



Semen quality of hair sheep supplemented with *Moringa oleifera* (Moringaceae) and *Trichanthera gigantea* (Acanthaceae)



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Abstract:

It was assessed the effect of the inclusion of *Moringa oleifera* Lam. and *Trichanthera gigantea* (Bonpl.) Nees in the diet of hair (Pelibuey) sheep on the quality of their semen. During 90 d, the diets of 15 sheep (24 kg ± 3.95) were divided into three treatments: T1: integral diet with 40 % of *M. oleifera* + Taiwan grass (*Pennisetum purpureum* Schumach.), T2: integral diet with 40 % of *T. gigantea* + Taiwan grass, and T3: 40 % commercial feed + Taiwan grass. The daily weight gain (DWG), carcass yield (CY), testicular development

(DT) determined by the diameter (AE) and the scrotal circumference (SC) of these sheep were determined, and the volume, concentration, motility (CASA), viability (SYBR-14/IP), mitochondrial activity (J-C1) and acrosomal integrity (FITC-PSA) of their sperm samples were assessed. No differences were found ($P>0.05$) in the GDP and RC. Differences were found ($P<0.05$) in DT, T1 (AE= 48.84 ± 5.99 mm, CE= 9.29 ± 1.13 cm) and T3 (AE= 48.83 ± 4.34 mm, C = 26.62 ± 1.27 cm) exhibited higher values than T2 (44.57 ± 5.59 AE= mm, CE= 25.42 ± 1.50 cm); as for viability, T2 (62.90 ± 6.10 %) and T1 (54.00 ± 6.61 %) had higher percentages than T3 (24.45 ± 7.56 %); in motility, T1 (93.9 ± 2.1 %) and T2 (88.6 ± 1.9 %) had a higher percentage than T3 (71.9 ± 4.0 %). The inclusion of *M. oleifera* and *T. gigantea* in the diet allows to obtain a GDP, RC and DT similar to those of commercial feed and increases the number of viable sperm cells by more than 20 %; it also improves certain parameters of motility, thereby improving the reproductive potential of the stallions.

Keywords: Viable sperm cells, motility, *Pennisetum purpureum*, channel performance.

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Introduction

The high demand for sheep meat forces producers to improve all its processes aimed at reducing production costs, to increase the production and reproduction parameters in order to increase the productivity per unit area, to improve animal welfare, and to propose a sustainable approach to ensure the permanence of the system through time⁽¹⁾.

In tropical areas, the atmosphere is a determining factor for livestock production, and in particular for sheep production, which must deal, among other factors, with a marked seasonality in the production of forage, forcing producers to implement supplementation strategies (mainly with commercial feed) in order to maintain optimal levels of production; this has generated a dependency on the feed, as well as a considerable increase in production costs⁽²⁾. Within this context, the use of tree species with fodder potential has become relevant in animal feed^(3,4,5), because its implementation allows to generate strategies of partial replacement of grains in the diet⁽⁶⁾. However, the use of tree species with fodder potential is conditioned by their high nutrient content, tolerance to pruning, steady production of biomass and content of secondary compounds that may affect the animals that consume them^(7,8). This is one of the main reasons that limit their use for animal feed⁽⁹⁾, since it can

generate estrogenic effects that inhibit, among others, the growth and maintenance of the reproductive system. In the reproductive system of males, the affectations by presence of secondary compounds can be: an increased number of immature sperm with a significant decrease in motility, a reduction of the lumen of seminiferous tubules, an increase in the apoptotic index of germ cells, prostatic atrophy, and affectation of spermatogenesis^(10,11).

Within this context, various species such as *Moringa oleifera* Lam. (Moringaceae) and *Trichanthera gigantea* (Bonpl.) Nees (Acanthaceae) are alternative dietary supplements⁽⁸⁾. The presence of secondary metabolites has been reported in these; for example, García⁽¹²⁾ recorded the presence of phenols, flavonoids and tannins that precipitate the protein and condensed tannins; on the other hand, Balercia *et al*⁽¹³⁾, Rajimakers *et al*⁽¹⁴⁾ and Ghasi *et al*⁽¹⁵⁾ observed in *M. oleifera* and *T. gigantea*, the presence of riboflavin, niacin, folic acid, pyridoxine, protein, vitamins A, B, C, and E, as well as amino acids and high levels of β -carotene; consumption of these antioxidants helps to improve semen quality by eliminating free radicals found in the seminal plasma. For this reason, it is necessary to study the effect that these plants may generate in the animals that consume them. Therefore, the objective was to evaluate the effect of the inclusion of *M. oleifera* and *T. gigantea* in the diet of Pelibuey sheep on the seminal quality.

Material and methods

The study was carried out in the agricultural and livestock production and research area of the Technological Institute of Conkal, Yucatán, Mexico (21°05' N and 89°32' W), which is located at an altitude of 7 meters above sea level and has an AW₀ climate, with an annual precipitation of 900 mm and an average temperature of 29 °C.

Treatments, animals and management

Fifteen (15) male pubertal Pelibuey sheep (aged 6 ± 0.5 mo) with an average weight of 24 kg \pm 3.95 were utilized and distributed in a fully random way in three treatments (n=5): T1= integral diet with 40 % *M. oleifera* + Taiwan grass (*Pennisetum purpureum* Schumach.). T2= integral diet with 40% *Trichanthera gigantea* + Taiwan grass. T3= integral diet + Taiwan grass. The amount of feed offered was 4.7 % of the live weight during the 90 days of the experiment (the consumption was adjusted every seven days). Table 1 shows the contents of the percentage composition of the experimental diets, and Table 2, the proximate analysis.

Table 1: Percentage composition of the experimental diets

Component	Diets (%)		
	T1	T2	T3
<i>Moringa</i>	16	0	0
<i>Trichanthera</i>	0	16	0
Molasses	4.24	3.72	7
Minerals	1.2	1.08	3
Canola	3.2	5.88	21
Corn	8.28	8	5
Sorghum	7.08	5.32	4
Taiwan grass	60.0	60.0	60.0

T1: integral diet with 40% *M. oleifera* + Taiwan grass (*Pennisetum purpureum* Schumach.), T2: integral diet with 40% *T. gigantea* + Taiwan grass, and T3: commercial feed + Taiwan grass.

Table 2: Proximal chemical analysis of the offered diets

Diets and grass	Dry matter	Crude protein	Neutral detergent fiber	Acid Detergent Fiber	Ashes
T1	90.83	13.81	20.22	16.18	1.01
T2	91.54	14.01	26.27	14.21	0.95
T3	98.82	23.16	22.83	13.47	0.98
Taiwan grass	90.96	8.00	67.26	42.21	0.97

T1: integral diet with 40 % *M. oleifera* + Taiwan grass (*Pennisetum purpureum* Schumach.), T2: integral diet with 40% *T. gigantea* + Taiwan grass, and T3: commercial feed + Taiwan grass

Development of lambs

To determine the development of lambs, the animals were weighed every seven days; prior to each weighing, the animals underwent a period of 18 h of fasting. They were weighed on a T31P (OHAUS) electronic platform scale with a capacity for 300 kg, with a margin of error of 0.1 kg. The daily weight gain was determined through the analysis of these weights⁽¹⁶⁾.

Testicular development

The measurement of the scrotal circumference was taken with a plastic measuring tape, while the testicular diameter was registered using a TRUPER brand digital caliper⁽¹⁷⁾.

Semen quality

For the assessment of the semen quality, after the 70-days experimental period (at age 8 ± 0.7 mo), six semen samples per animal (two per week) were collected in each treatment using an estrogenized female dummy and the artificial vagina method with a rubber sleeve (Liner). Warm water at 45 °C and air were introduced by blowing through a valve in order to obtain the temperature and pressure required to stimulate ejaculation⁽¹⁸⁾. Once the ejaculates were obtained, they were transported to the laboratory in isothermal conditions (35 to 37 °C) for evaluation. At the laboratory, the samples were placed in a double boiler at 37 °C for immediate evaluation.

The following were determined in the collected samples:

- 1) The volume of the ejaculate, with a graduated pickup tube.
- 2) The sperm concentration was determined with the CASA system (ISAS®v1 (Proiser R&D, Valencia, Spain) by diluting 5 µl of pure semen in 995 µl of distilled water (1:200); 9 µl of the diluted sample were then placed in a Bürker chamber for analysis. Sperm motility was evaluated by depositing 5 µl of diluted sample at a concentration of 30×10^6 spermatozoa/ml on a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel), and four recordings were made of each sample; the dilution was performed with the commercial diluent (Triladyl® + distilled water + 20 % egg yolk). The parameters evaluated for this variable were: percentage of total motility (TM), percentage of progressive motility (PM), curvilinear speed (CLS, µm/s), rectilinear speed (RLS, µm/s), average speed (AS, µm/s), linearity index (LIN), straightness index (STR), oscillation index (WOB), average amplitude of lateral displacement of the head (ALH), sperm head beat frequency (HBF).
- 3) The sperm viability was assessed by staining with the Fluorochrome SYBR-14/IP (Live/Dead® kit L-7011, Invitrogen™); 1 µL of SYBR-14 (10 µM) was mixed with 1 µl IP (12 µM) in 100 µl of diluted sperm sample and kept for 10 min at 37 °C; 5 µl of the stained sample were subsequently placed on a glass slide preheated at 37 °C for evaluation in a (CX-31, Olympus, Tokyo, Japan) fluorescence microscope with a wavelength of 488 nm.

4) The mitochondrial activity was determined using the J-C1 fluorochrome (153 μM , Molecular Probes® T-3168, Invitrogen™). 1 μl of the fluorochrome was mixed with 100 μl of diluted sperm sample and kept for 10 min at 37 °C; 5 μl of the stained sample were subsequently placed on a glass slide preheated to 37 °C for evaluation under a fluorescence microscope with a wavelength of 488 nm.

5) The integrity of the acrosomes was evaluated with the FITC-PSA fluorochrome (100 $\mu\text{g}/\text{mL}$, L-0770, Sigma-Aldrich™), adding 5 μl of the fluorophore in 100 μl of diluted sperm sample and was kept for 30 min at 37 °C; 5 μl of the incubated sample were subsequently placed on a slide and were evaluated in a fluorescence microscope with a wavelength of 485 nm.

The data obtained were analyzed using the PROC GLM procedure of the statistical package SAS 9.4 for Windows (Inst. Inc. Cary, NC, USA); a repeated means variance analysis (ANOVA) a Tukey mean comparison test were performed in order to evaluate the effect of diet on the sperm quality.

Results and discussion

Productive behavior

The daily weight gains of the studied animals did not exhibit any differences ($P < 0.05$) between treatments. These gains are greater than those recorded by Madera-Solis *et al*⁽¹⁹⁾, who with different levels of inclusion of *Leucaena leucocephala* (Lam.) de Wit found daily weight gains of 25, 41.67, 50, and 75 g in Pelibuey sheep. For their part, Rivers *et al*⁽²⁰⁾ reported profits of 54, 87 and 56 g/d when assessing the use of *Morus* sp. and *Gliricidia sepium* Kunth ex Walp. as substitutes for concentrated feed for growing lambs. Daily revenues of 45.5, 96.3, 124.5 and 106.4 g with different levels of inclusion of hay from *Hibiscus rosa-sinensis* L. are also reported in growing sheep⁽⁴⁾. The profits obtained in this study are less than those mentioned by others⁽¹⁶⁾, which recorded daily gains of 229 ± 10 g/d for Pelibuey-Katadin animals housed in elevated cages and fed a commercial concentrate with a 16 % crude protein content and *Cynodon nlemfuensis* Vandyerst hay.

When assessing the performance of the carcass, no differences were found ($P < 0.05$) between treatments: 50.79, 49.44 and 46.94 % for treatments T1, T2 and T3, respectively; these values are similar to those registered by Magaña-Monforte *et al*⁽¹⁶⁾, who obtained a yield of

49.1 ± 0.58 with crossbred animals of the Pelibuey-Katahdin breeds. The yields of this study were lower than those found in another research⁽²¹⁾, which cites a carcass output of 52.8 % for whole males, 56.64 % for castrated males, and 55.87 % for females. The proper nutritional balance present in the assessed diets generated an adequate, similar productive response in the study animals, compared to other researchers performed under similar conditions. These results in the evaluated rations (Table 2) can be attributed to the adequate level of protein and the improved nutritional balance⁽²²⁾; this stimulates an increase in the level of efficiency of nutrients produced by greater microbial activity in the rumen⁽²³⁾ and ensuring metabolic stability⁽²⁴⁾, positively affecting the various productive parameters evaluated in the present study (daily weight gains and carcass output).

Testicular development

Testicular volume is an indicator of the fertilizing ability of the male and of the highest fertility; also a smaller number of abnormal spermatozoa has been observed in animals with a larger scrotal circumference. In the study animals, the testicular development determined by means of the increase in scrotal circumference and diameter exhibited differences (P<0.05) between the treatments evaluated for both variables (Table 3). For the variable scrotal circumference, treatment T3 had a higher mean than T2, while T1 did not differ (p>0.05) from the other two treatments. For the scrotal diameter variable, there was no difference between treatments T1 and T3; however, both were larger than with T2.

Table 3: Scrotal circumference, scrotal diameter, and hair sheep seminal volume and concentration supplemented with *M. oleifera* and *T. gigantea* (mean ± standard error)

Treatments	Scrotal circumference (cm)	Scrotal diameter (mm)	Volume (ml)	Concentration
T1	26.48±0.33 ^{ab}	48.84±1.05 ^a	0.70 ± 0.05 ^a	1882.63 ± 202.66 ^a x 10 ⁶
T2	25.42±0.33 ^b	44.57±1.05 ^b	0.61 ± 0.05 ^a	1841.92 ± 182.34 ^a x 10 ⁶
T3	26.62±0.33 ^a	48.83±1.05 ^a	0.63 ± 0.06 ^a	1014.49 ± 482.44 ^a x 10 ⁶

T1: integral diet with 40% *M. oleifera* + grass (*Pennisetum purpureum* Taiwan), T2: integral diet with 40 % *T. gigantea* + Taiwan grass, and T3: commercial feed + Taiwan grass.

^(a,b) different letters between columns indicate significant differences (P<0.05).

The means for the scrotal circumference were: 26.4 ± 0.33 , 25.4 ± 0.33 and 26.6 ± 0.33 cm for treatments one two and three, respectively (Table 3); they are lower than those recorded by Luna *et al.*⁽²⁵⁾, who found averages of 28.4 cm in sheep supplemented with animal fat and palm oil; as well as those reported in other studies^(26,27), of 29.4 and 36.5 cm, respectively. The averages of scrotal diameter were lower than those recorded by Ballin *et al.*⁽²⁶⁾. Testicular development is determined by various factors, mainly age, race, and nutrition⁽²⁷⁾; in this regard the study animals, with an average age of 7 ± 0.5 months, received a diet with adequate levels of protein and nutrients required for meeting the nutritional requirements according to their physiological stage, which is reflected in the weight gain and in the obtained testicular development.

Seminal evaluation

The variables of the ejaculate volume and sperm concentration did not exhibit any differences ($P > 0.05$) between treatments (Table 3). The volume for the three treatments lies within the range considered normal, between 0.5 and 2.0 ml, and this volume varies, depending on the age, size, body condition of the animal, frequency of collection, and dexterity of the operator⁽²⁸⁾. Despite this, the values reported in this experiment were lower than those reported by Mansano *et al.*⁽²⁹⁾, who obtained volumes of 1.3 ± 0.4 ml; Carrillo and Hernández⁽³⁰⁾ obtained 1.41 ± 0.11 ml; for their part Córdova-Izquierdo *et al.*⁽³¹⁾ obtained 1.11 ml; however, these two studies were performed with mature males unlike the pubertal males (7 ± 0.5 mo of age) used in the present study.

The low ejaculate volume values obtained in this study, contain a greater concentration of spermatozoa per ml. Therefore, these values are higher than those recorded in other studies, which indicate ranges of 206.4 to $711.89 \times 10^{6(30,31)}$, but similar to those found by Domínguez *et al.*⁽³²⁾, of 2.19 ± 0.04 and $108 \pm 8.0 \times 10^6$ spermatozoa/ml, when assessing the semen quality of F1 Katahdin \times Pelibuey lambs supplemented with alfalfa. In contrast, the concentration values obtained here are lower than those found by Quintero *et al.*⁽³³⁾, who obtained $2,628.2 \times 10^6$ when assessing hair sheep aged 2 to 5 yr

There were no differences ($P < 0.05$), in total motility, curvaceous speed, and AD, where treatments T1 and T2 were better than T3; and in the variables AS and RLS, T1 exhibited the best results with respect to T3; however, no differences were observed between T1 and T2. For the rest of the analyzed variables (PM, LIN, IR, OI, and BF), no differences were found between experimental groups (Table 4).

Table 4: Seminal motility parameters of hair sheep supplemented with *M. oleifera* and *T. gigantea* (mean \pm standard error)

Variables	Treatments		
	T1	T2	T3
MT, %	93.9 \pm 2.1 ^a	88.6 \pm 1.9 ^a	71.9 \pm 4.0 ^b
AS, $\mu\text{m/s}$	100.4 \pm 3.7 ^a	88.6 \pm 3.2 ^{ab}	73.0 \pm 6.8 ^b
CLS, $\mu\text{m/s}$	157.8 \pm 5.0 ^a	146.6 \pm 4.4 ^a	117.3 \pm 9.2 ^b
RLS, $\mu\text{m/s}$	70.3 \pm 3.1 ^a	61.3 \pm 2.7 ^{ab}	54.6 \pm 5.8 ^b
AD, μm	4.8 \pm 0.1 ^a	4.6 \pm 0.1 ^a	3.7 \pm 0.3 ^b
PM, %	30.79 \pm 2.64 ^a	29.23 \pm 2.32 ^a	30.18 \pm 4.87 ^a
IR, %	64.72 \pm 1.31 ^a	64.98 \pm 1.15 ^a	69.62 \pm 2.42 ^a
WOB, %	61.96 \pm 1.63 ^a	59.85 \pm 1.43 ^a	60.78 \pm 3.01 ^a
HBF	10.32 \pm 0.25 ^a	10.57 \pm 0.22 ^a	9.89 \pm 0.47 ^a
LIN, %	42.27 \pm 1.9 ^a	40.85 \pm 1.67 ^a	44.35 \pm 3.5 ^a

T1= 40% of *M. oleifera* + Taiwan, T2= 40% of *T. gigantea* + Taiwan, T3= commercial feed + Taiwan.

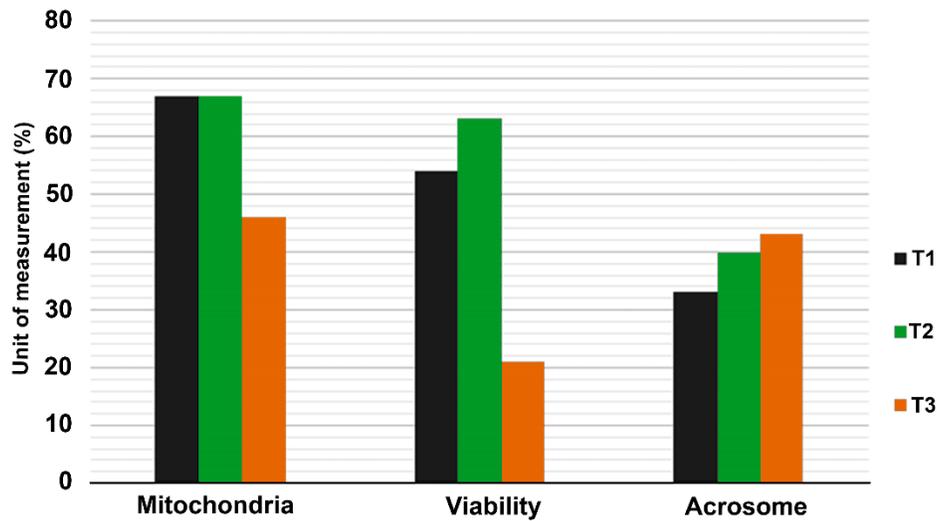
MT= total motility; AS= average speed; CLS= curvilinear; speed; RLS= rectilinear speed; AD= amplitude of displacement; PM= progressive motility; STR= straightness index; WOB= oscillation index; HBF= sperm head beat frequency; LIN= linearity index.

^{a,b} Values with different letters in the same row are different ($P < 0.05$).

The results for the motility variable are higher than those recorded by Mansano *et al*⁽²⁹⁾, of 52 %, but similar to those found in another research⁽³²⁾, which registered an 80 % total motility and over 20 % progressive motility; however, the results of this study are lower than those recorded by Quintero *et al*⁽³³⁾, who obtained 90.69 % progressive motility.

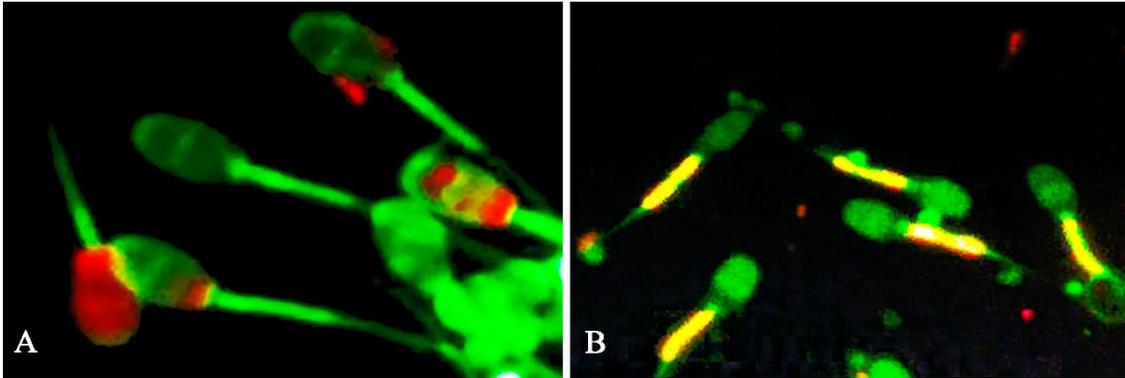
In the analysis of the plasma membrane viability variable, differences ($P < 0.05$) were found between the assessed treatments (T1: 54 %, T2: 63 % and T3: 21 %), T2 being the treatment that exhibited the highest percentage of viability, in contrast with T3, which obtained the lowest percentages (Figure 1).

Figure 1: Feasibility, mitochondrial activity and integrity of the acrosome of hair sheep supplemented with *Moringa oleifera* and *Trichanthera gigantea*



T1: integral diet with 30% *M. oleifera* + Taiwan grass (*Pennisetum purpureum* Schumach.), T2: integral diet with 30% *T. gigantea* + Taiwan grass, and T3: commercial feed + Taiwan grass.

The mitochondrial activity and acrosome integrity variables did not reveal any differences ($P > 0.05$) between treatments, while T1 exhibited the highest means for the mitochondrial activity variable, T3 showed the highest percentages for the mitochondrial activity variable (Figure 1). The integrity of the plasma and acrosome membranes and mitochondrial activity are major factors for fertilization, as an intact plasma membrane indicates that the spermatozoon is alive; an intact acrosomal membrane allows penetration of the oocyte, and the mitochondrial activity ensures the presence of energy required to move the spermatozoon (Figure 2). For this reason, it is essential to include it in the assessments on semen quality⁽³¹⁾. The results observed in this study agree with those recorded by Cordova-Izquierdo *et al*⁽³¹⁾ who obtained 68.5 % of the integrity of the plasma membrane by assessing sheep of the Suffolk race. They also match the results of Rubio-Guillen *et al*⁽³⁴⁾, who recorded 42 % when evaluating West African sheep. However, the percentages obtained in this study are lower than those found in other researches^(30,32,33) but are consistent with the values estimated by Domínguez *et al*⁽³²⁾ for acrosomal integrity.

Figure 2: Sperm cells from sheep fed with *Trichanthera gigantea* or *Moringa oleifera*

A. Living (*green*) and dead (red or orange) sperm heads stained with SYBER-14/IP fluorochromes; viability of the acrosome located in the apical region using the fluorochrome FITC-PSA (colorless: viable; green: corrupted). **B.** Mitochondrial activity located in the middle region of the tail stained with the fluorophore JC1, with activity (yellow or orange) and without activity (green).

The parameters recorded in the assessment of the semen quality as well as of the testicular development may be attributed to the adequate nutritional balance of the assessed diets, as sperm production and quality, and testicular size, are directly influenced by nutrition. An increase in the nutritional level increases the frequency of pulses of LH and increases levels of FSH⁽³⁵⁾, hormones that promote spermatogenesis and androgen secretion⁽³⁶⁾.

The animals assessed in the present study are pubescent, animals, and animals in this condition have a greater amount of immature, dead or defective spermatozoa, which in turn produce high amounts of free radicals that affect those spermatozoa that are in good conditions, since free radicals cause a cascade of lipid peroxidation affecting mainly the unsaturated fatty acids of the plasma membrane of the sperm, causing loss of sperm function and cell apoptosis, and therefore are one of the leading causes of death of sperm cells⁽³⁷⁾.

It has been observed that the inclusion of antioxidants in the diet improves the percentage of live sperm cells because antioxidants eliminate the free radicals that are found in the seminal plasma and confer protection to the spermatozoa^(13,14); Priyadarshani and Varma⁽³⁸⁾ observed a higher percentage of live cells when they used *M. oleifera* in the diet of mice. This result is similar to what has been observed in the present study and may be ascribed to the contribution of antioxidants (beta-carotene) contained in *M. oleifera* and *T. gigantea*⁽¹⁵⁾.

The secondary compounds present in both assessed species may be related to the values found in the present study; therefore, further studies determining the presence and concentration of these would contribute greater evidence in this regard.

Conclusions and implications

The replacement of 40 % of commercial feed by *M. oleifera* or *T. gigantea* in the diet of Pelibuey sheep promotes adequate testicular development, and improves the sperm viability by more than 20 %, as well as certain parameters of motility —such as total motility, average speed, curvilinear speed, rectilinear speed, and the average amplitude of lateral motility of the sperm head—, whereby the reproductive potential of the stallions may be improved.

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