Effect of free gossypol in whole cottonseed on the semen quality of Holstein bulls

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It is suspected that feeding large amounts of whole cottonseed and cottonseed meal to dairy cows can cause gossypol intoxication. It has also been shown that male antifertility is probably the first physiological effect of free gossypol. The objective of this investigation was to determine whether diets containing 0,185 or 0,254% free gossypol per kg dry matter would cause any spermatozoal defects at intakes of 64 or 75 mg free gossypol (as whole cottonseed) per kg live mass in two-year-old Holstein bulls. Twelve Holstein bulls were allotted to three comparable groups receiving complete diets containing 0; 27,9 and 39,1% whole cottonseed per DM which contained 0,64% free gossypol per DM. Semen was collected on days 60, 80, 100, and 120 and compared for volume, colour, motility, concentration, % spermatozoa live or dead, % spermatozoa with normal morphology, viability after deep freezing and swimming speed. Histological studies were also carried out on the testes. No parameters were influenced significantly ($p < 0,05$) and it was concluded that free gossypol intakes used in this study did not cause any spermatozoal defects and that the free gossypol was probably efficiently detoxified in the rumen.

Daar word vermoed dat die voeding van groot hoeveelhede heel katoensaad en/of katoensaadoliekoek aan melkkoeie moontlik tot gossipolvergiftiging kan lei. Dit is ook bekend dat manlike onvrugbaarheid die eerste fisiologiese effek van vry gossipol is. Die doel van hierdie studie was om vas te stel of diëte wat 0,185 of 0,254% vry gossipol per kg droë materiaal bevat enige spermdefekte teen innames van 64 of 75 mg vry gossipol (as heel katoensaad) per kg lewende massa in twee-jaar-oue Holsteinbulle sal veroorsaak. Twaalf Holsteinbulle is ewekansig in drie vergelykbare groepe ingedeel en vergelykbare diëte gevoer wat onderskiedelik 0; 27,9; en 39,1% heel katoensaad op 'n DM basis bevat het waarvan die vrygossipolinhoud 0,64% op 'n DM basis was. Semen is op dag 60, 80, 100, en 120 na aanvang van die proef versamel en ten opsigte van volume, kleur, beweeglikheid, konsentrasie, % sperme lewendig of dood, % morfologies normale sperme, lewensvatbaarheid na bevriesing en swemspoed vergelyk. Histologiese studies is ook op die testes uitgevoer. Geen van die gemete kenmerke het betekenisvol ($p < 0,05$) tussen die drie groepe verskil nie. Daar is tot die gevolgtrekking gekom dat die genoemde vrygossipolinnames geen spermdefekte veroorsaak het nie en dat die vry gossipol waarskynlik in die rumen ontgiftig is.

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Gossypol, a yellow phenolic pigment found in the seeds, stems and roots of the cotton plant (Gossypium sp.) has antispermatogenic effects in the human, rat, hamster, Stumptail monkey, Cynomulgus monkey and pig (National Coordinating Group on Male Antifertility Agents, 1978; Nadakavukaren, Sorensen & Tone, 1979; Zatuchni & Osborn, 1981; Shandilya, Clarkson, Adams & Lewis, 1982; Ling-Yun, Shu-Haum, Gia-Xiang, He-Ping, Xi-Yun, Qiang, Zhu-Kui, Te-Fu, Ke-Xian, Sheng-Rong & Xin-Min, 1984). The National Coordinating Group on Male Antifertility Agents (1978) reported that gossypol intake by humans caused a decrease in the percentage of motile spermatozoa followed by an increase in malformed spermatozoa and a gradual decline in spermatozoa concentration until azoospermia was reached. The antimotility effect of gossypol on spermatozoa was also demonstrated *in vitro* with the semen of boars (Tso & Lee, 1981), rodents (Hahn, Rusticus, Probst, Homm & Johnson, 1981), monkeys (Stephens, Critchlow & Hoskins, 1983) and guinea pigs (Shi & Friend, 1983). The antimotility effects are probably due to the detrimental interactions of gossypol with spermatozoal membranes, probably as an uncoupler of mitochondrial oxidative phosphorylation (Nadakavukaren *et al.*, 1979; Tanphaichitr, Chen & Bellvé, 1984; Reyes, Allen, Tanphaichitr, Bellvé & Benos, 1984) and on metabolic enzymes (Lee & Malling, 1981; Tso & Lee, 1981; Kennedy, van der Ven, Straus, Bhattacharyya, Waller, Zaneveld & Polakoski, 1983).

Although ruminants are considered to be relatively tolerant to gossypol intoxication, due to the binding of gossypol in the rumen by the ϵ-amino group of lysine (Reiser & Fu, 1962), iron (Jonassen & Demint, 1955), calcium, sodium, potassium (Berardi & Goldblatt, 1969) and certain amines, especially octylamine (Singleton & Kratzer, 1973), Lindsey, Hawkins & Guthrie (1980) showed that diets supplying 24 g free gossypol per lactating Holstein cow per day caused depressions in haemoglobin and total protein. Increased erythrocyte fragility was also found as was an increased respiration rate in hot weather. Smalley & Bicknell (1982) reported temporary infertility in bulls fed 3,63 kg ammoniated whole cottonseed and 1,36 kg cottonseed meal per day. In contrast to these findings, Coppock, West, Moya, Thompson, Rowe, Nave, La Bore & Gates (1985) did not find gossypol toxicity when using 13 blood metabolites as indicators in lactating Holstein cows fed diets containing 15 and 30% whole cottonseed. The same authors also found no evidence of gossypol toxicity using 11 blood metabolites as indicators when diets containing 15, 35, and 55% whole cottonseed were fed to dry Holstein cows.

Hoffer (1983) found that gossypol intakes severely damaged some seminiferous tubules in the rat testis. Using electron microscopy, severely affected Sertoli cells were found which exhibited many large vacuoles and an overall decrease in cytoplasmic ground substance, rough and smooth endoplasmic reticulum and Golgi apparatus.

Because gossypol inhibits spermatogenesis in experimental animals and humans with minimal or no side effects, the antispermatogenic effect is regarded as its first probable physiological detriment. This study was undertaken to determine whether high intakes of free gossypol (as whole cottonseed) causes spermatozoal or testicular defects in two-year-old Holstein bulls.

Materials and methods

Twelve, two-year-old Holstein bulls were allotted to three comparable groups receiving complete diets containing 0; 25; and 35% whole cottonseed (air dry) (Table 1) which contained 0,58% free gossypol. The free gossypol contents of the diets were 0,01; 0,17 and 0,23%, respectively, (air dry basis) (Table 2).

Semen was collected on days 60, 80, 100, and 120 of the feeding period with an artificial vagina (Cambridge type) and compared for volume, colour, motility, concentration, viability, morphology, viability after deep freezing and swimming speed after deep freezing. The more subjective methods of semen evaluation were included in the study because of their importance in practical semen evaluation. Semen colour as a possible indicator of sperm concentration was described as thick creamy, creamy, light creamy, milky, watery or clear (Haq, 1949).

Motility was scored on a basis of 0–5 with a light microscope. A score of 0 implied that spermatozoa are totally immotile and a score of 5 implied very vigorous motion with extremely rapid waves and eddies, indicating about 100% actively motile spermatozoa (Haq, 1949). After the initial scoring, semen was kept in a waterbath at a constant temperature of 32°C and rescored after incubation periods of 2, 4, and 6 hours.

Spermatozoa concentration was determined with a haemocytometer. The semen was diluted 1:200 with a gentian violet and alcohol solution, placed on the haemocytometer and covered with a cover-glass. Spermatozoa were counted with a light microscope and the concentration calculated (Salisbury, Beck, Elliot & Willett, 1943).

Percentage live/dead spermatozoa was determined by using an eosin-nigrosin stain (Swanson & Bearden, 1951) and counting 500 spermatozoa per ejaculation of each bull. This procedure was repeated after the semen had been deep frozen and thawed. The eosin-nigrosin stain was also used for the identification of morphologically abnormal spermatozoa. Abnormalities were classified as head, middlepiece or tail defects and 500 spermatozoa were scanned per ejaculate of each bull using a phase contrast microscope (Swanson,& Bearden, 1951).

Swimming speed of spermatozoa in deep frozen, thawed semen was estimated by means of frame lapse videography and computer analysis. A colour video camera was connected to a colour video monitor via a video tape recorder. Semen (7µl) was placed on a microscope slide, covered with a coverglass, preheated and maintained at a specific temperature (32°C) by means of a temperature-controlled stage. Ten different semen fields were chosen at random for each bull and taped. Motile sperm were followed and drawn with a pen on a transparency during replay of the tape. The videotape was advanced, frame by frame, using remote control and the respective points, 10 frames apart, were joined to one another. Each trace was followed by means of a 'light pen' on a graphics tablet coupled to an Apple II computer to determine the distance travelled by each spermatozoa. An algorhythm was used to trace velocity (swimming speed) in micrometer per second (µm/sec), the chord velocity (vector from starting point to end point) and the angle of curvature.

Samples for histological slides were taken at three different sites in each testis and fixed in a 10% formaldehyde in saline solution. Slides were stained according to the standard H.E. method and examined with a light microscope (McManus & Mowry, 1960).

Specimens for gossypol analysis were also taken at three different sites on each testis and frozen. Free and total gossypol were determined according to American Oil Chemists Society (1970).

Differences between treatments were tested by standard one-way analysis of variance procedures (Snedecor & Cochran, 1980).

Results and discussion

The ingredient and chemical compositions of the three experimental diets are presented in Tables 1 and 2. The three diets were formulated to be as similar as possible.

Table 1 Composition of experimental diet

Ingredient		Diet A	Diet B	Diet C
Whole cottonseed	(%)	0,0	25,00	35,00
Barley meal	(%)	62,0	49,50	44,50
Oat husks	(%)	5,0	5,00	5,00
Cottonseed husks	(%)	6,0	2,00	0,00
Cottonseed oilcake	(%)	12,0	3,50	0,50
Sugarcane molasses	(%)	13,0	13,00	13,00
Limestone	(%)	2,0	2,00	2,00
Monensin-Na		30,0 ppm	30,0 ppm	30,0 ppm
Vitamins and minerals		+	+	+*

* A standard vitamin and mineral premix for ruminants was added

Table 2 Chemical composition of experimental diets

Constituent*		Diet A	Diet B	Diet C
Dry matter	(%)	89,67	89,73	89,52
Crude protein	(%)	12,00	11,94	12,06
Ether extract	(%)	1,92	6,38	8,20
Metabolizable energy**	(MJ/kg)	10,50	10,83	10,99
Crude fibre	(%)	8,99	9,47	9,52
Calcium	(%)	0,90	0,91	0,91
Phosphorus	(%)	0,36	0,39	0,41
Free gossypol	(%)	0,0120	0,1660	0,2275

* On an air-dry basis

** Calculated according to ingredient values by Van der Merwe (1983)

Table 3 Semen parameters of Holstein bulls fed diets containing different levels of whole cottonseed (free gossypol)

Parameter		Treatment A	B	C
Free gossypol in diet DM	(%)	0,01	0,17	0,23
Semen volume	(ml)	5,06±0,70*	5,25±0,76	5,21±0,54
Motility index of fresh semen	($\bar{5}$)**	3,78±0,20	3,72±0,26	3,66±0,20
Motility index after two hours	($\bar{5}$)**	3,00±0,27	2,81±0,30	2,81±0,22
Motility index after four hours	($\bar{5}$)**	2,31±0,27	2,22±0,29	2,22±0,26
Motility index after six hours	($\bar{5}$)**	1,69±0,20	1,59±0,25	1,69±0,29
Sperm cell concentration	($\times 10^7$/ml)	98,44±2,70	97,81±6,95	98,44±4,54
Live sperm cells	(%)	89,38±1,80	89,06±3,19	90,31±1,93
Normal sperm cells	(%)	94,88±0,68	95,00±0,84	95,44±0,77

* Standard error of the mean

** ($\bar{5}$) — Parameter was scored out of 5

Whole cottonseed was included at 0; 25; and 35% on an air dry basis.

Diets B and C contained 6,38 and 8,20% ether extract, compared to 1,92% in the control diet (A) and also 0,1660 and 0,2275% free gossypol, compared to 0,0120% in diet A, respectively. All other chemical analyses were very comparable.

There were no differences between parameters measured consecutively on days 60, 80, 100, and 120 of the feeding period and only the mean value for each measurement over all four collection periods is given in Tables 3 and 4.

Semen colour varied between light creamy and creamy with no abnormal colours. The average volume of the ejaculate varied between 4 and 6 ml with no statistical differences ($p < 0,05$) between the three treatments (Table 3). The average ejaculate volume of bulls reported in the literature varies between 2 and 10 ml (Roberts, 1971; Hansel & McEntee, 1975).

The movement of sperm is a direct expression of a number of intracellular mechanisms associated with the processes of maturation, transport, capacitation and fertilization (Katz, Overstreet & Hanson, 1981). According to Sotelo, Montalvo, de la Luz Crail & Gonzalez-Garza (1982), motility is the first quality of spermatozoa that will be influenced by an excess of gossypol. A degree of motility of 3 or higher is the average requirement for good quality semen (Roberts, 1971). In this study, the average degree of spermatozoa motility was above 3,5. No statistical differences ($p < 0,05$) were found between the three treatments (Table 3). There were also no differences in motility index after incubation for two, four or six hours (Table 3) of spermatozoa. The swimming speed of bull sperm in this study was lower than the values reported by Samuels & van der Horst (1986) using the same technique. The values obtained for total swimming speed and progressive swimming speed in this study varied from 42,3 to 47,7 μm/s and 37,7 to 43,3 μm/s (Table 4) whereas Samuels & van der Horst (1986) reported 68,2 and 64,2 μm/s, respectively, for thawed bull semen. No statistical differences ($p < 0,05$) were found between the three treatments. There were also no statistical differences ($p < 0,05$) between the progressive ratios of the spermatozoa of the three bull groups (Table 4). All values were relatively high, ranging from 0,89 to 0,91 and in agreement with values reported in the literature (Samuels & van der Horst, 1986), indicating that bull spermatozoa swim relatively straight. The average radian of the swimming circle varied from 46 to 52 μm, with no statistical differences ($p < 0,05$)

Table 4 The sperm swimming speed of thawed semen from Holstein bulls fed diets containing different levels of whole cottonseed (gossypol)

Parameter		Treatment A	B	C
Free gossypol in diet DM	(%)	0,01	0,17	0,23
Swimming speed, total	(μm/sec)	42,34±3,55*	47,68±2,87	47,67±2,38
Swimming speed, linear	(μm/sec)	37,71±2,55	43,18±2,51	43,33±2,24
Progressive ratio	$\left(\frac{\text{linear } \mu m}{\text{total } \mu m}\right)$	0,89±0,04	0,91±0,07	0,91±0,10
Track radian	(rad μm)	46,11±3,12	52,52±4,57	52,84±2,69

* Standard error of the mean

between the three treatments (Table 4). No spermatozoa showed a swimming angle greater than 180°, meaning that none of the measured sperm swam in circles. In this study, free gossypol intakes of 64 and 75 mg per kg live mass did not effect the measured motility parameters.

The normal number of spermatozoa per ml in bull semen is given as 1 000 to 1 800 million by Hafez (1972) and 2 000 to 3 000 million by Hansel & McEntee (1975). In this study, the concentration varied between 800 to 1 200 million with no statistical differences ($p < 0,05$) between the three treatments (Table 3). Although the concentration of spermatozoa was not relatively high it was acceptable for young bulls (20–24 months).

The percentage of live spermatozoa varied between 89,1 to 90,3% (Table 3). No statistical differences ($p < 0,05$) were noted between treatments. The percentage live cells after deep freezing varied from 41 to 46%. Once again no differences were found between groups. Although the average percentage live spermatozoa after deep freezing was not high, it was acceptable. It was therefore concluded that a daily intake of 64 or 75 mg free gossypol per kg live mass did not effect the viability of sperm in fresh or deep frozen and thawed bull semen.

The percentage of morphologically normal spermatozoa (phase contrast microscopy) varied between 94,9 and 95,4%. No statistical differences ($p < 0,05$) were obtained between treatments. The values obtained in the study compared very favourably with those reported in the literature for normal semen (Hafez, 1972).

While Hoffer (1983) found that gossypol intakes severely damaged some seminiferous tubules in the rat testis, no damage could be detected in this study. Spermatogenesis and the Sertoli- and germ cells also appeared to be completely normal. No free or bound gossypol could be traced in the testes of the bulls.

None of the measured spermatozoal parameters in this study were influenced by free gossypol intakes of 64 and 75 mg per kg live mass per day in whole cottonseed. It was therefore concluded that the free gossypol was probably efficiently detoxified in the rumen. Because of the contradictory results reported in the literature on gossypol poisoning of ruminants, further research should be carried out to establish why gossypol intoxication is sometimes posible in mature ruminants and sometimes not. A possible reason can be the presence or absence of gossypol binding agents in the rumen.

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