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Article

Effects of Dietary Flaxseed Oil and Ascorbic Acid on the Reproductive Performance of South African Indigenous Sheep

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Abstract: The current study aimed to evaluate the effects of the dietary inclusion of flaxseed oil and ascorbic acid on the reproductive performance of South African indigenous sheep (*Ovis aries*). Twenty-two matured South African indigenous rams (eight BaPedi, nine Zulu, and five Namaqua Afrikaner; age of 6 years and body weight of 64.4 ± 1.6 kg) were randomly assigned into five treatment groups (NC (standard diet), PC (basal diet), FO (5% flaxseed oil), AA (4% ascorbic acid), and FO + AA (5% flaxseed oil + 4% ascorbic acid)). Semen samples were collected during the natural breeding season using an artificial vagina. Semen samples were evaluated for macroscopic (semen volume, pH, and sperm concentration) and microscopic (morphology, malondialdehyde, membrane permeability, and sperm cell motility parameters) characteristics. Eighty-one South African indigenous ewes were synchronized using controlled intravaginal drug devices impregnated with 0.3 g progesterone for 10 days and then injected with 300 IU of Pregnant Mare Serum Gonadotropin. These ewes were then hand-mated. The data were subjected to the General Linear Model (GLM) in Minitab[®] 2017. A cross-tabulated Chi-Squared (χ^2) test was used to track the frequencies of the conception rate. Treatment means were separated using Student's Least Significant Difference (LSD) and considered significantly different when the *p*-value was less than 0.05. No considerable breed effect was observed for semen volume, semen pH, sperm concentration, intact membrane, and non-intact membrane. FO + AA led to higher semen volume (1.05 ± 0.06 mL), intact sperm membranes ($88.83 \pm 1.27\%$), and low malondialdehyde levels (0.37 ± 0.04 nmol/mL) in comparison to other treatment groups. Total sperm motility was higher in FO + AA ($95.81 \pm 0.80\%$) compared to FO, AA, PC, and NC. The conception rate was higher in FO (94%), AA (94%), and FO + AA (100%) when compared to NC (71%) and PC (79%). The testosterone concentration did not differ among PC, FO, AA, and FO + AA. It was concluded that the inclusion of 5% flaxseed oil (FO and FO + AA) improves both the semen quality and the conception rate of South African indigenous sheep.

Keywords: docosahexaenoic acid; omega n-3; sperm quality; sperm improvement



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1. Introduction

South African indigenous sheep breeds include Zulu, BaPedi, Namaqua Afrikaner, and Damara sheep [1,2]. These breeds were named after their respective tribes where they predominate [3]. Furthermore, they are hardy and tolerant to harsh environmental conditions and graze marginal land not suitable for cropping [4]. These characteristics attracted rural breeding to these breeds and therefore are used for household income and food sources, thus playing a significant role in food security [5]. Despite their advantages over exotic breeds, they are characterized by small body frames, making them

unattractive to commercial farmers, and hence are endangered with an unknown population size [6]. Mavule et al. [7] reported uncontrolled breeding as the main factor leading to their extinction.

Flaxseed oil is a rich source of alpha-linolenic acid (ALA), synthesized de novo through delta-5 and -6 enzymes to form long-chain polyunsaturated fatty acids (LCPUFA) such as docosahexaenoic acid (DHA) [8]. Long-chain polyunsaturated fatty acids are vital for testes functioning in human beings [9] and spermatogenesis in broiler chickens [10]. Flaxseed oil appears also to contain about 58% linolenic acid (LA), which plays a vital antioxidant role to improve both animal reproductivity and health in domestic livestock animals [8]. Moreover, flaxseed oil increases the level of alpha-linolenic and eicosapentaenoic acids, which are important in the synthesis of reproductive hormones such as testosterone, luteinizing, and follicle-stimulating hormones in cattle [11]. Furthermore, flaxseed oil contains other fatty acids such as stearic, oleic, linoleic, and palmitic acids, containing a high content of natural antioxidants such as vitamin E, as shown in Mithun bulls [12]. Natural antioxidants are essential for the reduction of reactive oxygen species during semen handling and storage [13].

The dietary inclusion of omega n-3 sources such as fish oil and its influence on reproductive performance in domestic ruminant animals is well-described [14]. However, sheep cannot synthesize omega n-3 fatty acids due to the lack of ω 3 desaturase and Δ 12 desaturase, and hence can only synthesize polyunsaturated fatty acids (PUFAs) from precursors (18:2 n-6 and 18:3 n-3), with the assistance of both Δ 6 and Δ 5 desaturases [11]. Supplementing with flaxseed oil improves sperm motility in turkeys [15], cattle [16,17], pigs [18], goats [19], and sheep [20,21]. Ascorbic acid, on the other hand, is a natural water-soluble antioxidant that is highly concentrated in the epididymis of numerous species [22]. Its function is to act as a protective vitamin, scavenging lipid free radicals and oxygen species [23]. Ascorbic acid has been reported to improve semen quality, the dietary response of omega n-6 PUFAs [23], the maturity rate in pig oocytes, and reduce oxidative stress during the in vitro handling of gametes [24,25].

Despite the benefits reported following the feeding of flaxseed oil and ascorbic acid, it is still not clear if the dietary inclusion of flaxseed oil can improve reproduction performance in sheep. This can shed light and accelerate the conservation programs formed by various developing countries, including the South African Department of Agriculture, Rural Development, and Land Reform (DALRD), to improve the population size of indigenous breeds through in vivo conservation. Therefore, the aim of this study is to assess the effects of flaxseed oil and ascorbic acid on the reproductive performance of indigenous South African sheep.

2. Materials and Methods

2.1. Ethical Approval

The Agricultural Research Council (ARC-AREC, reference number: APAEC 2019/33) and Tshwane University of Technology Animal Research Ethics Committee (TUT-AREC, reference number: AREC2020/05/001) approved all study procedures used in this study.

2.2. Study Site and Animals

The study was conducted at the Agricultural Research Council, Irene, Animal Production South Africa (ARC, Irene). The ARC, Irene area is situated in the Highveld, 1525 m above sea level. This area extends between 25°53'59.6" South latitude and 28°12'51.6" East longitude. Study animals were maintained in the same environment with water provided ad libitum throughout the study. Study animals were selected based on their availability at the ARC since these are scarce breeds. Twenty-two matured South African indigenous rams (8 BaPedi, 9 Zulu, and 5 Namaqua Afrikaner; age of 6 years and body weight of 64.4 ± 1.6 kg) were randomly assigned into five treatment groups (NC (standard diet), PC (basal diet), FO (5% flaxseed oil), AA (4% ascorbic acid) and FO + AA (5% flaxseed oil + 4% ascorbic acid)). Study rams were randomly

allocated, grouped, and housed as per the five study diets (NC—4 rams; PC—4 rams; FO—5 rams; AA—4 rams and FO + AA—5 rams) with no access to the pasture throughout the study. Eighty-one matured African indigenous ewes (BaPedi = 28, Zulu = 28, and Namaqua Afrikaner = 25; age between 3 and 4 years old) were used in this study, with the total number per breed chosen as per availability. Matured breeding males (rams) received respective treatment diets for 60 days prior to the commencement of semen collection and hand-mating to improve semen quality.

Controlled Intravaginal Drug Release dispensers (CIDR) (Zoetis, CIDR[®], Sandton, South Africa) containing 0.3 g progesterone were inserted according to the manufacturer's instructions using a disinfected applicator [26]. At CIDR removal (10th day), the ewes were injected intramuscularly with 300 IU of eCG (Intervet Schering-Plough Animal Health, Kempton park, South Africa) to stimulate ovulation [27]. The neutral teaser ram from the flock was used to spot the estrus response and the twitching/shaking of the tail was observed as a sign of estrus response.

2.3. Experimental Design and Treatment Diets

Matured study rams were randomly allocated into five treatment groups (NC control—NC; PC control—PC; flaxseed oil—FO; ascorbic acid—AA; and flaxseed oil + ascorbic acid—FO + AA). The NC was a standard diet used at the Agricultural Research Council made of *eragrostis curvula* hay and 200 g/ram/day of pellets. Pellets were bought from the local feed supplier. The PC was the basal diet formulated according to the National Research Council [28]. The FO was the basal diet enriched with 5% flaxseed oil. The AA was the basal diet enriched with 4% ascorbic acid, whereas FO + AA was the basal diet enriched with 5% flaxseed oil + 4% ascorbic acid. Following feed formulation and mixing, feed samples were taken and stored in 500 g containers and labeled.

Feed samples were dried at 60 °C, then processed (1 mm screen using Cyclotech Mill, Tecator, Hoganas, Sweden) [29], and the dry matter, ash, ether extract, and crude protein were analyzed (Table 1) as described by AOAC [30]. In brief, the dry matter was determined by heating up the feed samples to evaporate the water content. The ash content was determined by heating up the feed samples at 600 °C to remove moisture and volatiles. Crude protein was determined using the Kjeldahl method described by AOAC [30].

Table 1. Proximate analysis of treatment diets.

Proximate Analysis	Treatment Diets				
	NC	PC	FO	AA	FO + AA
Dry matter (%)	88.23	88.93	92.20	90.42	93.52
Ash (%)	5.32	12.36	5.00	5.49	4.75
Crude protein (%)	10.73	10.36	10.74	10.29	10.59
Ether extract (%)	2.03	2.16	19.79	1.70	24.29
Crude fibre (%)	29.01	21.71	25.90	28.71	20.95

NC control (standard diet); PC (basal diet); FO (5% flaxseed oil); AA (4% ascorbic acid); FO + AA (5% flaxseed oil + 4% ascorbic acid).

2.4. Experiment I: Semen Sample Collection, Processing, and Evaluation

Semen samples were collected during the natural breeding season (autumn, April–May) with the aid of an artificial vagina following 21 days of the training period. After sixty days, semen was collected twice a week with a two-day gap between collections to provide enough of a rest period. Semen samples were then collected and evaluated immediately after collection, within 30 min. This was replicated 10 times.

2.4.1. Semen Volume, pH, and Sperm Concentration

The volume of semen was measured in milliliters (mL) using graduated tubes on the collection glass before dilution.

Semen pH was measured using the Oakton pH meter (Eutech Instrument, Cyber-Scan pH 11/110, Singapore). The Oakton pH meter was calibrated before use by (1) rinsing the pH meter electrode in water before placing it in a 7 pH buffer solution and calibrating it; (2) rinsing the electrode again and placing it in a 4 pH solution, calibrating it, then placing it in a 10 pH buffer solution; and (3) after calibration in a 10 pH buffer solution, the electrode was rinsed again, placed in the sample, and the reading was then taken. Semen concentration was measured using the 134 spectrophotometer (Jenway 6310) at 532 nm wavelength.

2.4.2. Sperm Motility Evaluation

Semen samples were evaluated for sperm motility using the Computer-Aided Sperm Analyzer (CASA) Sperm Class Analyzer system (SCA, 5.0 version, Microptic, Barcelona, Spain), whereby one hundred microliters of swim-up medium (tris-based extender without egg-yolk and glycerol) and 5 μ L of raw semen sample were mixed in an Eppendorf tube. Following a mixture of semen and swim-up medium, five μ L of the mixture was pipetted, placed on the warmed microscope slide, and gently mounted with a coverslip (22 \times 22 mm, Germany). The samples were evaluated under 10 \times Ph1 BM magnification. For each sample, three fields containing \pm 200 sperm cells were evaluated.

2.4.3. Sperm Cell Plasma Membrane Integrity (Hyper-Osmotic Swelling Test)

Sperm plasma membrane permeability was evaluated using the Hyper Osmotic Swelling Test (HOST), as described by Maxwell and Johnson [31], with a few amendments. In brief, 10 μ L of raw semen was mixed with 200 μ L of HOST solution (0.3675 g sodium citrate and 0.6755 fructose) and dissolved in a 50 mL falcon tube. Then, the mixture was incubated for 30 min at 37 $^{\circ}$ C in a 5% CO₂ incubator [32]. Thereafter, the semen samples were smeared on the microscope slide gently at a 45 $^{\circ}$ angle and air-dried in a darker place to avoid light. A phase-contrast microscope at 400 \times magnification was used to analyze the slides. Under the microscope, sperm cells with coiled tails were recorded as sperm cells with an intact membrane, whereas those with straight tails were recorded as sperm cells with non-intact membranes. A total of 100 spermatozoa were counted per slide and repeated for all treatment diets.

2.4.4. Malondialdehyde Level

Sperm cell lipid peroxidation was estimated by the absorbance levels of MDA. Malondialdehyde levels were determined using thiobarbituric acid (TBA), as described by Chatiza et al. [32], with a few modifications. In brief, semen samples were analyzed for concentration and standardized to $5.29 \times 10^9 \pm 0.36$ [33] using a non-egg-yolk and glycerol tris-based extender [34]. The protein from the standardized samples was precipitated by mixing them with 1 mL of trichloroacetic acid (TCA). Thereafter, the samples were centrifuged at 1500 \times g force for 10 min [32]. The suspension of the samples was removed, and the supernatant was then mixed with 0.67% TBA in a 1:1 ratio, boiled at 100 $^{\circ}$ C for 10 min, and cooled before analysis. The absorbance was recorded using the spectrophotometer at 534 nm.

2.4.5. Sperm Cell Abnormalities and Viability

Eosin–nigrosin (Onderstepoort Faculty of Veterinary Science' Pharmacy, South Africa) was used to evaluate sperm morphology and viability as described by Ngcobo et al. [35]. The eosin–nigrosin stain was pre-warmed on a warm plate to 37 $^{\circ}$ C for about 5 min before mixing with the semen sample. Twenty μ L of the stain was pipetted into an Eppendorf tube and mixed with 5 μ L of the raw semen sample. Five μ L of the mixture was pipetted and placed near the labeled end of the microscope slide (76 \times 26 \times 1 mm, Germany). The

second microscope slide was held at a 45° angle to the slide with the mixture of stain and semen. Thereafter, it was pulled gently to avoid destroying the spermatozoa, as described by Dolatpanah et al. [36], across the slide. The smeared microscope slides were air-dried for one day before evaluation.

The following day, 30 µm of immersion oil was pipetted and placed on the dried smeared slide, placed under a fluorescent microscope (Olympus BX 51TF, Japan), and magnified (UPlanFLN 100x/1.30) for analysis. A total of 100 sperm cells per slide were counted for viability and another 100 sperm cells were counted for normalities and abnormalities. Sperm abnormalities were subdivided into primary defects (misshapen head, knobbed acrosome, swollen head, small head, short tail, double tail, abaxial mid-pierce, and double head), secondary defects (bent tail, distal droplets, bent mid-pierce, detached head, and proximal droplets) and tertial defects (loose tail, terminal droplets, reacted acrosome, and coiled tail).

2.5. Experiment II: Hand-Mating and Pregnancy Diagnosis

A total of fifteen (Zulu = 5, BaPedi = 5, and Namaqua Afrikaner = 5) rams and eighty-one (BaPedi = 28, Zulu = 28, and Namaqua Afrikaner = 25) ewes were used for hand-mating in this study. The hand-mating started at a ratio of 1:5 (ram:ewes of the same breed, except 1:6 for FO, AA, and FO + AA for Zulu and BaPedi) per mating pen (Table 2).

Table 2. Allocation of study animals (rams and ewes) per treatment diets during mating.

Sheep Breeds	NC	PC	FO	AA	FO + AA
Zulu	One ram × Five ewes	One ram × Five ewes	One ram × Six ewes	One ram × Six ewes	One ram × Six ewes
BaPedi	One ram × Five ewes	One ram × Five ewes	One ram × Six ewes	One ram × Six ewes	One ram × Six ewes
Namaqua Afrikaner	One ram × Five ewes	One ram × Five ewes	One ram × Five ewes	One ram × Five ewes	One ram × Five ewes

NB—NC—negative control (standard diet); PC—positive control (basal diet); FO—5% flaxseed oil; AA—4% ascorbic acid; FO + AA—5% flaxseed oil + 4% ascorbic acid. Means with different superscripts within the same column and the same cell differ significantly ($p < 0.05$).

Thereafter, the mating activities were observed, and once a ewe was mated, it was then removed from the mating pen to avoid possible multi-mating and disturbances. Hence, the mating ratio fell off since ewes were removed once mated. In the case where ewe/s were not mated at all, a follow-up mating with the same ram was conducted on the following 18th–25th day; however, these ewes were recorded as non-estrus responders and were removed from the study. The pregnancy diagnosis was conducted after forty-five days after mating using an ultrasound scanner (Ibex pro—E.I. Medical Imaging, Loveland, CO, USA) fitted with a low-frequency, deep-penetrating convex transducer (CL3Ei Model). The conception rate was calculated according to Farrag, [37] using the following formula:

$$\text{Conception Rate} = \frac{\text{Number of Ewes Pregnant}}{\text{Number of Ewes Mated}} \times 100 \quad (1)$$

2.6. Statistical Analysis

Data were subjected to an appropriate General Linear Model (GLM) in Minitab 17®. Treatment means were separated using Student's Least Significant Difference (LSD) and considered significantly different when the p -value was less than 0.05. Scores were subjected to a 1:1 frequency table and a Chi-squared (χ^2) test was performed to test for equal proportions.

3. Results

The results on the influence of breed and the dietary flaxseed oil and ascorbic acid on semen quality are available in Table 3. There was no significant ($p > 0.05$) influence of breed (Zulu, BaPedi, and Namaqua Afrikaner) on the semen volume, semen pH, sperm concentration, intact sperm cells, or damaged sperm cells. FO + AA had a significantly higher semen volume (1.05 ± 0.06 mL) compared to NC (0.85 ± 0.69 mL) and PC (0.70 ± 0.07 mL). However, semen volume did not differ significantly ($p > 0.05$) between FO + AA (1.05 ± 0.06 mL), AA (1.01 ± 0.07 mL), and FO (0.90 ± 0.06). However, the semen pH did not differ significantly ($p > 0.05$) among the treatment groups. Sperm concentration was higher in FO + AA (0.74 ± 0.05), AA (0.73 ± 0.05), FO (0.79 ± 0.05), and PC (0.73 ± 0.05) than that of NC (0.62 ± 0.05). Nevertheless, there were no significant differences between FO, AA, FO + AA, and PC for sperm concentration.

For plasma membrane permeability, semen collected from the FO + AA group had significantly higher ($p < 0.05$) intact sperm cells (88.83 ± 1.27) than other groups, followed by FO (85.23 ± 1.27). AA (81.10 ± 1.42) did not differ significantly ($p > 0.05$) from PC (77.87 ± 1.37) for the intact sperm cells. It is noteworthy that NC was the lowest-scoring group for intact sperm cells (72.80 ± 1.42). Damaged sperm cells were higher in NC (27.20 ± 1.42) than in the rest of the groups. However, PC (22.13 ± 1.37) and AA (18.91 ± 1.42) did not differ significantly ($p > 0.05$) for the damaged sperm cells.

Table 3. The effect of breed and the dietary flaxseed oil and ascorbic acid on semen quality.

Breeds	Semen Volume (mL)	Semen pH	Sperm Conc. ($\times 10^9$)	Intact Sperm Cell Membrane (%)	Non-Intact Sperm Cell Membrane (%)
Zulu	0.97 ± 0.46^a	6.21 ± 0.07^a	0.70 ± 0.04^a	82.18 ± 0.95^a	17.82 ± 0.95^a
BaPedi	0.85 ± 0.47^a	6.06 ± 0.07^a	0.73 ± 0.04^a	80.76 ± 0.97^a	19.24 ± 0.97^a
Namaqua Afrikaner	0.89 ± 0.58^a	6.15 ± 0.07^a	0.72 ± 0.05^a	80.56 ± 1.20^a	19.44 ± 1.20^a
<i>p</i> -Values	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Treatment diets					
NC	0.85 ± 0.69^{bc}	6.18 ± 0.10^a	0.62 ± 0.05^b	72.80 ± 1.42^d	27.20 ± 1.42^a
PC	0.70 ± 0.07^c	6.07 ± 0.10^a	0.73 ± 0.05^{ab}	77.87 ± 1.37^c	22.13 ± 1.37^b
FO	0.90 ± 0.06^{ab}	6.11 ± 0.09^a	0.79 ± 0.05^a	85.23 ± 1.27^b	14.77 ± 1.27^c
AA	1.01 ± 0.07^{ab}	6.16 ± 0.10^a	0.73 ± 0.05^{ab}	81.10 ± 1.42^c	18.91 ± 1.42^b
FO + AA	1.05 ± 0.06^a	6.16 ± 0.09^a	0.74 ± 0.05^{ab}	88.83 ± 1.27^a	11.17 ± 1.27^d
<i>p</i> -Values	$p < 0.05$	$p > 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

NB—NC—negative control (standard diet); PC—positive control (basal diet); FO—5% flaxseed oil; AA—4% ascorbic acid; FO + AA—5% flaxseed oil + 4% ascorbic acid. Means with different superscripts within the same column within the same cell differ significantly ($p < 0.05$).

The influence of breed and the treatment diets on sperm parameters (progressive motility, non-progressive motility, total motility, static motility, rapid motility, medium motility, and slow motility) are found in Table 4. There was no significant ($p > 0.05$) influence of breed (Zulu, BaPedi, and Namaqua Afrikaner) on all sperm motility parameters observed. Progressive motility was higher (71.90 ± 1.91) in the FO + AA-treated group than that of AA (65.80 ± 1.91), FO (62.91 ± 2.31), PC (60.71 ± 2.31), and NC (50.80 ± 2.31). The AA-, FO-, and PC-treated groups did not differ significantly ($p > 0.05$) for progressive motility. The NC-treated groups produced the lowest progressive motility (50.80 ± 2.31) than other groups. Non-progressive motility followed an opposite trend, where NC was the highest (36.80 ± 2.11) compared to the other groups. However, AA (25.20 ± 1.80), FO (30.80 ± 2.11), and PC (30.10 ± 0.11) did not differ significantly ($p > 0.05$).

Total motility was significantly ($p < 0.05$) higher (93.60 ± 0.91) in the FO-treated group in comparison to AA (90.90 ± 0.80), PC (90.82 ± 0.91), and NC (87.63 ± 0.91); however, this did not differ significantly ($p > 0.05$) with the FO + AA (95.81 ± 0.80)-treated group. Moreover, the total motility did not differ significantly ($p > 0.05$) between AA (90.90 ± 0.80) and PC (90.82 ± 0.91). The NC-treated group had higher (12.51 ± 0.91) static sperm cells than all other groups. PC did not differ significantly ($p > 0.05$) from the AA (9.01 ± 0.80)-treated group for the static sperm cells. Furthermore, FO + AA (4.11 ± 0.80) had the lowest static sperm cells than other groups with no significant ($p > 0.05$) to that of FO (6.41 ± 0.91).

Table 4. Influence of breed and dietary flaxseed oil and ascorbic acid on sperm motility parameters.

Breeds	PM	NPM	TM	Static	RM	MM	SM
Zulu	65.55 ± 1.65^a	26.32 ± 1.30^a	91.87 ± 0.99^a	7.20 ± 0.73^a	60.54 ± 1.90^a	10.92 ± 1.28^a	21.31 ± 1.01^a
BaPedi	65.33 ± 1.68^a	27.11 ± 1.32^a	92.44 ± 0.99^a	7.56 ± 0.74^a	57.76 ± 1.92^a	13.21 ± 1.30^a	21.47 ± 1.02^a
Namaqua Afrikaner	68.35 ± 2.09^a	23.65 ± 1.64^a	92.01 ± 1.25^a	7.99 ± 0.92^a	60.85 ± 2.40^a	12.73 ± 1.62^a	18.43 ± 1.28^a
<i>p</i> -Values	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Treatment diets							
NC	50.80 ± 2.31^c	36.80 ± 2.11^a	87.63 ± 0.91^c	12.51 ± 0.91^a	41.91 ± 3.11^c	15.80 ± 2.11^{ab}	29.8 ± 1.52^a
PC	60.71 ± 2.31^b	30.10 ± 2.11^{bc}	90.82 ± 0.91^b	9.21 ± 0.91^b	50.71 ± 3.11^b	17.30 ± 2.11^a	22.90 ± 1.52^b
FO	62.91 ± 2.31^b	30.80 ± 2.11^b	93.60 ± 0.91^a	6.41 ± 0.91^c	52.52 ± 3.11^b	19.41 ± 2.11^a	21.70 ± 1.52^{bc}
AA	65.80 ± 1.91^b	25.20 ± 1.80^{cd}	90.90 ± 0.80^b	9.01 ± 0.80^b	56.31 ± 2.70^b	15.62 ± 1.91^{ab}	19.01 ± 1.31^{bc}
FO + AA	71.90 ± 1.91^a	23.80 ± 1.80^d	95.81 ± 0.80^a	4.11 ± 0.80^c	66.50 ± 2.70^a	11.50 ± 1.91^b	17.80 ± 1.31^c
<i>p</i> -Values	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

NB—NC—NC—negative control (standard diet); PC—positive control (basal diet); FO—5% flaxseed oil; AA—4% ascorbic acid; FO + AA—5% flaxseed oil + 4% ascorbic acid. PM—progressive motility; NPM—non-progressive motility; TM—total motility; RM—rapid motility; MM—medium motility; SM—slow motility. Means with different superscripts within the same column within the same cell differ significantly ($p < 0.05$).

For rapid sperm motility, the FO + AA treated group continued to outperform the other treated groups. In brief, FO + AA had a significantly ($p < 0.05$) higher (66.50 ± 2.70) rapid motility than AA (56.31 ± 2.70), FO (52.52 ± 3.11), PC (50.71 ± 3.11), and NC (41.91 ± 3.11). Among AA, FO, and PC, we could not spot any significant differences ($p > 0.05$) in rapid sperm motility. There was no significant difference ($p > 0.05$) between NC (15.80 ± 2.11), PC (17.30 ± 2.11), FO (19.41 ± 2.11), and AA (15.62 ± 1.91) for the medium sperm motility. Moreover, FO + AA (11.50 ± 1.91) did not differ from AA and NC for medium sperm motility. However, slow sperm motility was higher in NC (29.80 ± 1.52) than in the PC- (22.90 ± 1.52), FO- (21.70 ± 1.52), AA- (19.01 ± 1.31), and FO + AA (17.80 ± 1.31)-treated groups.

Sperm cell viability, morphology, and abnormalities were also evaluated among the breeds (Zulu, BaPedi, and Namaqua Afrikaner) and within the treatment diets (NC, PC, FO, AA, and FO + AA) and the results are available in Table 5. There were no significant differences ($p > 0.05$) in sperm cell viability, morphology, and abnormalities among the breeds. Live sperm cells were higher in FO + AA (93.93 ± 1.73) than in AA (87.40 ± 1.73), FO (86.40 ± 1.73), PC (76.20 ± 1.73), and NC (70.30 ± 1.73). However, FO (86.40 ± 1.73) and AA (87.40 ± 1.73) differed significantly ($p > 0.05$) for the live sperm cells. NC had more (29.71 ± 1.73) dead sperm cells compared to all other groups. FO + AA scored the lowest (6.07 ± 1.66) in dead sperm cells than any other group. The normal sperm cells did not differ significantly ($p > 0.05$) between the FO + AA- (91.07 ± 1.48) and AA (87.73 ± 1.48)-treated groups; however, they were significantly ($p < 0.05$) higher compared to the PC- (75.50 ± 1.48) and NC (68.11 ± 1.51)-treated groups.

Sperm cell abnormalities were classified into primary, secondary, and tertiary and these results are available in Table 5. No significant ($p > 0.05$) difference was observed for sperm abnormalities between breeds. However, treatment diets resulted in some differences. The primary, secondary, and tertiary abnormalities were higher in NC (10.71 ± 0.51 , 10.72 ± 0.51 , and 10.61 ± 0.51) than in other treatment groups. It is noteworthy that the FO + AA treated rams had fewer (2.98 ± 0.49 , 2.98 ± 0.49 , and 2.98 ± 0.49) abnormalities (primary, secondary, and tertiary) than FO (4.61 ± 0.49 , 4.55 ± 0.51 , and 4.59 ± 0.49), respectively. However, the FO + AA-treated group did not differ significantly ($p > 0.05$) from the sperm abnormalities in AA.

Table 5. Influence of breed and dietary treatments on sperm viability, morphology, and plasma membrane permeability.

Parameters	Sperm Viability (%)		Sperm Morphology (%)		Sperm Abnormalities (%)		
	Live	Dead	Normal	Abnormal	Primary	Secondary	Tertiary
Breeds							
Zulu	80.72 ± 0.93^a	19.29 ± 0.93^a	80.24 ± 1.07^a	19.76 ± 1.07^a	6.572 ± 0.36^a	6.58 ± 0.36^a	6.59 ± 0.36^a
BaPedi	80.05 ± 0.94^a	19.95 ± 0.94^a	80.39 ± 1.09^a	19.61 ± 1.09^a	6.55 ± 0.36^a	6.54 ± 0.36^a	6.56 ± 0.37^a
Namaqua Afrikaner	79.84 ± 1.17^a	20.16 ± 1.17^a	78.16 ± 1.36^a	21.84 ± 1.36^a	7.14 ± 0.45^a	7.29 ± 0.45^a	7.26 ± 0.46^a
<i>p</i> -Values	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$
Treatment Diets							
NC	70.30 ± 1.73^d	29.71 ± 1.73^a	68.11 ± 1.51^d	32.01 ± 1.51^a	10.71 ± 0.51^a	10.72 ± 0.51^a	10.61 ± 0.51^a
PC	76.20 ± 1.73^c	23.8 ± 1.66^b	75.50 ± 1.48^c	24.50 ± 1.48^b	8.21 ± 0.49^b	8.20 ± 0.51^b	8.22 ± 0.49^b
FO	86.40 ± 1.73^b	12.27 ± 1.66^c	86.27 ± 1.48^b	13.73 ± 1.48^c	4.61 ± 0.49^c	4.55 ± 0.51^c	4.59 ± 0.49^c
AA	87.40 ± 1.73^b	12.60 ± 1.66^c	87.73 ± 1.48^{ab}	12.27 ± 1.48^{cd}	4.11 ± 0.49^{cd}	4.14 ± 0.51^{cd}	4.10 ± 0.49^{cd}
FO + AA	93.93 ± 1.73^a	6.07 ± 1.66^d	91.07 ± 1.48^a	8.93 ± 1.48^d	2.98 ± 0.49^d	2.98 ± 0.51^d	2.98 ± 0.49^d
<i>p</i> -Values	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.05$

NB—NC—NC—negative control (standard diet); PC—positive control (basal diet); FO—5% flaxseed oil; AA—4% ascorbic acid; FO + AA—5% flaxseed oil + 4% ascorbic acid. Means with different superscripts within the same column within the same cell differ significantly ($p < 0.05$).

The concentration of reproductive (testosterone) hormones was also evaluated among the breeds (Zulu, BaPedi, and Namaqua Afrikaner) and the different treatment diets (NC, PC, FO, AA, and FO + AA), and the results can be found in Table 6. Testosterone concentration did not differ significantly ($p > 0.05$) among the breeds used in this study. Moreover, when the treatment diets were examined, a significantly ($p < 0.05$) higher testosterone concentration was observed in FO + AA (26.31 ± 3.02) than in NC (13.40 ± 3.02). However, the testosterone concentration on FO + AA (26.31 ± 3.02) did not differ significantly ($p > 0.05$) from that in AA (18.87 ± 3.24), FO (21.56 ± 3.02), and PC (21.15 ± 3.02).

The level of MDA was also evaluated following supplementation with dietary flaxseed oil and ascorbic acid, and the results are shown in Table 6. There were no significant differences ($p > 0.05$) among the breeds for the level of MDA. However, when the influence of diet was evaluated, the NC had a higher (0.54 ± 0.05) MDA level than PC (0.27 ± 0.04), FO (0.22 ± 0.04), and FO + AA (0.37 ± 0.04), with no difference from AA (0.43 ± 0.05).

The conception rate was also determined following the dietary inclusion of flaxseed oil and ascorbic acid in the rams' diet and the results can be found in Table 6. South African indigenous ewes were synchronized to limit the influence of females. However, three (Namaqua Afrikaner = 2 and Zulu = 1) ewes did not respond to the estrus synchronization and hence were not mated and removed from the study. There was no significant difference ($p > 0.05$) in the conception rate between the Zulu sheep (93%) and the BaPedi sheep (93%). However, the conception rates observed in Zulu and BaPedi sheep were significantly ($p < 0.05$) higher than those observed in Namaqua Afrikaner

sheep (78%). The inclusion of dietary flaxseed oil and ascorbic acid resulted in a significantly ($p < 0.05$) higher conception rate in FO + AA (100%), AA (94%), and FO (94%) than that of the NC- (71%) and the PC (79%)-treated groups. Nevertheless, the effects of breeds and the treatment diets did not yield any significant ($p > 0.05$) differences.

Table 6. Influence of breed and dietary treatments on testosterone concentration.

Parameters	Testosterone Concentration (ng/mL)	Malondialdehyde Level (nmol/mL)	Conception Rate (%)
Breeds			
Zulu	17.33 ± 2.41 ^a	0.37 ± 0.04 ^a	93 (25/27) ^a
BaPedi	23.17 ± 2.37 ^a	0.36 ± 0.04 ^a	93 (26/28) ^a
Namaqua Afrikaner	20.27 ± 2.34 ^a	0.37 ± 0.03 ^a	78 (18/23) ^b
<i>p</i> -Values	$p > 0.05$	$p > 0.05$	$p < 0.05$
Treatment diets			
NC	13.40 ± 3.02 ^b	0.54 ± 0.05 ^a	71 (10/14) ^b
PC	21.15 ± 3.02 ^{ab}	0.27 ± 0.04 ^{cd}	79 (11/14) ^b
FO	21.56 ± 3.02 ^{ab}	0.22 ± 0.04 ^d	94 (16/17) ^a
AA	18.87 ± 3.24 ^{ab}	0.43 ± 0.05 ^{ab}	94 (16/17) ^a
FO + AA	26.31 ± 3.02 ^a	0.37 ± 0.04 ^{bc}	100 (16/16) ^a
<i>p</i> -Values	$p < 0.05$	$p < 0.05$	$p < 0.05$

NB—NC—negative control (standard diet); PC—positive control (basal diet); FO—5% flaxseed oil; AA—4% ascorbic acid; FO + AA—5% flaxseed oil + 4% ascorbic acid. Means with different superscripts within the same column within the same cell differ significantly ($p < 0.05$).

4. Discussion

The current study is the first study to evaluate the effect of supplementation with flaxseed oil and ascorbic acid on the reproductive performance of South African indigenous sheep. Semen volume did not differ among the breeds (Zulu, BaPedi, and Namaqua Afrikaner) used in this study; however, they ranged from 0.85 ± 0.47 to 0.97 ± 0.46 . Semen volume is a sum of the seminal vesicle, prostate secretion, and spermatozoa [38]. The volume of semen observed in Zulu rams in this study (0.97 ± 0.46) was similar to that reported by Ngcobo et al. [32] of 0.97 ± 0.25 . However, Chella et al. [32] reported a higher volume of semen (1.1 ± 0.1) in Zulu rams following the use of an electro-ejaculator. Munyai [39] reported a lower semen volume (0.5 ± 0.3) in Zulu rams than that reported in the current study. FO + AA improved the semen volume compared to the controls, similar to that reported in Ravi bulls [40] and aged chickens [10]. It is known that, as rams grow up, particularly beyond 3 years, semen quantity and quality decrease [33]. Semen volume evaluation is vital because it allows the calculations of both spermatozoa and non-sperm cells in the ejaculate [38].

The current study found that semen volume, pH, and sperm concentration of BaPedi rams were 0.85 ± 0.47 , 6.06 ± 0.07 , and 0.73 ± 0.04 , respectively. These results were better than those reported by Munyai [39], where BaPedi produced 0.5 ± 0.1 , 7.3 ± 0.4 , and 0.9 ± 177.2 for semen volume, pH, and sperm concentration. The method used to collect semen does have an effect on the semen quality [39] and this might be the cause of these differences in these studies.

Namaqua Afrikaner in the current study produced semen volume, pH, and sperm concentrations of 0.89 ± 0.58 , 6.15 ± 0.07 , and 0.72 ± 0.05 , respectively. Previous studies reported better results for Namaqua Afrikaner, where it produced semen volume, pH, and sperm concentrations of 0.9 ± 0.02 , 7.3 ± 0.3 , and 1.2 ± 30.5 , respectively [9]. So, age might have played a critical role in these differences because old rams +5 years are associated with a decrease in semen quality and quantity [33]. However, the semen volume observed in all diets fell within a normal range [41]. The improvement in FO + AA might be due to the fact that flaxseed oil improves testicular development and spermatogenesis in mammals [17]. Furthermore, flaxseed oil is a rich source of alpha-linolenic acid, which is altered later to form docosahexaenoic acid, which is vital for testes function [10]. The effects of the breed and the treatment diets on semen parameters did not show a clear significant difference among the breeds or diets when interacted. The Zulu, BaPedi, and Namaqua Afrikaner are South African indigenous sheep differentiated through the places where they are found and named based on their owners' tribe or languages [3]. Therefore, non-significant differences observed for semen quality parameters were expected.

In the current study, semen pH did not differ significantly ($p > 0.05$) in all treatment groups (NC, PC, FO, AA, and FO + AA). Generally, semen pH measures the level of acidity in the semen and has been reported to be around 6.7 ± 0.2 in South African indigenous rams during the breeding season [33]. The acidic semen pH comes as a result of the decrease in natural buffering, leading to the death of some of the sperm cells in the ejaculate during in vitro semen handling, hence creating an acidic environment for other sperm cells, leading to shorter lifespan [41]. However, it is noteworthy that semen pH from this study fell within a normal range of semen pH in all treatment groups, justifying the proper semen handling post-collection [38]. Based on our knowledge, there is no direct or indirect effect of semen acidity on the quality of semen. Moreover, provided that the ratio of male to female during mating is very low, semen quality improvement remains vital for flock fertility [10], especially after 4 years of age in sheep [33] since semen quality declines with age [33].

There was no significant difference in sperm concentration in PC, FO, AA, and FO + AA, with NC scoring the lowest, although this did not differ significantly from that of PC, AA, and FO + AA. Sperm concentration can be described as the number of sperm cells per volume unit [42]. There is a clear relationship between sperm concentration and semen volume; hence, sperm concentration cannot be calculated without accurate semen volume [43]. Therefore, non-significant differences in semen volume throughout the treatment diets (NC, PC, FO, AA, and FO + AA) might explain the non-significant differences observed for sperm concentration in these treatment diets (except NC).

Plasma membrane permeability/integrity measured using HOST showed non-significance ($p > 0.05$) for sperm plasma membrane permeability between AA (81.10 ± 1.42) and PC (77.87 ± 1.37). Notably, FO + AA had higher intact sperm cells than the rest of the groups. The role of supplementing ascorbic acid on sperm membrane integrity has been reported previously [22]. Flaxseed oil contains alpha-linolenic acid (± 50.20), which is then synthesized de novo to form longer PUFAs such as docosahexaenoic acid (DHA). DHA and EPA influence the membrane structure of the sperm cells, making the sperm membrane smooth and participating in the response mediated by the protein. This could, in turn, affect the production of lipid-mediated conductors, cell signal transduction, and gene expression. Therefore, the current study observed a clear improvement in intact membranes following supplementing the diets with flaxseed oil, ascorbic acid, and flaxseed oil + ascorbic acid. This was supported in the current study, where the groups NC and PC had higher damaged membranes when compared to the FO and FO + AA groups.

The sperm cell motility parameters (progressive, non-progressive, total, rapid, medium, and slow motility) were evaluated by CASA as influenced by the breed (Zulu, BaPedi, and Namaqua Afrikaner). In all these parameters measured, there were no significant differences among the breeds. Nevertheless, the sperm cell motility parameters for Zulu rams and BaPedi fell within the normal range that has been reported before [33,35,44,45]. Goshme et al. [46] classified sperm motility as (1) very poor when motility is around 10%, (2) poor when motility is 20–40%, (3) fair when motility is 40–70%, (4) very good when motility is 75–90%, and (5) very good when motility is >90%.

The FO + AA improved progressive motility, total motility, and rapid motility compared to other diets. These results were better than those reported in Zulu rams [33,35], BaPedi [45], and Namaqua Afrikaner [39] when fed on pastures. Our results were in line with those reported elsewhere in bulls [19,47], pigs [48,49], and avians [50], where flaxseed oil/linseed oil improved sperm motility parameters. Sperm motility is known to be a critical mark of good-quality sperm cells [51].

Sperm vitality was also evaluated in this study and showed better improvement in FO, AA, and FO + AA when compared to NC and PC. Sperm vitality refers to the integrity and permeability of the sperm cell membrane [52]. Sperm viability, on the other hand, refers to the total percentage of live sperm cells per semen ejaculate [52]. These factors are critical to know whether static spermatozoa are alive or dead, and hence should be evaluated together with the sperm motility evaluation [38]. In this study, rams fed NC and PC produced higher dead and damaged sperm cells compared to FO, AA, and FO + AA. Therefore, the FO-, AA-, and FO + AA-treated groups had higher live and intact sperm cells. High live and intact sperm cells per ejaculate are important relative to a successful pregnancy [38]. Significantly higher dead and damaged sperm cells in the NC and PC groups might indicate the structural defects in the flagellum [53]; high static and non-viable sperm cells thus indicate an epididymal pathology [54].

Sperm cell morphology was also evaluated after dietary enrichment with flaxseed oil. Sperm morphology can be defined as the actual shape of the sperm cell [55] and has a direct impact on fertility and embryo development [56]. Sperm morphology was divided into primary, secondary, and tertiary abnormalities. Primary sperm abnormalities are abnormalities that occur during spermatogenesis [55], secondary abnormalities occur as the sperm transits in the epididymis [57], and tertiary abnormalities occur during the *in vitro* handling of the sperm cells. In this study, there were no significant differences in primary abnormalities except that of double heads, where PC produced more sperm with double heads. The presence of any sperm abnormalities above 30% might cause fertility failure [55]. It is noteworthy that none of the treatment diets used in this study led to higher sperm abnormalities above 30%. Nevertheless, enriching diets with flaxseed oil did not cause any significant differences in primary sperm abnormalities except for small heads, which were higher in the flaxseed oil + ascorbic acid group. The primary cause of sperm abnormalities has been linked to many genes, including that of biallelic SUN5 mutations, the homozygous deletion of SUN5, or the mutation of BRDT [56,58,59]. However, the FO + AA group significantly reduced the double tail and double head. This is very important since mucus from the female reproductive tract itself filters out spermatozoa, principally those with impaired quality and morphology [60].

Testosterone is a male hormone responsible for secondary sex characteristics [61]. Testosterone, which is responsible for spermatogenesis, was also improved after supplementing with flaxseed oil elsewhere [62]. In the current study, FO + AA was found to improve testosterone concentration. Leydig cells are the main steroidogenic cells that are critical for the synthesis of testosterone to induce secondary male characteristics. Previous studies have associated testosterone with age in sheep [61]. Moreover, it is well known that testosterone concentration declines with age, especially after 5 years of age [33]. In the current study, six-year-old rams were used, and the testosterone level observed after supplementation with flaxseed oil and ascorbic acid suggests that the treatment reversed the effect of age on both semen quality and testosterone. These results were similar to

those found in Wistar rats, where supplementing ascorbic acid increased testosterone concentrations significantly [22].

The concentration of MDA in sperm cells following dietary enrichment was evaluated. Breed did not influence the MDA level; however, FO and FO + AA had the lowest concentration of MDA. The MDA concentration is the main product correlated to lipid peroxidation [63]. Lipid peroxidation is a reaction of reactive oxygen species (ROS) with sperm plasma membrane lipids [63]. The high lipid bilayer in the plasma membrane jeopardizes the sperm cell, making it vulnerable to reactive oxygen species [64]. Reactive oxygen species (ROS) drastically reduce sperm freezability by lipid peroxidation, impairing the capacity to fertilize ova [65,66] by oxidation stress. In this study, a clear role of flaxseed oil and ascorbic acid was evidenced through the low level of MDA found. These results were in line with those reported by Ezazi et al. [23]. However, high concentrations of ascorbic acid can be detrimental to the sperm membrane, causing lipid peroxidation [67].

The lipids around the plasma membrane in the form of PUFAs are attacked by ROS because they consist of unconjugated double bonds separated by methylene groups [68]. This occurs due to a lower concentration of scavenging enzymes and antioxidants confined within the sperm cytoplasm [68]. Therefore, the massive reduction in MDA levels following supplementation with ascorbic acid and flaxseed oil is due to the antioxidant quantities in these feeds. Ascorbic acid is a nonenzymatic antioxidant and is regarded as a water-soluble ROS scavenger with high vigor [69], while flaxseed oil contains vitamin E [12].

The conception rate observed in Zulu and BaPedi sheep was significantly ($p < 0.05$) higher than that observed in Namaqua Afrikaner sheep (72%). Our results were similar to those reported by Gizaw et al. [70] in Ethiopian sheep breeds. The inclusion of dietary flaxseed oil and ascorbic acid resulted in a significantly ($p < 0.05$) higher conception rate in FO + AA (100%), AA (94%), and FO (94%) than in the NC- (71%) and PC (79%)-treated groups. Similar fertility results were reported by Ezazi et al. [23] previously. Nevertheless, the effect of breed and the treatment diets did not yield any significant ($p > 0.05$) differences.

5. Conclusions

The dietary inclusion of flaxseed oil and ascorbic acid improves the reproductive performance of indigenous South African sheep. Moreover, based on the results, this study paved the way to improving semen quality and hence might lead to promising sperm post-thawed survival for the application of artificial insemination in the future. Furthermore, the dietary inclusion of flaxseed oil and ascorbic acid can be used to improve testosterone concentration and reduce MDA levels, thus improving the conception rate. However, it is still recommended that more studies be performed to evaluate post-thawed sperm quality and fertility following flaxseed oil and ascorbic acid supplementation.

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