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Sheep spermatogenesis is affected by the nutritional levels of cocoa meal, a byproduct of cocoa butter manufacturing rich in theobromine and caffeine

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ABSTRACT - The objective of this study was to evaluate the effects of cocoa meal in feed concentrate on the spermatogenesis in sheep. Twenty-five uncastrated males were divided into four groups and fed concentrated feed, supplemented with 0, 10, 20, and 30% cocoa meal, respectively, for 150 days. At the end of this period, the animals were slaughtered, the testicles were collected for histological slides, and testicular morphometric assessments were conducted, including cell type quantification in the seminiferous epithelium, intrinsic spermatogenesis yield, Sertoli-cell index, total number of Sertoli cells (TNSC), TNSC per testicular gram, daily sperm production (DSP), DSP per testicular gram, testicular sperm reserve (TSR), and TSR per testicular gram. Data were subjected to normality analysis using the Shapiro-Wilk test, followed by the tests for each condition, at a significance level of 5%. The administration of cocoa meal did not alter the germinal epithelial cell population, except for the number of type-A spermatogonia, which was lower in the control group than in the group that received 20% supplementation. A difference was found in the ratio between the number of type-A spermatogonia and primary spermatocytes in pre-leptotene/leptotene, and in the ratio between Sertoli cells and primary spermatocytes in pre-leptotene/leptotene, the control group and the group that received 10% cocoa meal significantly varied from each other, but not in relation to the other evaluated percentages. The TNSC, TNSC/g, DSP, and TSR levels were diminished in the group that consumed 30% cocoa meal. The TNSC, DSP, and TSR exhibited a negative quadratic tendency. Supplementation with 10% cocoa meal improved the TNSC, DSP, and TSR of sheep.

Keywords: Sertoli cells, spermatogonia, testicular morphometry

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1. Introduction

To increase reproductive indices, the feed of reproducing male livestock should be supplemented with concentrates (Deng et al., 2012). The increased production costs associated with the increase in the energy level of the diet of ewes has a positive response on the performance of ewes and increased spermatogenesis rates and other reproductive aspects, therefore, with economic return (Souza et al., 2019; Santos et al., 2022).

The use of alternative supplement sources in concentrated sheep feed has become commonplace. Considering product availability and the consequent unwarranted shipping of supplements, such alternative sources may increase the profit (Silva et al., 2014). However, diminished costs alone do

not justify the use of a particular feed ingredient; the supplement should also provide the necessary nutrients required by the animals to reach sufficient reproductive potential (Souza et al., 2019).

Cocoa meal has excellent potential as a feed supplement in sheep farming, and yields good results in relation to productivity (Carvalho et al., 2006; Campos et al., 2017). However, regarding reproduction, more specifically spermatogenesis, the effects of cocoa meal-based supplementation remain understudied.

Cocoa meal, a byproduct of cocoa butter manufacturing, often used in livestock meal production, is an important source of theobromine and caffeine and is comprised of 2.2 and 0.1% theobromine and caffeine, respectively (Adamafio, 2013). At high concentrations, these substances adversely affect the testicular parenchyma, and consequently, spermatogenesis, thus reducing the reproductive potential of animals (Funabashi et al., 2000; Park et al., 2015).

Studies on laboratory rodents have confirmed that high theobromine concentrations (between 250-700 mg/kg/day in the diet) promote adverse effects, including degeneration and necrosis of spermatids and spermatocytes and multinucleate giant cell formation (Tarka Jr. et al., 1979; Funabashi et al., 2000; Eteng et al., 2005) by interfering with germ cell kinetics and Sertoli cell toxicity (Ettlin et al., 1986). Wang and Walter (1994) concluded that Sertoli cells are the primary target cells of theobromine toxicity. The destructive effects of high theobromine concentrations in large segments of rat seminiferous tubules are irreversible (Tarka Jr. et al., 1981).

According to Park et al. (2015), excessive peripubertal caffeine (120 and 180 mg/kg/day in rats) intake can interfere with testis maturation and function, possibly by interrupting endogenous testosterone secretion and reducing the sensitivity of Leydig cells to gonadotrophic stimulation. In addition, pubertal caffeine administration reduced testis growth and altered testis histomorphology (Park et al., 2015). However, moderate caffeine doses (50 μM/day) are reportedly beneficial to spermatogenesis in humans (Dias et al., 2015).

Industries have attempted to utilize cocoa waste materials as feed resources. However, when the dietary concentrations of these materials exceed 10-15%, the reproductive indices of laboratory rodents are diminished. To date, these deleterious effects on reproduction have not yet been proven in ruminants. Therefore, the objective of this study was to evaluate the effects of feed concentrate, supplemented with cocoa meal, on sheep spermatogenesis.

2. Material and Methods

This study was conducted in Cruz das Almas, Bahia, Brazil (12°675'422" S latitude and 39°089'580" W longitude). The study region has an average altitude of 220 m above sea level with an average rainfall of 1,200 mm (INMET, 2016) and hot tropical climate type Af, according to the Köppen-Geiger classification (Köppen and Geiger, 1928). During the experimental period, the maximum and minimum temperatures were 29.3 and 27.8 °C, respectively. Research on animals was conducted according to the institutional committee on animal use (23007.003272/2015-91).

In this study, 25 uncastrated male Santa Ines × Dorper sheep, with an average age of four months, average initial weight of 25.09±4.19 kg, and body condition score of 2.5±0.5, were selected for the experiment. The animals were divided into four groups using a completely randomized design. Each group was subjected to different treatments; they were fed dry food concentrates, containing varying percentage of cocoa meal, which partially substituted the corn and soybean meal content (Table 1): control group (n = 6), no cocoa meal; 10% cocoa meal (n = 6); 20% cocoa meal (n = 7); and 30% cocoa meal (n = 6).

The animals were maintained in a semi-intensive production system, in an Aruana grass (*Panicum maximum* 'Aruana') rotational grazing area; feed concentrate (1.6% of body weight) was offered once per day in private feeders for 150 days, and water was provided *ad libitum*. The concentrate was calculated to meet the nutritional requirements of energy, protein, and minerals for sheep with an average body weight of 30 kg and an average daily weight gain of 200 g, according to the NRC (2007) (Table 1), and the roughage:concentrate intake ratio was 60:40.

Table 1 - Percentage of corn, cocoa, and soybean meal in experimental diets of male sheep

Ingredient	Cocoa meal supplement (in the dry matter of the concentrate)			
	0% (control)	10%	20%	30%
Soybean meal	25.00	20.00	15.00	15.00
Cocoa meal	0.0	13.30	26.50	39.70
Ground corn	73.00	64.70	56.50	43.30
Nucleus	2.00	2.00	2.00	2.00
Chemical composition (%)				
Dry matter	88.13	87.76	87.40	87.07
Crude protein	18.87	17.79	16.68	17.59
NDF	13.82	18.77	23.73	28.75
TDN	83.83	80.50	77.17	73.53

NDF - neutral detergent fiber; TDN - total digestible nutrients.

The estimated grass intake was calculated from the dry matter intake equation:

$$DMI\ (kg/day) = \{[(FP \times NDFiF) \times SI] / CIFO\} + DMIS, \quad (1)$$

in which DMI = dry matter intake (kg/day), FP = fecal production (kg/day), NDFiF = neutral detergent fiber indigestible concentration (kg/kg DM) in the feces, SI = supplement intake (kg/day), CIFO = NDF_i forage (kg/kg DM), and DMIS = dry matter intake of the supplement (kg/day).

At the end of the experimental period, the animals were slaughtered, and histological slides were prepared following the procedure presented by Morais et al. (2012). The collected testes were weighed and fixed in 10% buffered formalin for 24 h and then transferred to 70% alcohol. Testicular fragments were dehydrated with an increasing ethanol series — 70, 80, 90, 95, and 100%— with concentration augmented every 30 min, followed by inclusion in glycol-methacrylate solution (Historesin, Leica™, Switzerland). Sections of 3-µm thick were cut with a rotating microtome (RM 2245, Leica™, Switzerland) using polytetrafluoroethylene (PTFE) coated microtome high profile blades (TBS). The sections were stained with toluidine blue/1% sodium borate for 25 s, mounted with Alkolan, and analyzed under a light microscope (Olympus, Tokyo, Japan).

Five cross-sections of seminiferous tubules in stage 1 of the seminiferous epithelium cycle (SEC) were evaluated and analyzed. The sections were chosen randomly, and the spermatogenic lineage cell nuclei and Sertoli-cell nucleoli were counted, using a Nikon™ microscope coupled with micrometric eyepiece, under a 40X magnification.

The counting of each cellular type was corrected for the average nuclear diameter (AND) and thickness of each section using the formula modified by Amann and Almquist (1962). The correction was grounded in the average diameter of the nucleoli in relation to the morphological irregularity of the Sertoli-cell nuclei. The correction was performed using the following equation:

$$\text{Corrected number} = \text{Total counting} \frac{\text{Thickness of cut (µm)}}{\text{Thickness of cut (µm)} + \sqrt{\frac{(\text{AND})^2}{2} - \frac{(\text{AND})^2}{4}}} \quad (2)$$

The spermatogonial mitosis efficiency coefficient was calculated by dividing the number of type-A spermatogonia (A) by the number of primary spermatocytes in pre-leptotene/leptotene (PL). The ratio between the number of rounded spermatids (Ar) and primary spermatocytes in pachytene (PQ) was calculated. The general yield of spermatogenesis was defined as the ratio between the number of Ar atoms and A.

The ratio between the number of PL and PQ determined the occurrence of cellular losses during meiotic prophase. The Sertoli-cell indices were determined from the ratio between the number of each germ-cell type and the sum of germ cells in each cross section.

To calculate the total population of Sertoli cells, the corrected number of nucleoli per seminiferous tubule cross-section was used. Based on the methodology used by Hochereau-de Reviers and Lincoln (1978), we extrapolated the amount of Sertoli cells in each gram of testis, total organ weights, and across the total seminiferous tubule length, using the following equation:

$$TNSC = \frac{TLST\ (m) \times Corrected\ number\ of\ Sertoli\ cells\ per\ transversal\ section}{Histological\ thickness\ (m)} \quad (3)$$

in which TNSC is the total number of Sertoli cells, and TLST is the total length of seminiferous tubules.

The following equation was used to calculate the spermatic reserve based on quantitative histology:

$$TSR = \frac{TTL\ (m)}{Thickness\ of\ cut\ (m)} \times average\ of\ Ar \quad (4)$$

in which TSR is total spermatic reserve, TTL is total tubule length and Ar is rounded spermatids per transverse section. As with the total Sertoli-cell population, the TSR per gram (TSR/g) value was extrapolated to the total weight of the testicles.

The daily sperm production (DSP) was calculated based on the testicular quantitative histology, as proposed by Amann and Almquist (1962), using the following equation:

$$DSP = \frac{TVST \times No.\ Ar}{DSEC \times TCSA \times HST} \quad (5)$$

in which TVST is the total volume of seminiferous tubules, No. Ar is the corrected average number of Ar in stage 1 of SEC, DSEC is the duration of SEC in days, TCSA is the tubule cross-sectional area in meters, and HST is the histological section thickness in meters.

The data were subjected to a normality analysis using the Shapiro–Wilk test. The variables that met the normality assumptions were subjected to variance analyses, regression, and Dunnett's tests. The variables that did not meet the criteria were subjected to the Kruskal–Wallis tests. Statistical analyses were conducted using SPSS version 23.0 (IBM Corp., Armonk, NY, USA), with a significance level set at 5%.

3. Results

The administration of cocoa meal did not alter the germinal epithelial cell population (P>0.05), except for the number of type-A spermatogonia, which was lower in the control group than in the group that received 20% supplementation (Table 2).

In relation to the spermatogenesis yield, the ratio of the number of type-A spermatogonia to the number of primary spermatocytes in pre-leptotene/leptotene was greater in the control group than in the group treated with 10% cocoa meal. The other variables, in relation to the treatments, did not have any observable effect (P>0.05) (Table 3).

Table 2 - Corrected cell populations of the seminiferous epithelium at stage 1 (SEC) in male sheep fed different percentage of cocoa meal in partial substitution of corn and soybean meal in concentrate

Variable	Cocoa meal supplement (in the dry matter of the concentrate)				P-value
	0% (control)	10%	20%	30%	
A[1]	1.82±0.35*	2.24±0.29	2.39±0.31*	2.11±0.40	0.048
S[1]	4.70±0.72	5.43±0.86	5.53±0.58	5.01±0.75	0.185
PL[2]	30.94±5.61	25.96±10.41	34.84±5.51	32.06±11.05	0.066
PQ[1]	56.90±11.95	53.54±10.94	65.39±6.60	58.89±10.76	0.220
Ar[1]	139.35±20.94	159.06±40.65	170.69±21.49	152.09±20.54	0.239

A - type-A spermatogonia; S - Sertoli cells; PL - primary spermatocytes in pre-leptotene/leptotene; PQ - primary spermatocytes in pachytene; Ar - rounded spermatids.
[1] Normal data distribution, presented as the mean ± standard deviation.
[2] Abnormal data distribution, presented as the median ± interquartile range.
* ANOVA and Dunnett's test treatments variations at 5% significance.

The Sertoli-cell index was different for Sertoli cells and PL (P = 0.008). The relationship between the Sertoli cells and other seminiferous epithelium cell types showed no significant variation among the groups (P>0.05) (Table 4).

Variables TNSC, TNSC/g, DSP, and TSR exhibited a negative quadratic tendency, presenting an optimum maximum level of 12.27, 12.27, 12.45, and 12.45% of cocoa meal, respectively (P<0.05). The DSP/g and TSR/g variables were similar among all treatments (P>0.05) (Table 5).

Table 3 - Intrinsic spermatogenesis yield of male sheep fed cocoa meal in partial substitution of corn and soybean meal in concentrate

Variable	Percentage of cocoa meal (ratio)				P-value
	0% (control)	10%	20%	30%	
A:PL	1:16.42±2.58*	1:12.34±2.03*	1:14.58±1.67	1:15.24±0.98	0.010
PL:PQ	1:1.99±0.64	1:1.94±0.09	1:1.89±0.11	1:1.84±0.12	0.870
PQ:Ar	1:2.54±0.66	1:2.96±0.25	1:2.61±0.21	1:2.63±0.44	0.350
A:Ar	1:77.24±9.19	1:71.31±16.53	1:71.89±8.80	1:74.02±15.37	0.846

A - type-A spermatogonia; PL - primary spermatocytes in pre-leptotene/leptotene; PQ - primary spermatocytes in pachytene; Ar - rounded spermatids.
Variables were normally distributed and are presented as the cell-type ratio mean ± standard deviation.
* ANOVA and Dunnett's test treatment variations at 5% significance.

Table 4 - Sertoli-cell index of male sheep fed different percentages of cocoa meal in partial substitution of corn and soybean meal in concentrate

Variable	Percentage of cocoa meal (ratio)				P-value
	0% (control)	10%	20%	30%	
S:A	1:0.39±0.02	1:0.41±0.04	1:0.43±0.02	1:0.42±0.06	0.249
S:PL	1:6.32±0.81*	1:5.06±0.43*	1:6.26±0.36	1:6.45±1.03	0.008
S:PQ	1:12.23±2.76	1:9.82±0.78	1:11.85±0.85	1:11.81±1.55	0.078
S:Ar	1:29.83±3.49	1:29.14±4.60	1:30.89±2.90	1:30.70±4.10	0.834
S:CG	1:48.77±4.33	1:44.43±5.61	1:49.43±3.67	1:49.38±4.94	0.213

A - type-A spermatogonia; S - Sertoli cells; PL - primary spermatocytes in pre-leptotene/leptotene; PQ - primary spermatocytes in pachytene; Ar - rounded spermatids; CG - total germ cells.
Variables were normally distributed and are presented as the cell-type ratio mean ± standard deviation.
* ANOVA and Dunnett's test treatment variations at 5% significance.

Table 5 - Estimates of sperm production and total Sertoli cells per gram of testis, calculated from the testicular histology analysis of male sheep fed cocoa meal in partial substitution of corn and soybean meal in concentrate

Variable	Cocoa meal supplement (in the dry matter of the concentrate)				P-value
	0% (control)	10%	20%	30%	
TNSC ($\times10^9$)**	2.87±0.67	4.24±1.10	3.74±0.54	1.32±0.28	0.000
TNSC/g ($\times10^6$)	20.58±3.27*	28.79±5.76*	27.25±3.35	25.44±6.13	0.036
DSP ($\times10^9$)**	8.21±2.34	11.71±3.28	11.03±1.99	3.86±0.97	0.000
DSP/g ($\times10^6$)	58.12±9.10	80.35±23.37	80.59±14.89	74.26±19.23	0.110
TSR ($\times10^9$)**	86.26±24.59	122.97±34.46	115.85±20.94	40.59±10.24	0.000
TSR/g ($\times10^6$)	610.29±95.55	843.77±245.45	846.25±156.44	779.81±201.99	0.110
Regression equation					R^2
TNSC ($\times10^9$)**	$Y = -281323970X^2 + 7004245168X + 84962299219$				0.738
DSP ($\times10^9$)**	$Y = -9468491X^2 + 232345451X + 2875810701$				0.675
TSR ($\times10^9$)**	$Y = -26792759X^2 + 667070968X + 8091647544$				0.675

TNSC - total and per gram number of Sertoli cells; DSP - total and per gram of daily sperm production; TSR - total and per gram of testicular sperm reserve; R^2 - coefficient of determination.
Variables were normally distributed and are presented as the cell-type ratio mean ± standard deviation.
* ANOVA and Dunnett's test treatments variations at 5% significance.
** Negative quadratic effect obtained by the Regression test at 5% significance.

4. Discussion

Despite the well-reported degenerative effects of caffeine on spermatogenesis (Wang and Waller, 1994; Cavalcante et al., 2014; Park et al., 2015), Dias et al. (2015) showed that caffeine, at determined concentrations in humans (5-50 μM), can stimulate lactate production in Sertoli cells, offering greater nutritional support for germline cells, thus positively affecting reproduction. This is a possible explanation for the spermatogonia increase in the group treated with 20% cocoa meal. In a previous study, at the highest concentration (500 μM), caffeine stimulated high density lipoprotein (HDL) activity to sustain lactate production (Dias et al., 2015).

The treatment with 20% cocoa meal increased the number of type-A spermatogonia; however, other cellular types did not show any difference. According to Wrobel et al. (1995), the type-A spermatogonia degeneration may not affect primary spermatocyte production due to the myriad of cellular divisions that occur among spermatogonial classes and other subsequent cell types.

The reduced PL count in the group treated with 10% cocoa meal does not seem to have a biological justification. It is common for degenerative processes to occur in germline cells (Amann, 1962), and PL are usually the most vulnerable (Andreussi et al., 2014; Rocha et al., 2020). However, for this reasoning to be valid, the groups treated with higher cocoa meal concentrations would also have to exhibit reduced PL levels.

Studies have suggested a common variation in the spermatogenesis of small ruminants (Leal et al., 2004; Sousa et al., 2014; Rocha et al., 2020), which can be justified by degenerative alterations or nutritional (Souza et al., 2014) and genetic factors (Andreussi et al., 2014). This variation may influence the dynamics of spermatogenesis growth waves (Perey et al., 1961), thus promoting a wide range of said waves within the species and even in different seminiferous tubules of the same animal.

The presence and functionality of Sertoli cells greatly influence the beginning of the spermatogenic process and the mitigation of associated degeneration (Edelsztein and Rey, 2020). According to Boujrad et al. (1995), Sertoli cells reach numeric stability in puberty, and alterations, which generally occur due to metabolic dysfunctions, are not usually related to the reduction of total cellular number, excluding cases of gonadal tissue degeneration.

Despite the presence of substances considered toxic in the cocoa meal (Adamafio, 2013), it was administered to animals after the numerical establishment of the cellular type. The cocoa meal did not affect the multiplication process of the Sertoli cells of the animals. Although there was no numerical impact, the high cocoa meal concentration altered the Sertoli-cell function, compromising the spermatogenic process.

The continuous intake of cocoa meal, and consequently theobromine and caffeine (2.2 and 0.1%, respectively) (Adamafio, 2013; Berndtson et al., 1987), representing the daily average ingestion of 105 mg kg^{-1} theobromine and 4.8 mg kg^{-1} caffeine per animal, may explain the reduced spermatogenesis presented by the group treated with 30% cocoa meal.

Previous studies have shown dose-dependent deleterious effects of cocoa anti-nutritional factors (Funabashi et al., 2000; Dias et al., 2015), demonstrated here as the negative quadratic behavior observed for the TNSC, TNSC/g, DSP, and TSR variables.

According to Wang and Waller (1994), high theobromine concentrations reduce the weight of the testicular parenchyma, with Sertoli cells being the main target of the substance. According to Funabashi et al. (2000), intake of 250 mg kg^{-1} theobromine per day, for four weeks, may induce testicular necrosis and peeling of the seminiferous epithelium, affecting the products of spermatogenesis. In this study, theobromine intake was 105 mg kg^{-1} per day; however, the treatment was administered for 21.4 weeks, far surpassing the four-week period mentioned by Funabashi et al. (2000).

In addition to theobromine, high caffeine concentrations can alter spermatogenesis, as it modifies the oxidative profile of the Sertoli cells, making their proteins vulnerable and compromising their

development and existence, resulting in reduced lactate availability for the spermatogonia and, consequently, the entire spermatogenic process (Dias et al., 2015).

5. Conclusions

Feed concentrate supplemented with 10% cocoa meal improves the total number of Sertoli cells and total number of Sertoli cells per gram of testicle of reproducing male sheep. Additionally, 10% cocoa meal supplement improves the daily sperm production and total spermatic reserve. Concentrations exceeding this level adversely affects the spermatogenic process of these animals and are thus not recommended.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Formal analysis: D.S. Macedo, A.L.A. Santana and L.P. Barbosa. Methodology: D.S. Macedo, L.F. Rocha, E.E.G. Pinheiro, A.L.A. Santana, R.S. Souza, A.R. Bagaldo, P.P.A. Bezerra and L.P. Barbosa. Project administration: L.P. Barbosa. Supervision: L.P. Barbosa. Writing – original draft: D.S. Macedo and A.L.A. Santana. Writing – review & editing: A.L.A. Santana and L.P. Barbosa.

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