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Inclusion of soybean oil in the diet on cryopreservation of ram semen

Inclusión de aceite de soya en la dieta sobre la criopreservación del semen ovino

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ABSTRACT

The objetive was to evaluate the effect of soybean oil (SO) supplemented diet on the cryopreservation of Pelibuey ram semen. Twenty-seven rams were divided three treatments (n= 9): T1: 0% SO (control); T2: 3% SO and T3: 6% SO, for a period of 60 days. The frozen-thawed semen was assessed for sperm motility parameters, plasma membrane integrity, mitochondrial activity, acrosome integrity and tail membrane integrity (Host). The results were analyzed by ANOVA, followed by Tukey's comparison test. T1 and T3 were similar and superior (P<0.05) to T2 in total motility and progressive motility. Mean velocity and acrosome integrity in T1 were higher (P<0.05) compared to T2 and T3. Viability in T1 was similar to that of T2 and T3, however, T2 and T3 were different (P<0.01). Mitochondrial activity in T3 was higher, compared to T1 and T2 (P<0.05). No differences (P>0.05) were found in the other motility parameters and in host. The inclusion of soybean oil in the diet did not improve the cryopreservation of ram semen, although the inclusion of 6% soybean oil in the diet increased mitochondrial activity.

Keywords: fatty acid, spermatozoa, frozen-thawed.

RESUMEN

El objetivo fue evaluar el efecto del aceite de soya (AS) en la dieta, sobre la criopreservación del semen ovino. Durante 60 días, 27 ovinos permanecieron divididos en tres tratamientos (n=9): T1: 0% AS (control); T2: 3% AS y T3: 6% AS. Se analizaron los parámetros de motilidad, viabilidad, actividad mitocondrial, integridad del acrosoma y de la membrana de la cola (Host) a la descongelación. Los datos se analizaron con un ANOVA y una prueba de Tukey para la comparación de medias. El T1 y T3 fueron similares y superiores (P<0.05) al T2 tanto en la motilidad total como en la motilidad progresiva. La velocidad media y la integridad de los acrosomas del T1 resultó mayor (P<0.05) respecto al T2 y T3. La viabilidad del T1 fue similar al T2 y T3, aunque el T2 y T3 fueron diferentes (P<0.01). Por otra parte, la actividad mitocondrial del T3 fue mayor respecto al T1 y T2 (P<0.05). No se encontraron diferencias (P>0.05) en los demás parámetros de motilidad y en Host. La inclusión de aceite de soya en la dieta no mejora la criopreservación del semen ovino a pesar de que la adición de un 6% favorece la actividad mitocondrial.

Palabras clave: ácidos grasos, espermatozoides, congelado-descongelado.

INTRODUCTION

Nutrition and reproduction are generally linked, because the reproductive success of the animal depends on its nutritional status. The effect of this association has been studied over the years, often by altering diets in various ways in order to observe the resulting changes in the reproductive parameters of animals (Yeste *et al*., 2011). One of the most significant changes is the addition of polyunsaturated fatty acids (often referred to by their acronym PUFAs (Polyunsaturated Fatty Acids) to their diet. Different sources of PUFAs have been studied in the diet of mammals, such as omega-3 and omega-6, and it has been observed that their consumption influences some reproductive functions of the female, such as an increase in the diameter and number of follicles present in the ovary (Nurlatifah *et al*., 2020); as well as a shorter period for the first ovulation (Salehi *et al.,* 2016) and a positive effect on fertility (Rebollar *et al*., 2014). In males, it produces increased libido (Castellano *et al.,* 2010), testicular size (Van Tran *et al*., 2017; Mínguez-Alarcón *et al.,* 2017), sperm concentration (Alizadhe *et al.,* 2014; Fair *et al.,* 2014; Rodrigues *et al.,* 2017), motility (Alizadeh *et al.,* 2014; Liu *et al.,* 2015; Rodrigues *et al.,* 2017) and sperm viability (Alizadhe *et al.,* 2014; Esmaeili *et al.,* 2012[b]). Likewise, in the composition and structure of sperm mitochondria membranes (Gürler *et al*., 2015), which are responsible for producing the energy needed to produce flagellar movement, increasing their fluidity and plasticity and thus their function (Sullivan *et al*., 2018).

In most mammalian tissues, the most predominant fatty acid is linoleic acid (18:2 n-6), where its concentration reflects dietary intake (Guyenet & Carlson, 2015). Linoleic acid is obtained through dietary vegetable oils, such as sunflower, soybean, corn, and canola oils; as well as nuts and seeds (Sullivan *et al*., 2018). Soybean oil, is composed of several substances, mainly polyunsaturated fatty acids, particularly linoleic acid (LA, C18: 2), an omega-6 (ω6) fatty acid that constitutes ~ 55% of soybean oil (Deol *et al*., 2017) and also linolenic acid (ALA, C18: 3), an omega-3 (ω3) fatty acid that constitutes ~ 5-9% (Sullivan *et al.,* 2018).

On the other hand, the most abundant PUFAs in the membranes of most mammalian spermatozoa is omega-3, which has an important role in sperm cell functionality by providing fluidity and permeability; which are related to the ability to carry out fertilization (Esmaeili *et al.,* 2012[b]), and to an increase in cryoresistance (Towhidi *et al*., 2012; Fair *et al.,* 2014).

Ram sperm have been described to be more susceptible to cold shock during cryopreservation than other mammals (Grötter *et al*., 2019). These differences are attributed, in part, to lipid composition (Mandal *et al*., 2014; Chunrong *et al*., 2019) and the content of PUFAs present in sperm membranes (Mandal *et al.,* 2014). Also, mammals are unable to synthesize de *novo* fatty acids with double bonds at the n-6 (linoleic series) and n-3 (linolenic series) positions because they lack desaturase enzymes (Byrne *et al*., 2017), so PUFAs must be included in the diet.

The objective of the present study was to evaluate the effect of soybean oil rich in n-6 and n-3 PUFAs added in the diet in amounts of 3 and 6%, on the cryopreservation of Pelibuey ram semen.

MATERIAL AND METHODS

Location

The study was conducted during the months of September to November 2018 at the Pelibuey and Blackbelly Ram Germplasm Bank of the National Institute of Forestry, Agricultural and Livestock Research (INIFAP) in Mocochá municipality, Yucatán, Mexico, located in the north-central region of the state, between parallels 21º 05' and 21º 10' North Latitude and meridians 89º 27' and 89º 30' West Longitude, at an altitude of 9 m above sea level. The predominant climate in the region is warm sub-humid (Aw0) with rainfall in summer, with a pluvial precipitation of 997 to 1132 mm, and an average annual temperature of 26.5 °C.

Animals

Twenty-seven male ram of the Pelibuey breed were used, with an average age of 2.0 ± 0.5 years, average live weight of 42.5 ± 2.9 kg and body condition of 3.5 on a scale of 1 to 5 points. Likewise, it was verified that the animals did not present any physical anomaly that could exclude them from the study.

Feeding

Rams were randomly distributed in three groups (n=9 per group), supplying each group with different percentages of soybean oil (SO) in the diet: Control treatment (T1): (0% SO); Treatment 2 (T2): 3% SO and Treatment 3 (T3): 6% SO, supplemented with 30% of commercial feed (NUTRIMYN Borrego 14% PC, MYN Distribuidora de S. A de C.V.) and 70% CT-115 grass (*Pennisetum purpureum*) (Table 1). Diets were provided free access at a rate of 450 $g^{-1}/a^{-1}/d^{-1}$ in the morning (11:30h), for 60 days (spermatogenesis in ram: 58 days; Díaz *et al*. 2017), including an adaptation period of 14 days, where soybean oil (Bakers & Chefs®, Hermosillo, Mexico) was gradually supplied in the diet until reaching the established in each treatment.

Table 1. Chemical composition of experimental diets

Components	T1	T2	T3
Protein (%)	14.00	14.00	14.00
Energy (Mcal ME/kg DM.) *	2.71	2.79	2.64
Calcium (g).	6.3	6.3	6.3
Phosphorus (g).	3.2	3.2	3.2
Soy oil 3 % (g).	0	300	0
Soy oil 6 % (g).	0	0	550

*Estimated based on NRC (2007)

Obtaining seminal samples

Using an artificial vagina and with the help of a ewe that served as a dummy, 162 ejaculates were selected (6 ejaculates/ram-1; 54/group-1) that met the following criteria: volume >0.5, mass motility >4 (scale 0-5), motility >70% and sperm concentration >3,000 x 106 sperm/mL (Arando *et al.,* 2019, 2020). Semen was collected at a frequency of twice per week (one ejaculate/day/stallion) in the morning hours (8:00), and for 3 weeks following the supplementation period.

Sperm dilution

The selected ejaculates were diluted with Triladyl® + double distilled water + 20% egg yolk to a final concentration of 400 x 10^6 spermatozoa/mL, and then packed in 0.25 mL French straws (Minitüb®, Tiefenbach, Germany).

Freezing of semen

Freezing of the samples was performed by placing the straws 4 cm above the surface of liquid nitrogen (LN2), for 10 minutes; immediately afterwards the straws were immersed in LN2 and stored until evaluation.

Thawing of semen

The thawing procedure was performed by immersing the straws in a water bath at 37°C for 30 seconds.

Sperm concentration

5 µL of the semen sample was diluted in 995 µL of distilled water. Subsequently, 9 µL of the diluted sample was placed on each of the two sides of the Bücker chamber and 4 fields from each side were captured with the AI Station computerized sperm analysis system (SPERM.TECH®, Valencia, Spain).

Sperm motility

Motility was analyzed with the AI Station system by placing 5 µL of thawed semen diluted to ~30 x10^6/mL spermatozoa on a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel) preheated to 37°C; and at least five fields with a minimum of 300 spermatozoa/sample were captured. The motility parameters analyzed were: Total motility (TM, %), Progressive motility (PM, %), Curvilinear velocity (VCL, µm/s), Rectilinear velocity (VSL, µm/s), Average velocity (VAP, µm/s), Linearity index (LIN= VSL/VCL x 100), Straightness index (STR= VSL/VAP x 100), Oscillation index (WOB= VAP/VCL x 100), Amplitude of lateral head displacement (ALH, µm) and Tail beat frequency (BCF, Hz).

Sperm viability

It was assessed by SYBR-14 and propidium iodide (PI) staining (Live/Dead® kit L-7011, Invitrogen™). Samples were stained with 1 µL of SYBR-14 (10 µM) and 1 µL of PI (12 µM) and allowed to incubate for 10 minutes at 37°C in the dark. Subsequently, 5 µL of the sample was placed on a pre-warmed slide at 37°C and evaluated using an epifluorescence microscope (LWScientific i40-DNA). The percentage of viable cells was determined by counting 200 spermatozoa per sample. Cells stained with SYBR 14 (Green fluorescence) were considered viable; whereas cells stained with PI (Red fluorescence) were considered dead.

Acrosome integrity

It was assessed by FITC-PSA staining (100µg/mL, L-0770, Sigma-Aldrich™). Samples were stained with 5 µL of FITC-PSA and allowed to incubate for 30 minutes at 37°C in the dark; 5 µL of the sample was immediately placed on a slide and evaluated. The percentage of cells with intact acrosome was determined by counting 200 spermatozoa per sample. Spermatozoa stained with FITC-PSA (green fluorescence) were considered with damaged acrosome; while those without fluorescence were considered intact.

Mitochondrial activity

It was analyzed with JC-1 staining (153 µM, Molecular Probes® T-3168, InvitrogenTM). Samples were stained with 1 µL of JC-1 and allowed to incubate for 10 minutes at 37°C in the dark; 5 µL of the sample was then placed on a slide and analyzed.

The percentage of spermatozoa with mitochondrial activity was determined by counting 200 cells per sample. Spermatozoa with mitochondrial activity were considered to be those that showed orange fluorescence in the middle part of the flagellum; while those that did not show fluorescence were considered to have no mitochondrial activity.

Tail plasma membrane integrity (HOST).

It was evaluated with a dilution of 5 µL of sperm sample in 50 µL of endosmosis solution (0.735 g of sodium citrate dihydrate and 1.351 g of fructose in 100 mL of distilled water) at 100 mOsm/L and allowed to incubate for 1 hour at 37 °C. Subsequently, 5 µL of the sample was placed on a slide and analyzed with a phase contrast microscope. The percentage of spermatozoa with HOST was determined by counting 200 cells per sample. Spermatozoa with coiled tails were considered to have intact tail membrane (positive endosmosis); while those without coiled tails were considered to have damaged tail membrane (negative endosmosis).

Statistical analysis

Variables expressed as percentages: total motility, progressive motility, viability, mitochondrial activity, intact acrosomes and Host, were transformed to arcsine√(variable)/100 before analysis. Subsequently, they were analyzed with an ANOVA; and to find statistical differences between treatments, Tukey's test at $P \leq 0.05$ was used through the Statistical Analysis System statistical package (SAS Inst. Inc., 2011).

RESULTS AND DISCUSSION

Numerous studies have shown that the inclusion of PUFAs in the diet can improve sperm cell functionality (Sullivan *et al*., 2018), as well as their cryoresistance (Towhidi *et al*., 2012; Fair *et al.,* 2014), by influencing the composition and structure of their membranes. On the other hand, other studies mention that diets conformed with PUFAs produce an increase in the incorporation of DHA in the plasma membrane lipids of spermatozoa (Gholami *et al.,* 2010), causing a decrease in cholesterol content and consequently a greater susceptibility of spermatozoa to cold shock (Díaz *et al*., 2017). In this sense, the values obtained from treatments T1 and T3 were similar and superior ($P<0.05$) to T2, both in total motility (57.8±2.3;59.6±2.6% vs 46.8±3.3%) and progressive motility (27.0±0.3;30.2±1.2% vs 14.0±2.1%), respectively. These results are similar to those recorded by Losano *et al.* (2017) in thawed semen from bulls that were previously fed with 6% palm oil for 60 days; as well as those reported by Díaz *et al*. (2017) in thawed sperm samples from Araucano breed ram previously supplemented with 3% fish oil, where total motility decreased and the other motility parameters did not present differences with respect to the control treatment.

For their part, Khoshvaght *et al.* (2016), observed higher progressive motility in thawed semen from Holstein bulls previously supplemented with 3.5% fish oil for 11 weeks, being similar to what was reported in thawed semen samples from Holstein breed bulls supplemented with 50 g of conjugated linoleic acid (Lutrell®) for 10 weeks (Karimi *et al.*, 2016). In mean velocity (VAP), T1 (71.4±4.7µm/s) resulted higher (P<0.05) with respect to T2 and T3 (53.8±3.0 and 50.3±6.8µm/s, respectively); while for the rest of the other motility parameters analyzed (LIN, STR, WOB, ALH and BCF) no differences were found (P>0. 05) between treatments (Table 2); this being similar to what was found by Byrne *et al*. (2017) in thawed sperm samples from pubescent Holstein bulls, fed with safflower oil; where motility parameters were not affected, but the amplitude of head displacement (ALH), which was higher with respect to the control, was.

It is known that oils of animal origin, such as fish oil, contain higher concentrations of n-3 PUFAs (Omega-3) than those of vegetable origin (Omega-6); it has even been shown that the supplementation of n-3 PUFAs in the diet modifies the fatty acid composition of the sperm membrane, improving semen quality. These effects have been reported in ram (Samadian *et al*., 2010; Díaz *et al*., 2017), horse (Brinsko *et al.,* 2005), pig (Rooke *et al.,* 2001), bull (Khoshvaght *et al.*, 2016; Byrne *et al.,* 2017), dog (Alonge *et al*., 2019) and human (Martínez-Soto *et al*., 2012) spermatozoa.

Table 2. Effect of dietary soybean oil supplementation on the motility of thawed ram semen

Motility parameters	Treatment (soy oil %)		
	T1 (0 %)	T2 (3 %)	T3 (6 %)
TM (%)	57.8 ± 2.3[a]	46.8 ± 3.3[b]	59.6 ± 2.6[a]
PM (%)	27.0 ± 0.3[a]	14.0 ± 2.1[b]	30.2 ± 1.2[a]
VAP (µm/s)	71.4 ± 4.7[a]	53.8 ± 3.09[b]	50.3 ± 6.8[b]
VCL (µm/s)	113.3 ± 6.0[a]	91.7 ± 3.50[a]	82.1 ± 4.7[a]
VSL (µm/s)	53.9 ± 1.9[a]	36.8 ± 2.76[a]	36.5 ± 3.2[a]
LIN (%)	44.4 ± 0.5[a]	36.1 ± 1.94[a]	41.3 ± 1.7[a]
STR (%)	69.0 ± 0.8[a]	59.0 ± 2.80[a]	65.0 ± 2.9[a]
WOB (%)	61.9 ± 2.5[a]	57.0 ± 1.55[a]	59.6 ± 1.4[a]
ALH (µm)	3.6 ± 0.2[a]	3.2 ± 0.10[a]	2.9 ± 0.1[a]
BCF (Hz)	11.1 ± 1.6[a]	8.8 ± 3.51[a]	8.7 ± 6.7[a]

([ab]) Different superscripts on the same line indicate significant differences (p <0.05). TM, total motility; PM, progressive motility; VAP, average velocity; VCL, curvilinear velocity; VSL, rectilinear velocity; LIN, linearity index (LIN= VSL/VCL x 100); STR, straightness index (STR= VSL/VAP x 100); WOB, oscillation index (WOB= VAP/VCL x 100); ALH, amplitude of lateral head displacement; BCF, tail beat frequency

Regarding acrosome integrity, T1 (66.6±7.6%) resulted higher (P<0.05), with respect to T2 and T3 (41.3±3.8 and 29.2±5.2%, respectively). This is similar to that reported by Losano *et al.* (2017) in thawed sperm samples from bulls that were previously fed 6% fatty acids for 60 days; however, it differs to that reported by Byrne *et al.* (2017) in thawed semen from pubescent Holstein-Fresian bulls fed safflower and fish oil for 10 days. Contrary to that described by Díaz *et al*. (2017) in thawed sperm samples from Araucano breed ram, which were fed fish oil for 60 days. This result was probably due to the increased oxidative stress caused by the freezing and thawing processes of the samples, which was exacerbated by the effect of PUFAs on the increased susceptibility of the sperm membrane to lipid peroxidation.

The viability of T1 (43.3±6.5%) was similar to T2 (36.6±3.1%) and T3 (53.0±4.6%); although T2 and T3 were different (P<0.01) (Table 3). These results were similar to those obtained by Moallem *et al.* (2015), Losano *et al.* (2017) and Byrne *et al.* (2017) in thawed sperm samples from bulls fed fatty acids and omega-3 for 13 weeks; as well as in bulls fed fatty acids (Megalac®) for 60 days and in thawed semen samples from pubertal Holstein-Fresian bulls that were fed safflower and fish oil for 12 days relative to the control treatment, respectively. However, these results differ to that reported by Díaz *et al*. (2017), where the viability of thawed ovine sperm samples was higher in the control treatment with respect to the dietary supplementation treatment with fish oil for 60 days. On the other hand, Khoshvaght *et al.* (2016) reported higher sperm viability in thawed semen samples from Holstein bulls, which were previously supplemented with fish oil for 11 weeks.

Different studies indicate that increasing the concentration of bovine sperm long-chain n-3 PUFAs does not produce appreciable improvements in plasma membrane fluidity compared to a basal diet (Moallem *et al.* 2015); Byrne *et al.* (2017), which could have happened with n-6 PUFAs from soybean oil in the hair ram spermatozoa of this study.

Mitochondrial activity at T3 (65.3±6.5%) was higher, with respect to T1 and T2 (37.3±7.3 and 33.6±3.5), respectively (P<0.05). These results differ to that reported by Losano *et al.* (2017) where mitochondrial activity did not present differences between treatments of sperm samples thawed from bulls that were previously fed 6% fatty acids for 60 days. It has been shown that the inclusion of n-6 PUFAs in the diet can produce an integration of phospholipids to the plasma membrane, replacing saturated fatty acids that are more linear and rigid, generating an increase in the fluidity and plasticity of membranes, facilitating the interaction with membrane enzymatic proteins and, therefore their function (Sullivan *et al*., 2018). Likewise, reduction in the amount of these phospholipids, which make up 10% of mitochondrial phospholipids, alters membrane potential (Jiang *et al*., 2000). In most mammalian tissues, the most predominant fatty acid is linoleic acid (18:2 n-6) (Guyenet & Carlson, 2015), which is obtained through dietary vegetable oils, such

as sunflower, soybean, corn and canola oils; as well as nuts and seeds. This acid is necessary for the correct functioning of several complexes of the mitochondrial respiratory chain, so it is related to such essential processes as ATP generation (Mileykovskaya *et al*., 2005), proton conduction through cytochrome bc1 (Lange *et al*., 2001); as well as in the prevention of osmotic instability and uncoupling of the chain, when the respiration rate is high (Koshkin & Greenberg, 2002).

In tail plasma membrane integrity (host test), no significant differences were found (P>0.05); which is in agreement to that reported in thawed semen from Holstein bulls fed 50 g linoleic acid (Karimi *et al.*, 2016). However, Gholami *et al.* (2010) mention that n-3 PUFA increase the incorporation of DHA in the head and tail head piece of spermatozoa, which influences the biochemical composition of the sperm membrane, giving it better plasticity and stability.

Table 3. Effect of dietary soybean oil supplementation on motility, mitochondrial activity, intact acrosomes and Host of thawed ovine semen

Treatment	Soy oil (%)	Viability (%)	Mitochondrial activity (%)	Intact acrosomes (%)	Host (%)
T1	0	$43.3 \pm 9.5^{ab**}$	$37.3 \pm 7.3^{b*}$	$66.6 \pm 7.6^{a*}$	29.3 ± 1.6^{a}
T2	3	36.6 ± 3.1^{b}	33.6 ± 3.5^{b}	41.3 ± 3.8^{b}	21.5 ± 2.1^{a}
T3	6	53.0 ± 4.6^{a}	65.3 ± 6.5^{a}	29.2 ± 5.2^{b}	25.0 ± 2.5^{a}

$^{(ab)}$ Different superscripts in the same column indicate significant differences ($p<0.05$*) ($p<0.01$**)

Finally, these contradictory results obtained in this study with that of other authors may be associated with many factors, such as: the species and animal breed, supplementation time, reproductive status of the animal, amount of PUFAs present in the sperm membrane, concentration and type of PUFAs added to the diet, among others (Esmaeili *et al*., 2012[a]; Liu *et al.,* 2015; Martínez-Soto *et al.,* 2012).

In rams, dietary n-3 PUFAs cause modifications in sperm fatty acid profiles, playing an important role on semen quality and its tolerance to cold shock, this is due to the incorporation of DHA into cell membrane phospholipids (Esmaeili *et al.,* 2012[b]), since it has been shown that there is a relationship of the amounts of DHA in the sperm membrane with their cryoresistance(Martínez-Soto *et al*., 2012). On the other hand, it has been documented that dietary n-3 PUFAS promote sperm membrane susceptibility to lipid peroxidation, affecting sperm fertilizing capacity. Despite this, it has been reported that a diet enriched with vitamin E, zinc, selenium, folic acid and n-3 PUFA for at least two months, in adequate amounts, improves sperm quantity and quality, especially sperm count and motility, modifying the physical and functional properties of the sperm cell membrane (Alonge *et al*., 2019). In this sense, a future study with supplementation based

on PUFAs n-6 fatty acids and their association with antioxidants would be desirable to prevent possible damage caused by the effect of lipid peroxidation.

CONCLUSION

The inclusion of soybean oil in the diet based on commercial feed and CT-115 grass does not improve the cryopreservation of Pelibuey ram semen even though the addition of 6% enhances mitochondrial activity.

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Errata Erratum

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