root bark. Gossypol has molecule weight of 518.54 and structure of (2,2"-binophthelene)-8,8'-dicarboxaldehyde - 1,1', 6,6',7,7'-hexahydroxy-5,5'-diisoprophyl (Qian and Wang, 1984). In seed, gossypol is contained in pigment glands and constitutes 30–40 per cent of their weight. All of the gossypol in whole seed is in the free form (Pons *et al.* 1953), but cottonseed meal, unlike the raw seed, has had the oil removed by processing and most of gossypol has been converted to "bond form" by cooking (Edwards, 1960).

Low level of gossypol intake in lactating dairy cows had no effect on packed cell volume, copper in plasma, activities of transaminase and gross composition of milk. However, cows consuming direct solvent extracted or screw-pressed cottonseed meal had decreased hemoglobin, increased protein, erythrocyte fragility and gossypol in plasma and liver (Linasay *et al.*, 1980). Erythrocyte fragility was increased in a linear fashion in lambs injected with 5, 10, 20 and 30 mg of gossypol acetic acid (GAA). Free gossypol in diets was less toxic than those by direct injection of GAA (Danke *et al.*, 1965; Houston *et al.*, 1990).

Gossypol, the toxic constituent of cottonseed products, has shown to cause infertility in monogastric male including man (Qian and Wang, 1984). The studies in bulls and rams also have shown the specific effects on gonadal tissues. However, the detrimental effects on reproduction of female of ruminant animals received scant attention. Thus, the objectives of this experiment were to determine

the effects of cottonseed products on estrous cycle length and corpus luteum (CL) function in cycling sheep, early embryonic development in pregnant sheep and hematological effects on red blood cells.

Materials and Methods

Twenty-nine Rambouillet sheep (avg wt, 58 ± 1.5 kg; 2 to 5 yr of age) were randomly assigned to three treatment groups. The treatment groups were: a control diet containing no cottonseed ingredients (n = 10); a diet containing cotton seed meal/cotton seed hull (CSM/CSH; n = 9), and a diet containing whole cotton seed (WCS/CSH; n = 10; Table 1). The diets were formulated to be isocaloric and isonitrogenous and to meet NRC requirements (NRC, 1975). The percentage of free gossypol in the cottonseed products was determined by Pope Testing Laboratories, Dallas, TX. The results were: CSH, .045%; CSM, .258%; and WCS, .480% free gossypol. Sheep were fed 1.5 kg of their respective diets daily. By calculation, the sheep were offered the following amount of free gossypol: control diet, (T0 = 0 g/d); CSM/CSH diet (T1 = 0.96 g/d); and WCS/CSH diet (T2 = 2.42 g/d). Experimental diets were fed for an average of 87 d (pooled SE, 3.9).

Estrous cycles were monitored daily using raddled rams equipped with marking harnesses. Weekly blood samples were obtained to monitor lutea function. Concentrations of serum progesterone were determined using a kit (Coat-A-Count TKPG5; Diagnostic Products Corp., Los Angeles, CA). After an average of 68 d on their respective diets, the sheep were synchronized using sychro-mate B (SMB) implants (Ceva Lab., Overland Park, KS) implanted subcutaneously in the ear as describe by Ruttle *et al.*, 1988. The size of implant was obtained by cutting a commercially sold 6-mg bovine implant into two parts. The implants remained in place for 12 d at which time they were removed. For superovulation, sheep were injected with using follicle stimulating hormone (FSH; Schering Co., Kenilwort, NJ) in a series as follows: day 10 after implantation, 4 mg FSH twice daily; day 11, 3 mg FSH twice daily; and day 12, 2 mg FSH-P twice daily (total dose = 18 ng) as described by Ruttle *et al.*, 1988. After implant removal, the sheep were placed with rams. The breeding activity was observed at 12-h intervals for 72 h.

Five days after estrus (day 0), the embryos were surgically flushed via a mid-ventral incision. The sheep were anesthetized with xylazine (Rompun; Lloyd Lab., Shenandoah, IA). They were also given atropine (Anthony Products, Arcodia, CA) to prevent salivation. The number of CL on each ovary were recorded. Embryos were flushed by inserting a curved laboratory animal incubation needle (Baxter Scientific, Dallas, TX) into the oviduct and forcing Dulbecco's phosphate buffered saline (American Embryo Systems, Grand Prairie, TX) containing 2% fetal calf serum (American Embryo Systems) into the oviduct and out through a glass cannula inserted into the uterine horn just anterior to the internal bifurcation. The flush media was collected in a petri dish (Falcon Integrid; Becton Dickinson and Co., Lincoln Park, NJ).

The embryos were located under a stereomicroscope (Nikon, Japan) and evaluated using a phase contrast microscope (Leitz Fluovert, West Germany). Embryos were evaluated for their morphological quality using a 5-point scale (Ruttle *et al.*, 1988): 1 = EXCELLENT - perfectly symmetrical with even granulation and no blastomere extrusion; 2 = GOOD - occasionally slightly asymmetric in shape, even granulation with some blastoderm extrusion; 3 = FAIR - slightly asymmetric in shape, blastomere extrusion and some degeneration; 4 = POOR - uneven granulation, much blastomere extrusion and degeneration; and 5 = UNFERTILIZED.

After the sheep were on their respective diets for an average of 87 d (pooled SE = 3.9), a blood sample was obtained for analysis of

Table 1. Composition of diets

Ingredient	Treatments [a]		
	Control	CSM/CSH	WCS/CSH
	%		
Sorghum, dry rolled	28.78	28.78	28.78
Cottonseed hulls		48.15	39.94
Sudan grass hay [b]	53.31		
Cottonseed meal [c]		16.32	
Whole cottonseed			29.92
Soybean meal	10.50		
Animal/vegetable oil	6.05	5.39	
Urea	0.71	0.71	0.71
Salt	0.50	0.50	0.50
Ammonium chloride	0.50	0.50	0.50
Dicalcium phosphate	0.89	0.89	0.89
Potasium chloride	0.07	0.07	0.07
Trace mineral premix	0.50	0.50	0.50
Vitamin A, D, and E premix	0.19	0.19	0.19

[a] Formulated on dry matter basis, except whole cottonseed and fat sources.
[b] Ground in tube grinder and mix with other ingredients.
[c] Direct solvent extracted.

serum hemoglobin and met-hemoglobin. Serum was obtained from the samples via centrifugation, frozen and stored at -20° C until analysis. The serum hemoglobin and met-hemoglobin were determined using a Co-Oximeter (Model 282, Instrumentation Laboratory, Lexington, MA).

The end points evaluated by statistical analysis were: ADG, estrous cycle length, interval to onset of estrus after synchronization, number of CL after superovulation, number and quality of embryos collected per ewe, and serum hemoglobin and met-hemoglobin. The data were analyzed by a completely randomized design analysis of variance (Steel and Torrie, 1980) using the General Linear Models procedure (SAS, 1985). The two degrees of freedom for treatment were resolved into a set of orthogonal polynomials (linear and quadratic) based on the grams of free gossypol fed per day.

Results and Discussion

The ADG of the sheep over the first 56 d of the experiment was 0.11 kg for the group receiving 2.42 g/d dietary free gossypol, 0.12 kg for the group receiving 0.96 g/d dietary free gossypol, and 0.13 kg for the group receiving 0 g/d dietary free gossypol (P> 0.70). The feeding of cottonseed products to sheep in this experiment did not affect ADG. Numerous studies have shown that the feeding of cottonseed products had no effect on ADG in bulls (Chase *et al.*, 1990; Jimenez *et al.*, 1989), sheep (Danke *et al.*, 1965; Arshami and Ruttle 1989; Calhoun *et al.*, 1990) and goat (Menges *et al.*, 1991). Also GAA treatment did not affect body weight in rats (Gafvels *et al.*, 1984; Wang *et al.*, 1984; Beaudoin, 1988).

The results of reproductive characteristics are summarized in Table 2. The length of the first estrous cycle was not different (P>0.05) among the treatment groups (T0 =

Table 2. Effects of gossypol on reproductive characteristics

	T0	T1	T2	Difference*
n	10	9	10	
First estrous cycle length, d	15.4	15.3	15.4	ns
Second estrous cycle length, d	15.9	15.2	18.7	ns
Interval to estrus after implant removal, h	28.2	27.0	26.7	ns
Number of corpus luteum after superovulation	10.3	11.8	10.7	ns
Number of embryos recovered	4.8	5.2	4.8	ns
Embryo quality score	2.5	2.6	2.4	ns

ns, not significant difference (P>0.05).

15.4 d, T1 = 15.3 d, and T2 = 15.4 d) when the sheep had been on their respective diets for an average of 42 d (pooled SE,1.5 d). Likewise, the length of the second estrous cycle was not different (P>0.05) among the treatment groups (T0 = 15.9 d, T1 = 16.2 d, and T2 = 16.7) when the sheep had been on their respective diets for an average of 58 d (pooled SE, 2.1 d).

The interval to estrus after removal of the SMB implants (26.8 h) was not different (P>0.05) among the three levels of dietary free gossypol. The number of corpora lutea present on the ovaries (10.6) at the synchronized estrus was also not different (P>0.05) among the treatment groups. Five days after the synchronized estrus, 44 % of the ovulated ova were recovered by flushing the oviducts and uterus for embryos. The number of embryos recovered was not different (P>0.05) among the levels of dietary free gossypol, after recovering the embryos, they were scored on a five-point scale (see materials and methods) for their quality. The quality scores of the recovered embryos were not different (P>0.05) among the treatment groups.

The data indicated that embryo score was not affected by gossypol treatment. In another study (Wyse *et al.*, 1991b), quality grade and developmental stage score of heifer embryos were also not affected by diets containing gossypol but the proportion of degenerating embryos was affected by diets containing gossypol. However, study in rats found an effect of embryonic development when rat embryos were cultured in GAA (Lin *et al.*, 1989).

In sheep of this experiment and heifers (Gray *et al.*, 1990), cottonseed products did not affected the length of the estrous cycle or the serum concentrations of progesterone. However, when granulosa cells are cultured *in vitro* and stimulated with forskolin, the progesterone secretion into the media was less in heifers fed diets containing CSM or WCS then those fed a control diet that contained no cottonseed products. In addition, free gossypol from CSM had an effect on the total number of follicles > 4 mm in diameter (Wyse *et al.*, 1991).

The concentration of progesterone were measured in serum samples obtained at weekly interval from the sheep in each of the treatment group. As shown in Figure 1, luteal activity, as measured by progesterone concentrations, was observed in the sheep within each treatment group. These concentrations are considered to be normal and the pattern of progesterone concentrations in sheep. In non-ruminants, GAA-treated rats showed more irregular and longer estrous cycle than control rats (Lagerlof and Tone, 1985). In human females, cooking with crude cottonseed oil led to prolonged menstrual cycles (Fen *et al.*, 1980). In contrast, this study found that gossypol did not affect the normal pattern of estrous cycle length.

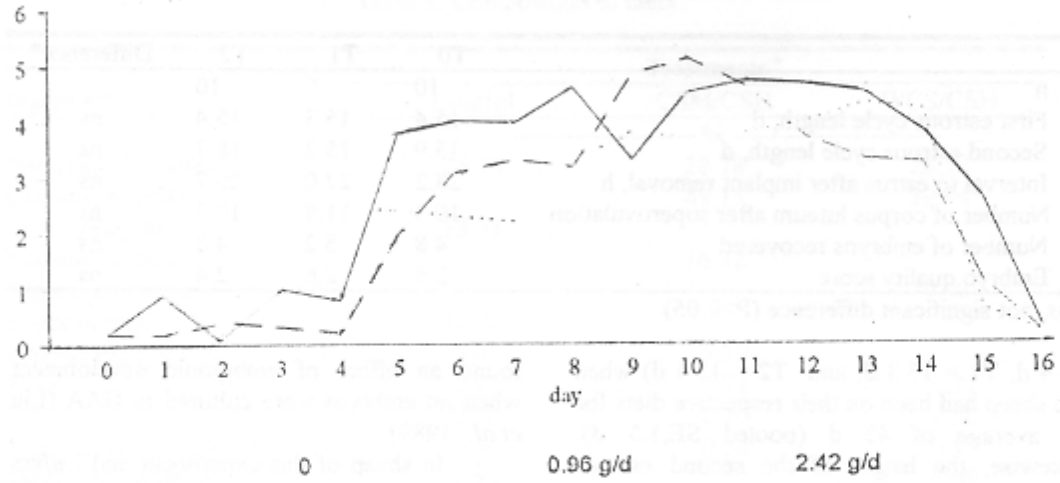

Figure 1. Progesterone concentration of the sheep.

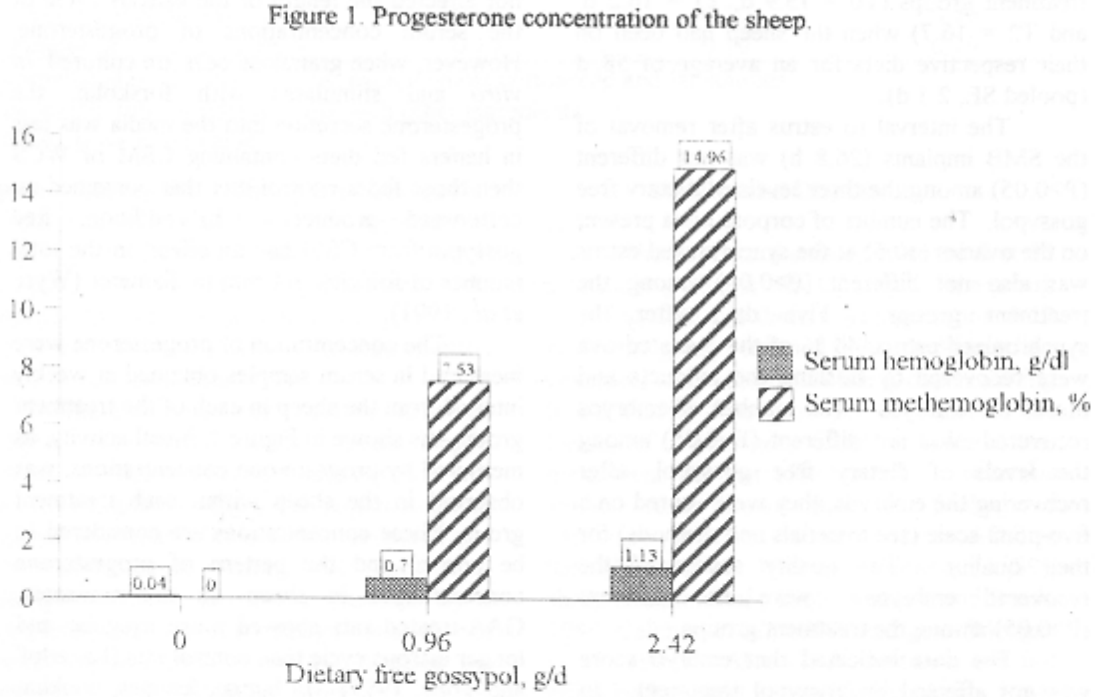

Figure 2. The linear relationship between different levels of gossypol fed per day and serum hemmoglobin and methemoglobin.

Past experiments (Ruttle *et al.*, 1988) and this study indicated progesterone implant and FSH-P injection could achieve synchronization and superovulation in sheep. This study also agreed with study in rats indicating gossypol did not inhibit ovulation (Hahn *et al.*, 1981).

After the sheep had been on their respective diets for 82.5 d (pooled SE, .85), serum hemoglobin and met-hemoglobin were measured. As shown in figure 2, the serum hemoglobin ($P< 0.005$) and met-hemoglobin ($P<0.001$) increased in a linear fashion as the levels of dietary free gossypol increased. This study indicated that erythrocyte fragility was increased as the amount of free gossypol fed per day increased. Increased osmotic fragility of RBC associated with gossypol intake has been observed in sheep and goat (Calhoun *et al.*, 1990) and cows (Grey *et al.*, 1990). Erythrocyte fragility was resulted from either that not all free gossypol could be detoxified in the rumen or that a portion of the bound gossypol was released during digestion in the small intestine of sheep (Calhoun *et al.*, 1990a) and cows (Gray *et al.*, 1990).

Conclusion

The sheep fed diets containing free gossypol for an average of 87 d had no detrimental effects on the reproductive end point. However, hematological evidence was observed indicating that gossypol had an effect on the erythrocyte fragility.

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