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Evaluation of the seminal quality of rabbits fed diets containing different inclusion levels of flaxseed (*Linum usitatissimum*)

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Abstract

The aim of this study was to evaluate the effect of flaxseed (*Linum usitatissimum*) on the physical and morphological quality of fresh and refrigerated rabbit semen. Twenty New Zealand males were used, and distributed in four groups according to the level of inclusion of flaxseed in the total diet for 100 days: 0 %, 3 %, 6 %, and 9 %. Fifteen days after starting the supply of the diet, seminal collections were made once a week with an artificial vagina and a female as a dummy. For the physical and morphological evaluations, the semen was diluted in Tris-Egg Yolk medium and cooling at 5 °C; progressive sperm motility, and spermatic vigor and morphology were assessed at 0, 2, 12, 24, 48, and 72 post-cooling hours. This information was analyzed through analyses of variance and regression at 5 % significance. For the morphology, volume, color, and seminal appearance of fresh semen ($p > 0.05$), there was no difference. For volume, turbidity, motility, vigor, and concentration, average values of 0.92 ± 0.24 mL; 0.86 ± 0.35; 76.58 ± 7.13 %; 3.37 ± 0.28, and 205.75 ± 20.77 x 10^6 sptz were recorded, respectively. There were differences for motility and vigor at 0, 2, 12, 24, 48, and 72 post-cooling hours ($p > 0.005$). In conclusion, the inclusion of up to 9 % flaxseed in the rabbit diet did not alter the physical and microscopic parameters evaluated in this study in fresh and refrigerated rabbit semen.

Keywords: animal reproduction, diet, linseed, rabbit, spermatozoa

Evaluación de la calidad seminal de conejos alimentados con dietas que contienen diferentes niveles de inclusión de semillas de linaza (*Linum usitatissimum*)

Resumen

El objetivo de este trabajo fue evaluar el efecto de la semilla de linaza (*Linum usitatissimum*) en la calidad física y morfológica del semen fresco y refrigerado de conejos. Se emplearon 20 machos Nueva Zelandia distribuidos en grupos según niveles de inclusión de semilla en su dieta durante 100 días: 0 %, 3 %, 6 % y 9 %. 15 días después de iniciar el suministro de la dieta se realizaron colectas seminales una vez por semana con vagina artificial y una hembra como maniquí. Para la evaluación física y morfológica se diluyó en medio Tris-yema y refrigerado a 5 °C; se valoró la motilidad espermática progresiva, el vigor espermático y la morfología espermática a las 0, 2, 12, 24, 48 y 72 horas post-refrigeración. La información fue analizada mediante análisis de varianza y de regresión al 5 % de significancia. Para la morfología, volumen, color y aspecto seminal del semen fresco ($p > 0,05$) no hubo diferencia. Para volumen, turbidez, motilidad, vigor y concentración, hubo valores medios de 0,92 ± 0,24 mL; 0,86 ± 0,35; 76,58 ± 7,13 %; 3,37 ± 0,28 y 205,75 ± 20,77 × 10^6 sptz, respectivamente. Hubo diferencia para motilidad y vigor espermático a las 0, 2, 12, 24, 48 y 72 horas post-refrigeración ($p < 0,005$). Se concluyó que la inclusión de hasta 9 % de semilla de linaza en la dieta de conejos no alteró los parámetros físicos y microscópicos evaluados en este estudio en semen fresco y refrigerado de conejo.

Palabras clave: conejo, dieta, espermatozoide, linaza, reproducción animal

Introduction

Multiple investigations have been interested in the mechanisms that operate in the manipulation of diets and their reproductive effects in various species. Among them, the effect of energy on the reproductive processes of males and females can be cited (Rigolon et al., 2003).

Different sources of lipids have been supplied to production animals, including fats, as byproducts obtained in processing plants. Likewise, these have been provided from oilseeds, such as sunflower, soybean, canola, cotton, and flaxseed, among others (Wathes et al., 2007).

Other studies have shown that the relationship between type 3 and type 6 polyunsaturated fatty acids have a degree of responsibility, since they improve fluidity in the sperm membrane (due to compositional lipids), i.e., improving semen quality (Freitas et al., 2014; Strzezek et al., 2004).

In the context previously described, flaxseed is shown as an important alternative to raise reproductive indices. This is due to the linolenic acid in approximate amounts of more than 50 g/100 g of the sum of fatty acids (Cunnane et al., 2003) and linoleic acid (16 %) (Kennelly, 1996). Therefore, it is interesting to analyze the use of flaxseed, especially for omega-3 supply, since it can be an alternative to improve fertility rates.

For this reason, the aim of this study is to establish the effect of the flaxseed (*Linum usitatissimum*) added in the food ration of rabbits on the physical and morphological quality of fresh and refrigerated rabbit semen.

Materials and methods

The project was approved by the Animal Use Ethics Committee (CEUA, for its acronym in Portuguese) of Universidad Federal del Recóncavo de Bahia, Brazil. Twenty adult rabbits of the New Zealand breed were distributed, with an average age of 8 months and a mean weight of 3.0 ± 0.4 kg. They were housed in individual 1 m^2 wire-covered cages with galvanized steel feeders and ceramic manual drinkers.

The animals were randomly distributed into four groups according to the level of brown flaxseed inclusion in the dry matter of the total diet: 0 %, 3 %, 6 %, and 9 % (table 1). The experimental rations were proposed based on the recommendations of Villamide et al. (2010) for males of reproductive age. Besides, Tifton hay was used as bulky with a volume: ration ratio of 70:30 and an *ad libitum* water supply.

Table 1. Proportion of components of the experimental diets based on dry matter

Ingredients	Flaxseed levels (%)			
	0	3	6	9
Flaxseed	0.000	3.000	6.000	9.000
Tifton hay	31.689	32.850	35.346	36.872
Wheat bran	24.559	17.625	8.960	1.758
Corn	15.641	19.106	23.396	26.833
Soybean bran	20.102	20.198	20.513	20.645
Soybean oil	4.251	2.941	1.417	0.100
Calcareous	1.658	1.494	1.278	1.104
Dicalcium phosphate	0.790	1.024	1.320	1.564
Salt	0.552	0.553	0.556	0.557
Premix of vitamins and minerals	0.500	0.500	0.500	0.500
DL-Methionine 99	0.146	0.147	0.151	0.154
Lasalocid 20 %	0.063	0.063	0.063	0.063
Washed sand	0.050	0.500	0.500	0.850
Total	100	100	100	100

Source: Adapted from Villamide et al. (2010)

The diets were offered *ad libitum* in 20 × 20 × 10 feeders, with an option of 10 % for leftovers. This was done daily at 08:00 and 15:00 hours for a total of 100 days.

Semen collections were carried out to evaluate the physical and microscopic semen quality. These began 15 days after starting the provision of the diets (adaptation phase) and were carried out once a week from 07:00 hours. The ejaculates were obtained by the artificial vagina method (Andrade et al., 2002) and the use of a female as a dummy. A total of 67, 67, 72, and 69 ejaculates were obtained, corresponding respectively to the groups fed 0 %, 3 %, 6 %, and 9 % flaxseed.

At the end of the seminal collection, the gel from the ejaculate was removed immediately with the help of a forceps. The ejaculate was then stored in a water bath at 37 °C. The following criteria were considered to perform the seminal physical examination: ejaculate volume without gel (mL); seminal appearance (1-2), where 1 = watery white, and 2 = milky white; spermatic turbidity (0-5), where 0 is the absence of turbidity (does not imply the absence of motility), and 5 is the maximum value given to a marked mass movement; progressive sperm motility (0-100 %), and sperm vigor (0-5), where 0 is the absence of movement with weak and inexpressive lateral tail displacement, and 5 is vigorous, fast and progressive movement of the sperm. A semen sample (20 µL) was placed on a slide previously heated to 37.5 °C and observed under a microscope with 10x to 20x objectives to assess turbidity. The previous procedure was used to observe sperm vigor and motility, and at the end, a coverslip previously heated to 37.5 °C was placed on top and observed at 40x. For the evaluation of sperm concentration, semen was diluted (20 µL) in distilled water (1 mL) and counted in a Neubauer counting chamber (improved double grid, Germany). The ejaculates with vigor 3 and with 70 % motility were subjected to the cooling process.

The ejaculates were diluted in a modified Tris-Egg yolk medium (Castellini et al., 2002): 2.42 g of Tris (C_4HNO_3), citric acid (1.34 g), D-fructose (1 g), gentamicin (1 mg/mL) in distilled water (80 mL), and egg yolk (20 mL). A first dilution was made at a ratio of 1: 1 and, subsequently, a second dilution in which the remaining diluent calculated was added up to a final concentration of 100 million spermatozoids. The semen was then placed in a BotuFLEX® box (Botucatu, Brazil) allowing its refrigerated transport. Afterward, it was stored in a refrigerator stabilized at 5 °C and refrigerated. Sperm motility and vigor were evaluated at 0, 2, 12, 24, 48, and 72 post-refrigeration hours.

Major, minor and total defects were evaluated using the wet mount technique utilizing a phase interference microscopy at 400x, where an aliquot of semen (20 µL) was preserved in distilled water to be immediately evaluated in terms of sperm morphology (major, minor and total defects). Two hundred cells were counted and classified, according to Rao et al. (1980).

A digital environmental thermometer (Supermedy®, São Paulo, SP, Brazil) and a digital thermo-hygro lux meter anemometer (THAL-300 Thermo-hygro Digital/Instrutherm®) were placed in the experimental housing to register the bioclimatic data of the environmental temperature and relative air humidity. These were positioned at the level of the back of the animals, and the indices were checked at 08:00 and at 15:00 hours throughout the experimental phase.

The experimental design used was completely randomized (CRD). Analyses of variance and regression were performed at 5 % of significance prior to the comparison test of the effects of the treatment (or time); the data showed a normal distribution.

For the variables fresh and post-refrigeration semen evaluated, the means test for treatments was carried out through the PROC GLM procedure of the SAS 9.0 program. The regression equations were generated through PROC REG. An $\alpha = 0.05$ was accepted.

The PROC MIXED COVTEST procedure of the SAS 9.0 program was used to compare the effects of treatment and time, and evaluate progressive sperm motility and post-cooling sperm vigor. The means were compared through orthogonal contrasts, and the regression equations were generated. An $\alpha = 0.05$ was accepted.

Results and discussion

Fresh semen

The flaxseed incorporated in the rabbit feed ration did not alter the seminal appearance (coloration and seminal consistency); the white coloration and the aqueous consistency predominated ($p > 0.05$) (table 2). Similar results were obtained by Andreazzi et al. (2004) with the addition of 3 % of oils (canola, corn, and soybean) in rabbit diets. According to Campos et al. (2012), the milky coloration represents normality for rabbits, as it demonstrates good seminal quality, and there are also other colorations such as citrine-yellow that indicate a lower sperm concentration.

Table 2. Appearance and turbidity of fresh semen of rabbits supplemented with flaxseed (*Linum usitatissimum*) in the diet

Parameters	Flaxseed levels (%)				CV (%)	*p*-value	Average
	0	3	6	9			
Appearance	0.215 ±0.414	0.138 ± 0.348	0.215 ± 0.414	0.169 ± 0.377	17.3484	0.725	0.18 ± 0.03
Turbidity	0.907 ±0.909	0.692 ± 0.629	0.892 ± 0.831	0.961 ± 0.830	13.8755	0.801	0.86 ± 0.11

Note: CV: coefficient of variation. abc: Averages with different letters between columns differ significantly ($p < 0.05$).

Source: Elaborated by the authors

The current investigation showed no difference ($p > 0.05$) for seminal volume with a mean value of 0.92 ± 0.22 mL for all groups (table 3). An experiment carried out by Gliozzi et al. (2009) mentions a similar conclusion. This study evaluated fish oil (source of omega 3) and vitamin E in the supplementation of rabbit diets and their effects on semen quality. However, the authors mention that they found no difference in the seminal volume of the animals.

No difference was found ($p > 0.05$) for sperm turbidity, with a mean value of 0.86 ± 0.35 (table 3). Authors as Campos et al. (2012) evaluated the seminal quality of rabbits supplemented with royal jelly (0.5 and 1.0 g). It should be noted that this jelly is a source of docosahexaenoic acid (DHA) (omega-3 fatty acid). Among the results, values of 3.33 ± 1.12 and 3.11 ± 1.17 were found, which exceed the ones obtained in this experiment.

In another experiment where hypercholesteremia and its effects on the endocrine and exocrine functions of the rabbit testicle were measured, diets with and without 3 % cholesterol were implemented for 12 weeks. This yielded values of 2.3 ± 0.4 for the treated group and 3.3 ± 0.3 for the control group (Shimamoto & Sofikitis, 1998).

For progressive sperm motility, there was no difference ($p > 0.05$) since mean values of 76.59 ± 6.94 % were found for all groups (table 3). Mourvaki et al. (2010) also found no differences in this parameter in a study with a supply of 5 % flaxseed in the diet of rabbits. The authors obtained 72.4 % for the control group and 75.5 % for the one that received flaxseed.

Values below what is usually found for progressive sperm motility in rabbits may be related to altered body temperature. According to Jimoh and Ewuola (2018), sperm motility decreases significantly when the environmental temperature rises above 27 °C, making it difficult to achieve an optimal reproductive capacity. The average temperature during the experimental phase was 27.08 °C, where the average maximum and minimum recorded were 29.6 °C and 23.9 °C, respectively. The average relative humidity was 67.87 %, and the average maximum and minimum recorded was 74.5 % and 57.7 %, respectively.

As evidenced in table 3, the mean value for sperm vigor was 3.37 ± 0.28 for the groups, allowing us to affirm that there was no significant difference ($p > 0.05$). To characterize a good semen quality, Alvarino (2000) reported that for rabbits, sperm vigor values must be equal to or higher than 3.

On the other hand, Castellini et al. (2004) showed that it is possible to improve the seminal parameters with the supply of flaxseed in the diet, promoting improvements in the progressive sperm motility of the spermatozoa. Mourvaki et al. (2010) reinforced this statement with their study on the effect of flaxseed supplementation on the level of fatty acids of the seminal components and the prostate granules. The authors concluded that the sperm tail is the region most affected by diets with the addition of flaxseed, after the acrosome. This may be due to the increased amounts of omega-3 fatty acids in the tail and the decrease in cholesterol in this same region, since higher membrane fluidity in that region increases the curvilinear velocity of the spermatozoid.

No significant differences were observed for sperm concentration ($p > 0.05$) with a mean value of 206.22 ± 44.86 × 10^6sptz/mL for the evaluated groups (table 3). Nonetheless, Alvarino (1993) reported values in the range of 150 to 900 × 10^6 sptz/mL, with a mean of 250 × 10^6 sptz/mL.

Table 3. Macroscopic and microscopic parameters of fresh semen of rabbits supplemented with flaxseed (*Linum usitatissimum*) in the diet

Parameters	Flaxseed levels (%)				CV (%)	*p*-value	Average
	0	3	6	9			
Volume (mL)	0.98 ± 0.28	0.96 ± 0.25	0.85 ± 0.20	0.89 ± 0.21	25.7	0.803	0.92 ± 0.22
Turbidity (0-5)	0.90 ± 0.50	0.69 ± 0.30	0.89 ± 0.41	0.96 ± 0.18	43.1	0.686	0.86 ± 0.35
Motility (%)	76.5 ± 9.05	74.45 ± 7.50	76.15 ± 7.41	79.23 ± 4.59	9.5	0.779	76.59 ± 6.94
							0.93 ±0.22
Vigor (0-5)	3.37 ± 0.43	3.33 ± 0.18	3.32 ± 0.28	3.46 ± 0.25	9	0.883	3.37 ± 0.28
Concentration (Spz x10^6)	205 ± 24.4	187 ± 21.6	210 ± 21.0	221 ± 16.1	22.8	0.732	206.22 ± 44.86

Note: CV: coefficient of variation. abc: Means with different letters between columns differ significantly ($p < 0.05$).

Source: Elaborated by the authors

For the sperm morphological alterations of fresh semen, no differences were observed ($p > 0.05$), since the groups showed means in the respective order of 13.00 ± 3.46 % for major defects; 9.45 ± 2.08 % for minor defects, and 22.46 ± 4.61 % for total defects (table 4). The pathology of the primary defects most observed in this experiment was proximal cytoplasmic gout, similar to what was observed by Campos et al. (2012).

The values found are within those mentioned by Foote and Carney (2000), indicating 75 % normal sperm morphology for rabbit semen. Similar conclusions were also mentioned by Andreazzi et al. (2004) using rabbit diets handling 3 % canola, corn, and soybean oil.

Table 4. Morphological aspects of sperm from fresh semen of rabbits fed with flaxseed (*Linum usitatissimum*) in the diet

Parameters	Flaxseed levels (%)				CV (%)	*p*-value	Average
	0	3	6	9			
Major defects	11.67 ± 2.52	13.60 ± 11.90	14.13 ± 2.67	12.60 ± 5.83	27.8	0.72	13.00 ± 3.46
Minor defects	10.89 ± 1.86	8.70 ± 2.11	8.78 ± 2.47	9.47 ± 1.64	21.6	0.334	9.45 ± 2.08
Total defects	22.56 ± 2.64	22.29 ± 3.93	22.91 ± 4.83	22.07 ± 7.40	22.3	0.994	22.46 ± 4.61

Note: CV: coefficient of variation. abc: Means with different letters between columns differ significantly ($p < 0.05$).

Source: Elaborated by the authors

Refrigerated semen

There was no difference in the progressive sperm motility and sperm vigor of refrigerated semen ($p > 0.05$) depending on the treatments (table 5). Several aspects can interfere with the post-dilution semen quality; one of them is the difference of rabbit semen compared to that of most species, since it has a low coefficient of permeability to water (Curry et al., 1995), hindering efficiency when using diluents for rabbit semen (Mocé & Vicente, 2009).

Table 5. Progressive sperm motility and sperm vigor in refrigerated rabbit semen per inclusion level of flaxseed (*Linum usitatissimum*) in the diet

Variables	Inclusion levels of flaxseed (%) in the diet				Linear *p*-value
	0	3	6	9	
Motility (%)	29.2 ± 3.8	25.8 ± 3.8	28.3 ± 4.0	29.9 ± 4.0	0.4614
Vigor (0-5)	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.1708

Note: CV: coefficient of variation. abc: Means with different letters between columns differ significantly ($p < 0.05$).

Source: Elaborated by the authors

A better result is obtained with diluents that are based on Tris compared to other diluent options (Cortell & De Castro, 2008). On the other hand, egg yolk gets better results compared to other substances for seminal preservation after dilution (Andrade et al., 2008). The speed of semen cooling can also interfere with semen quality and, consequently, on fertility and the number of rabbit kits born (Mocé et al., 2009).

There was a decreasing linear behavior ($p < 0.05$) for the progressive sperm motility of the refrigerated semen as a function of time with values of 63.80 ± 1.60 (0 h), 46.20 ± 2.33 (2 h), 30.40 ± 1.47 (12 h), 17.60 ± 1.47 (24 h), 11.60 ± 0.83 (48 h), and 5.18 ± 2.54 % (72 h). Likewise, there was a decreasing linear

behavior for the sperm vigor of the refrigerated semen with values of 2.20 ± 0.08 (0 h), 1.54 ± 0.11 (2 h), 1.15 ± 0.07 (12 h), 0.55 ± 0.08 (24 h), 0.22 ± 0.05 (48 h), and 0.06 ± 0.05 (72 h) (table 6).

Table 6. Progressive sperm motility and sperm vigor per cooling time of rabbits fed with flaxseed (*Linum usitatissimum*) in the ration

Variables	Cooling time (h)						*p*-value Linear
	0	2	12	24	48	72	
Motility (%)	63.80 ± 1.60^{a}	46.20 ± 2.33^{b}	30.40 ± 1.47^{c}	17.60 ± 1.47^{d}	11.60 ± 0.83^{e}	5.18 ± 2.54^{f}	0.0001
Vigor (0-5)	2.20 ± 0.08^{a}	1.54 ± 0.11^{b}	1.15 ± 0.07^{c}	0.55 ± 0.08^{d}	0.22 ± 0.05^{e}	0.06 ± 0.05^{f}	0.0001

Note: CV: coefficient of variation. abc: means with different letters between columns differ significantly ($p < 0.05$).

Source: Elaborated by the authors

The values differ from those mentioned by Andrade et al. (2008), who contrasted several diluting means in rabbit semen: lactate ringer solution, citrate-egg yolk, and skim milk. In their study, they found progressive sperm motility values of 64.5 ± 8.7 (lactate ringer), 67.0 ± 5.3 (citrate-egg yolk), and 65.9 ± 5.3 % (skim milk) at the beginning of incubation (0 min). At 120 minutes of incubation, they found values of 50.3 ± 16.9 (lactate ringer), 57.5 ± 14.0 (citrate-egg yolk), and 55.8 ± 8.0 % (skim milk). However, there are differences in the abilities of the media to maintain the viability of the semen for prolonged periods (Seed et al., 1996). These differences can be visualized in fertility and in the number of rabbit kits born, according to López and Alvarino (1998). The same authors evaluated semen refrigerated with commercial diluent at 2, 24, 48, 72, and 96 hours of refrigeration, and found a significant decrease in fertility with 72 and 96 hours of refrigeration. There are also reports of fertility with a rate higher than 60 % in inseminations with a sperm concentration of 6×10^{6} per dose, but depending on the breeder, the breed, and the degree of selection (International Rabbit Reproduction Group, 2005). The recommended dose is 20×10^{6} million sperm per doe (Rebollar, 2000).

There was no difference for major defects with a mean of 12.01 ± 3.74 % between the groups. For minor defects, the mean was 20.11 ± 5.56 %, and for total defects, the mean was 32.39 ± 5.76 % in semen after 72 hours of refrigeration (table 7).

Table 7. Morphological aspect of the 72-hour-post-refrigeration semen of rabbits fed with flaxseed (*Linum usitatissimum*) in the diet

Parameters	Flaxseed levels (%)				CV (%)	*p*-value	Average
	0	3	6	9			
Major defects (%)	13.34 ± 1.57	11.92 ± 1.17	11.96 ± 1.92	10.82 ± 2.24	32.9	0.797	12.01 ± 3.74
Minor defects (%)	20.24 ± 2.77	18.30 ± 2.60	19.76 ± 1.87	22.14 ± 3.10	29.1	0.779	20.11 ± 5.56
Total defects (%)	33.58 ± 2.50	30.22 ± 2.87	31.72 ± 2.58	34.04 ± 2.85	18.6	0.738	32.39 ± 5.76

Note: CV: coefficient of variation. abc: Means with different letters between columns differ significantly ($p < 0.05$).

Source: Elaborated by the authors

The parameters that show values higher than those of the consulted literature are those of sperm morphology of fresh semen and semen after 72 hours of post-refrigeration, according to Foote and Carney (2000). A high percentage of sperm abnormalities in a collection may not reflect a decrease in fertility, but it may interfere with prolificacy values (Lavara et al., 2005).

Conclusions

The inclusion of up to 9 % flaxseed in the rabbit diet did not alter the physical and microscopic parameters of fresh semen. However, it altered the progressive sperm motility, and the vigor of the semen evaluated post-cooling, which indicates that there is still a challenge in the search for lipid sources in the diets to optimize the seminal quality in rabbits.

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Disclaimers

The authors made significant contributions to this document, agree with its publication, and state that there are no conflicts of interest in this study.

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