EFFECT OF SHEEP DIETS WITH MACADAMIA BY-PRODUCT AND PROTECTED FAT ON THE QUALITY OF FRESH AND FROZEN SEMEN[1]

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ABSTRACT**:** The ruminant diet is characterized by low lipid concentration, resulting from traditional diets composed by forage species. The use of agro industrial byproducts in animal feed may be interesting, once it reduces production costs and reduces environmental contamination. Among them, macadamia is known for interesting protein and carbohydrate contents; however, it is the amount of lipids that make it different. Fat supplementation can raise concentrations of blood cholesterol, a precursor metabolite of steroid hormones, which constitute biological membranes and possess specific and essential biological activities. The semen characteristics should be taken into account in the selection of the breeding herds, and the semen analysis makes it possible to evaluate the fertility of the sheep and allows obtaining important conclusions based on its results. The objective was to evaluate the seminal quality of Morada Nova sheep breed consuming diets supplemented with macadamia residue and protected fat. The experiment was carried out with 24 rams aged 18 or 30 months, distributed in four treatment groups: control (C), 50 g (MAC50) or 150 g (MAC150) of macadamia industrial byproduct; and 50 g of protected fat (Megalac®), added to the concentrate. Semen was collected at four intervals: before supplementation (day 0), 30, 60 and 75 days after the beginning of supplementation, and it was taken the measurements of volume, appearance, motility, vigour, turbulence, concentration and morphology. At days 60 and 75, semen was frozen for determination of plasma membrane integrity, acrosome integrity and mitochondrial activity after thawing. Analysis of variance was performed and the means were compared by the SNK test. In the analysis of fresh semen, a significant effect ($p<0.05$) of the treatments on motility was observed. For cryopreserved semen, there was no significate difference ($p>0.05$). The inclusion of 50 or 150 g of macadamia residue or 50 g of Megalac in the diet does not alter the quantitative and qualitative aspects of fresh and post-thawed semen.

Keywords**:** fatty acids, *Macadamia* spp., sheep, calcium salts, seminal biotechnology.

EFEITO DE DIETAS DE OVINOS COM COPRODUTO DE MACADÂMIA E GORDURA PROTEGIDA NA QUALIDADE DE SÊMEN FRESCO E CONGELADO

RESUMO**:** A dieta dos ruminantes é caracterizada por baixa concentração de lipídeos, resultante de dietas tradicionais compostas por espécies forrageiras. A utilização de coprodutos agroindustriais na alimentação animal pode ser interessante, pois além de reduzir custos na produção, reduz a contaminação ambiental. Dentre eles a macadâmia é conhecida por teores interessantes de proteína e de carboidratos; entretanto é a quantidade de lipídeos que a torna diferenciada. A suplementação de gordura pode elevar as concentrações de colesterol sanguíneo, metabólito precursor dos hormônios esteroides, que constituem membranas biológicas e possuem atividades biológicas específicas e essenciais. As características seminais devem ser levadas em consideração na seleção dos reprodutores, sendo que a análise do sêmen possibilita avaliar a fertilidade do carneiro e permite obter importantes conclusões a partir dos seus resultados. O objetivo foi avaliar a qualidade seminal de carneiros Morada Nova consumindo dietas suplementadas com resíduo de macadâmia e gordura protegida. O experimento foi conduzido com 24 carneiros, com idade entre 18 e 30 meses, distribuídos em quatro grupos de tratamento: controle (C), 50 g (MAC50) ou 150 g (MAC150) de subproduto industrial da macadâmia; e 50 g de gordura protegida (Megalac®), adicionados ao concentrado. O sêmen foi coletado em quatro intervalos: antes da suplementação (dia 0), 30, 60 e 75 dias após o início da suplementação. O sêmen foi coletado para avaliação do volume, aparência, motilidade, vigor, turbilhonamento, concentração e morfologia. Nos dias 60 e 75, o sêmen foi congelado para determinação da integridade da membrana plasmática, integridade do acrossoma e atividade mitocondrial após o descongelamento. A análise de variância foi realizada e as médias comparadas pelo teste SNK. Na análise do sêmen fresco, foi observado efeito significativo ($p<0,05$) dos tratamentos na motilidade. Para o sêmen criopreservado, não houve diferença ($p>0,05$). A inclusão de 50 ou 150 g de resíduo de macadâmia ou 50 g de Megalac na dieta não altera os aspectos quantitativos e qualitativos do sêmen fresco e pós-descongelado.

Palavras-chaves: ácidos graxos, *Macadamia* spp., ovinos, sais de cálcio, biotecnologia do sêmen.

INTRODUCTION

Several factors influence semen production and quality, nutrition being one of the most relevant. The energy level in the diet is one of the most studied components in terms of the influence in nutrition and on reproduction (COSTA et al., 2011). In general, the diet of ruminants is characterised by low lipid concentrations.

The incorporation of lipids, such as polyunsaturated fatty acids, in ruminant diets, besides being a nutritional strategy that allows increasing the energy density of the diet, also provides fundamental components for the physical and functional structure of the cells. In addition to increase blood concentrations of cholesterol (BIANCHI et al., 2014), a precursor metabolite of hormones (ARTUNDUAGA et al., 2010) and steroids, cholesterol constitutes biological membranes and has other specific and essential biological activities.

Polyunsaturated fatty acids are also precursors of prostaglandins and leukotrienes, which are important for sperm motility and the inflammatory process. The higher concentration of polyunsaturated fatty acids in the spermatic head and tail membrane is also involved in the sperm capacitation and interaction between the spermatozoa and the uterine surface (GHOLAMI et al., 2010) and oocytes (WONNACOTT et al., 2010).

The main function of spermatozoa is fertilisation, involving a series of processes associated with multiple cellular attributes. Motility, mitochondrial activity, plasma membrane integrity and an intact acrosome must be present (VAN TRAN et al., 2017), as well as patterns of whirling, vigour, osmotic balance and membrane peptide integrity, all of which influence the spermatozoid quality for fertilisation.

The process of semen cryopreservation is one of the factors responsible for decreasing fertility, caused by changes in sperm membrane quality and cellular structures (CÂMARA et al., 2011).

The lipid composition of the sperm plasma membrane, a highly dynamic structure that regulates extracellular changes and facilitates fertilisation, is the main determinant of motility, sensitivity to low temperatures and viability of sperm cells. Spermatozoa of various animal species has a higher ratio of polyunsaturated fatty acids in the phospholipid layer (MOTLAGH et al., 2014). The presence of polyunsaturated fatty acids may increase plasma membrane fluidity, providing greater resistance to ice crystal formation during cryopreservation (SELVARAJU et al., 2012). To contribute to the fluidity and flexibility of sperm membranes, polyunsaturated fatty acids participate in the acrosome reaction and are also involved in the anchoring of membrane receptors (GHOLAMI et al., 2010). They also increase the tolerability of the lipid bilayer to the stress caused by constant flagella movement (GHOLAMI et al. 2010).

The use of by-products and/or co-products of agro-industries in the diets of small ruminants can provide benefits such as provision of fatty acids, in many cases leading to greater food availability, better productive and reproductive results and lower costs, thereby increases production efficiency and minimizing the problem of waste disposal by agricultural industries (GERON et al., 2012).

Reports of the use of macadamia (*Macadamia integrifolia*) residues in the diet of animals are incipient in the literature. Although animal studies suggest toxicity, promising results in animal husbandry have been obtained from its introduction in the diets of broilers (ACHEAMPONG-BOATENG et al., 2016) and cattle (ACHEAMPONG-BOATENG et al., 2017). However, studies of the impacts of macadamia residue on seminal quality are lacking.

Due to the activity of ruminal microorganisms (bio hydrogenation) on fatty acids from high-fat supplementation, there may be a negative effect on sperm motility, viability and acrosome activity in sheep (VAN TRAN et al., 2017).

To reduce bio hydrogenation, one of the strategies is the use of protected fat (inert or by-pass), characterised by its minimal inhibitory effect on the metabolism of ruminal microorganisms, without affecting intestinal digestibility, which ensures the stability of the composition until it is absorbed by the intestine (ESMAEILI et al., 2012).

In view of the above, the objective of this study was to evaluate the influence of dietary supplementation with macadamia and protected fat (Megalac®) on weight, cholesterol, aspartate aminotransferase and

the quantitative and qualitative aspects of fresh and frozen semen of Morada Nova rams.

MATERIAL AND METHODS

Location and animals

The experiment was registered with the Ethics Committee on Animal Experimentation (CEUA/IZ – no. 187/13).

The experiment was carried out at Instituto de Zootecnia, Nova Odessa located at 22°42'S and 47°18'W, in the State of Sao Paulo, Brazil. The climate is defined as Cwa (Köppen), with dry winters and hot and rainy summers. Average annual rainfall in the municipality is about 1,270 mm.

Twenty-four Morada Nova rams, with a mean initial weight of 42.06 ± 5.87 kg, were housed in individual stalls. The animals were aged 18 (n=12) or 30 (n=12) months, with testicular symmetry and unchanged scrotum, normal prepuce and penis, good libido. All animals were clinically healthy and had been previously conditioned to mounts.

Treatments

The animals were divided by age into one of the following four treatments (Table 1): 1) control, receiving 450 g of concentrate; 2) MAC 50, receiving 500 g of concentrate containing 50 g of macadamia industrial by-product; 3) MAC 150, receiving 620 g of concentrate containing 150 g of macadamia industrial by-product; 4) ME, receiving 500 g of concentrate containing 50 g of Megalac®. The animals received corn silage, mineral salt for sheep and water *ad libitum*. The macadamia industrial by-product was obtained from a macadamia processing company and had no commercial value.

Bromatological analyses

Weekly sample collections of the concentrates were carried out for conventional chemical-bromatological analyses in the Bromatology Laboratory of the Instituto de Zootecnia. The samples were analysed in terms of chemical-bromatological composition (dry matter - DM, ash, neutral detergent fibre - NDF, acid detergent fibre - ADF, lignin, crude protein - CP, ethereal extract - EE), as defined by Goering et al. (1970) and Van Soest et al. (1991) (Table 1).

Blood collection and biochemical analyses

Blood samples were collected biweekly by venepuncture of the jugular vein and stored in 5 mL vacutainer tubes (Vacuette, Americana, Brazil). With the aid of a centrifuge, the serum was separated, transferred into 1.5 mL microtubes via a variable volume pipette and stored in a freezer until biochemical determination of cholesterol (Liquiform Cholesterol Kit, Labtest, Lagoa Santa, Brazil) and aspartate aminotransferase (AST kit, Labtest), using the Bioplus L200 semi-automatic equipment (Bioplus, Barueri, Brazil).

Animal weight and cholesterol were determined at the following times: day 0 (D0), at the beginning of the experiment, day 30 (D30), day 60 (D60) and day 75 (D75), considered the final day of the experiment, while AST was determined at the beginning (D0) and at the end of the experiment (D75).

Semen collection and evaluation

Semen was collected via an artificial vagina (MIES FILHO, 1987) with the help of a sheep in natural heat, as a dummy, placed in a containment trunk. The samples were collected on days 0, 30, 60 and 75.

The collected semen was placed in a water bath at 37°C to preserve the spermatozoa during analysis. Volume, colour, turbulence ability, motility (0-100%) and vigour (0-5), concentration (total and per mL) and sperm morphology were evaluated according to the Manual of the Brazilian College of Animal Reproduction (CBRA, 2013).

The rapid-green/rose Bengal (POPE et al., 1991) smear technique was used to evaluate the structural integrity of the acrosome membrane. To evaluate the integrity of the plasma membrane, a smear stained with eosin-nigrosine (E/N) (BARTH & OKO 1989) was used.

The cytochemical activity of the sperm mitochondria was measured by incubation with 3-31-diaminobenzidine, as described by Hrudka (1987).

Freezing and thawing of semen

Semen samples from the 60- and 75-day experiments were frozen with Bovimix® commercial medium. The semen was packed in 0.25mL straw at a concentration of 200×10^6 sptz/mL flasks and submitted cryopreservation performed using the P3 S1

Table 1 - Proportion of ingredients and bromatological analysis of the rations in the control treatment and in the treatments receiving 50 g (MAC 50) or 150 g (MAC 150) of macadamia industrial by-product and 50 g of protected fat (ME).

	Control (%)	MAC 50 (%)	MAC 150 (%)	ME (%)
Corn	64	57.6	48.51	57.6
Soybean meal	32	28.8	24.26	28.8
Limestone	1	0.9	0.76	0.9
Mineral mix	2	1.8	1.52	1.8
White salt	1	0.9	0.76	0.9
Macadamia	-	10	24.19	-
Megalac®	-	-	-	10
DM	91.8	91.65	92.28	91.99
CP	25.53	24.59	22.56	23.42
CF	4.28	4.97	2.76	4.17
EE	0.87	4.06	7.76	2.29
MM	6.31	5.66	4.77	7.16
ENN	63.00	60.72	62.14	62.94
ADF	5.47	5.27	4.81	5.16
NDF	13.52	12.98	10.74	13.48
Hemi-C	8.05	7.71	5.93	8.33
C	4.91	4.81	4.16	4.67
Lig	0.26	0.29	0.42	0.26

Amount of concentrate offered to sheep receiving 50 g (MAC 50) or 150 g (MAC 150) of macadamia industrial by-product or 50 g (ME) of protected fat (Megalac®). DM = dry matter; CP = crude protein; CF = crude fibre; EE = ethereal extract; ENN = non-nitrogenous extractive; MM = mineral matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; C = cellulose; Lig = lignin; Hemi-C = hemicellulose.

curve option of an automated cooling and freezing system (Tetakon® – TK 3000, TK Tecnologia em Congelação Ltda., Uberaba, MG, Brazil). Straws were cooled from 32 to 5◦C at a rate of 0.25◦C/min, kept at 5◦C for 120 min, immersed in liquid nitrogen vapor, until −120◦C (20◦C/min) and then plunged into liquid nitrogen and stored at −196◦C and stored for future evaluations.

Thawing was carried out in a water bath at 37ºC for 20 seconds; two straws were thawed from each treatment. After thawing, the semen was deposited in a micro tube, maintained in a 37°C water bath and submitted to analyses of sperm kinetic parameters using computer-assisted sperm analysis (CASA) set up for sheep, as described by Azevedo (2006), membrane integrity (E/N), acrosome integrity (POPE) and mitochondrial integrity (DAB I, II, III and IV). The sperm kinetic parameters of the spermatozoa were: spermatic velocity (VCL), curvilinear velocity in a uniform path, neglecting somatic cell lateral displacement (VAP), rectilinear velocity considering the spermatic trajectory a straight line (VSL), lateral displacement of the head (ALH), beat frequency (BCF), linearity (LIN), stratigraphic index of spermatic movement (STR) (MOSES et al., 1995).

Statistical analysis

The data collected during the field work were recorded and tabulated in files. Data consistency analyses were performed using the PROC MEANS and PROC UNIVARIATE subroutines for the normality test, included in the SAS statistical software.

To verify the fixed effects of age, treatment and time, analysis of variance was performed for animal weight, cholesterol, aspartate aminotransferase and the physical characteristics of the semen, spermatozoa morphology and E/N, DAB and POPE variables of the males (PROC GLM) by comparing the means by the SNK test at 5% probability. The simple interactions between the effects were tested, but did not present significant differences ($p>0.05$) and were therefore excluded from the final data analysis model, which was also the case for the triple interaction of the fixed effects.

To obtain the simple correlation coefficients, the CORR procedure (SAS, SAS Inst., Inc., Cary, NC) was used.

RESULTS

Mean initial and final weights were, respectively, 42.06 ± 5.87 kg and 47.61 ± 5.73 kg, ($p<0.05$). Table 2 presents the means of weight in relation to age, treatment and time. The control treatment presented a response similar to that of the other treatments, but the mean of the ME group significantly differed from those of MAC 50 and MAC 150 ($p<0.05$).

Cholesterol concentrations for the treatments receiving macadamia (MAC 50 and MAC 150) and Megalac (ME) were similar ($p>0.05$), but with mean values higher than the control ($p<0.001$). Cholesterol levels increased gradually during the experiment, ranging from 25.10 ± 5.67 mg/dL (D0) to 40.22 ± 10.96 mg/dL (D75), but only at D0 were the concentrations lower ($p<0.001$; Table 2). Concentrations of AST differed between the treatments MAC150 and ME (105.92 ± 34.45 x 81.58 ± 27.68 U/L, respectively) and between the period, with mean values of 80.58 ± 20.59 U/L at D0 and 109.17 ± 26.40 at D75 ($p<0.0001$).

In fresh semen, the treatments had significant impacts on motility ($p<0.05$). The average value in MAC 50 was significantly lower than that in MAC 150, but similar to those in the control and in ME. There were no effects of the treatments on volume, appearance, turbulence, vigour, concentration/mL, normal spermatozoa, minor, major and total defects (Table 3). Age did not influence the volume, concentration, percentage of normal spermatozoa and major defects ($p>0.05$) (Table 3). The mean values of vigour, motility and minor defects differed between ages, although these differences were not significant.

There was no influence of collection time on the averages of the variables volume, vigour, turbulence, motility, concentration/mL, major defects and minor defects ($p>0.05$) (Table 3).

Cryopreservation had no significant impact on acrosome integrity (POPE), mitochondrial activity (DAB) and plasma membrane integrity (E/N) (Table 4).

The treatments had no significant impact on sperm kinetic variables (Table 5).

DISCUSSION

Alternative sources of lipids and vegetable protein, readily available at low cost, may be of great value for supplementation of ruminant diets. In the present study, animals in the control treatment and in the treatment group receiving 50 g of protected fat presented the highest average live weight. Average weight differed with age, with increasing values over time.

Average cholesterol values increased in the treatments with the addition of macadamia by-product or protected fat. Homem Junior et al. (2010) stated that the energy present in the animals' diet can be evaluated by blood indicators such as cholesterol. Fat supplementation may raise the levels of blood cholesterol (BIANCHI et al., 2014), a precursor metabolite of steroid hormones, cholesterol constitutes biological membranes and has other specific and essential biological activities. In the present experiment, higher cholesterol levels were found in the treatments with macadamia supplementation (MAC 50 and MAC 150) (Table 2), but although the levels increased over time, the values were lower than those considered normal for ovine species (49 to 76 mg/dL, KANECO et al. 1997; or 57 to 63 mg/dL, ESMAEILI et al., 2012).

The increase in plasma cholesterol concentrations could be due to the higher intestinal absorption of some fatty acids contained in chylomicrons and low-density

Table 2 - Mean values and standard deviation of weight, cholesterol (mg/dL) and AST (U/L)related-to the effects of treatments, age and time throughout the experiment on Morada Nova sheep in Sao Paulo, Brazil.

Treatment	Control	MAC 50	MAC 15 0	ME
Weight	46.26 ± 6.01^{ab}	42.62 ± 5.03^{b}	42.91 ± 5.28^{b}	47.61 ± 5.73^{a}
Cholesterol (mg/dL)	29.13 ± 6.39^{b}	38.27 ± 9.37^{a}	36.54 ± 11.27^{a}	34.36 ± 11.13^{a}
AST (U/L)	99.42 ± 24.28^{ab}	92.58 ± 18.38^{ab}	105.92 ± 34.45^{a}	81.58 ± 27.68^{b}
Age	18 months	30 meses	30 months	
Weight	43.52 ± 5.86^{b}	1.06 ± 0.34	46.71 ± 6.21^{a}	
Cholesterol (mg/dL)	35.80 ± 10.58^{a}	2.90 ± 0.37	33.50 ± 9.80^{a}	
AST (U/L)	100.11 ± 28.00^{a}	43.58 ± 13.39^{b}	89.67 ± 26.57^{a}	
Period	D0	D30	D60	D75
Weight	42.05 ± 5.87^{b}	45.67 ± 5.96^{a} 5.96^{a}	46.10 ± 5.7^{a} 5.7^{a}	47.61 ± 5.25^{a}
Cholesterol (mg/dL)	25.10 ± 5.67^{b}	35.00 ± 8.36^{a} 8.36^{a}	38.23 ± 8.1^{a}	40.22 ± 10.96^{a}
AST (U/L)	80.58 ± 16.26^{b}	-	-	109.17 ± 26.40^{a}

Aspartate aminotransferase (AST). Treatment with addition of 50 g of macadamia industrial by-product (MAC 50), treatment with addition of 150 g of macadamia industrial by-product (MAC 150) and treatment with 50 g of protected fat (ME). Different letters in the row indicate significant differences (SNK test; $p < 0.05$).

lipoproteins (STAPLES et al., 1998). Inside the tests, specifically in the Leydig cells, under the influence of LH cholesterol is the initial substrate that participates in a sequence of enzymatic reactions, culminating in the synthesis of testosterone.

The level of aspartate aminotransferase (Table 2) is obtained through serum biochemical examination and used to evaluate if there is any lesion in the liver or pancreas. The AST activity differed between diets (P<0.05), with the lowest value in treatment ME (81.58 IU/L) and the highest in treatment MAC 150 (105.92 IU/L). With the exception of the mean for ME, the values remained above the physiological standard considered normal for the species (0 to 90 IU/L), according to Kaneko et al. (1997).

With respect to the physical characteristics of semen and sperm morphology, higher motility value was observed for MAC150. This is possibly associated with the positive effect of supplementation with macadamia residue, which increased the levels of blood cholesterol, the precursor of steroid hormones, facilitating spermatogenesis (FARAJI et al., 2012). There was no influence (p>0.05) of the treatments on volume, vigor, concentration (C/mL), normal spermatozoa, major defects and minor defects. Although normally associated with environmental influences (BARTH & OKO, 1989), estimates of minor defects, regardless of the treatments, were above 40%.

Regarding the nutritional aspect, the libido and the volume of the ejaculate are influenced by the alimentary level. No differences were found in the aspects related to the superiority of seminal quality with the inclusion of macadamia residue or protected fat. Sheep at 18 months presented the best results for vigour and motility characteristics compared to those of 30 months, but a higher percentage of minor defects was found (Table 3). Kargar et al. (2016), working with flax seed oil, vitamin E and these combinations, verified that both treatments can improve sperm motility, vitality and number of sperm with intact plasma membrane following

Table 3 - Mean values and standard deviation of fresh semen characteristics and sperm morphology according to treatment, age and time (D0-D75) in Morada Nova sheep, Sao Paulo, Brazil.

Treatment	Control	MAC 50	MAC 150	ME
Volume (mL)	1.07 ± 0.28	0.95 ± 0.21	1.06 ± 0.25	1.10 ± 0.32
Appearance	2.96 ± 0.21	2.92 ± 0.28	2.87 ± 0.45	2.96 ± 0.20
Turb	3.35 ± 1.07	3.17 ± 1.34	3.75 ± 0.85	3.46 ± 1.02
vigour(0-5)	3.35 ± 0.77	3.29 ± 1.0	3.58 ± 0.65	3.54 ± 0.66
Motili (0-100%)	78.26 ± 15.57^{ab}	70.42 ± 25.28^{b}	82.50 ± 8.47^{a}	79.79 ± 12.55^{ab}
Concentration	3.95 ± 1.31	3.87 ± 1.56	3.64 ± 1.45	4.31 ± 2.86
C total	4.04 ± 1.07	3.78 ± 1.17	3.98 ± 1.23	4.14 ± 1.27
Normal (%)	51.74 ± 14.56	51.46 ± 15.69	57.17 ± 12.02	52.75 ± 14.72
D major (%)	2.96 ± 4.99	2.62 ± 4.66	1.62 ± 1.86	3.00 ± 4.43
D minor (%)	45.30 ± 15.15	45.75 ± 15.41	41.21 ± 11.90	44.25 ± 14.31
D total	48.26 ± 14.56	48.37 ± 15.91	42.83 ± 12.02	47.25 ± 14.72
Age		18 months	30 months	
Volume (mL)		1.03 ± 0.17	1.06 ± 0.34	
Appearance		2.96 ± 0.20	2.90 ± 0.37	
Turb		3.72 ± 0.83^{a}	3.15 ± 1.24^{b}	
vigour (0-5)		3.70 ± 0.59^{a}	3.19 ± 0.89^{b}	
Motili (0-100%)		83.72 ± 7.48^{a}	71.87 ± 21.30^{b}	
Concentration		4.04 ± 1.57	3.85 ± 2.17	
C total		4.21 ± 1.42^{a}	3.77 ± 0.84^{b}	
Normal (%)		50.19 ± 14.69^{a}	56.33 ± 13.30^{b}	
D major (%)		1.81 ± 3.48	3.27 ± 4.62	
D minor (%)		48.00 ± 13.97^{a}	40.31 ± 13.37^{b}	
D total		49.81 ± 14.69^{a}	43.58 ± 13.39^{b}	
Period	D0	D30	D60	D75
Volume (mL)	1.17 ± 0.36	0.99 ± 0.23	1.01 ± 0.20	1.01 ± 0.25
Appearance	3.0 ± 0.0	2.87 ± 0.45	2.87 ± 0.34	2.96 ± 0.20
Turb (0-5)	3.30 ± 0.88	3.29 ± 1.04	3.62 ± 1.13	3.50 ± 1.28
vigour (0-5)	3.48 ± 0.85	3.08 ± 0.97	3.62 ± 0.58	3.58 ± 0.65
Motili (0-100%)	73.26 ± 22.03	75.00 ± 17.19	82.50 ± 11.89	80.00 ± 15.04
Concentration	4.19 ± 1.56	3.40 ± 2.79	4.40 ± 1.33	3.78 ± 1.44
C total	4.53 ± 1.11^{a}	3.25 ± 1.14^{b}	4.28 ± 1.01^{a}	3.91 ± 1.11^{a}
Normal	55.04 ± 16.08^{ab}	58.33 ± 14.25^{a}	47.29 ± 11.04^{b}	52.58 ± 13.86^{ab}
D major (%)	3.43 ± 5.74	1.42 ± 2.39	3.58 ± 4.60	1.79 ± 2.78
D minor (%)	41.35 ± 16.59	40.25 ± 14.65	49.12 ± 10.99	45.62 ± 12.89
D total	44.78 ± 16.26^{ab}	41.67 ± 14.25^{b}	52.71 ± 11.04^{a}	47.42 ± 13.86^{ab}

(MAC 50) Treatment with addition of 50 g of macadamia industrial by-product; (MAC 150) treatment with addition of 150 g of macadamia industrial by-product; and (ME) treatment with addition of 50 g of protected fat. Turbulence (Turb), motility (Motili), concentration/mL (C/ml x 10^9), normal spermatozoa (Normal), major defects (D major) and minor defects (D minor) and total defects (D total). Time D0 (day 0), 30 days (D30), 60 days (D60) and 75 days (75). Different letters in the row indicate significant differences (SNK test; $p < 0.05$).

Table 4 - Mean values and standard deviation of the effects of cryopreservation on semen acrosome integrity (POPE), mitochondrial activity (DAB I, II, III and IV) and plasma membrane integrity (E/N) in Morada Nova sheep, Sao Paulo, Brazil.

	Control	MAC 50	MAC 150	ME
E/N	20.66 ± 7.07	20.83 ± 5.70	24.50 ± 5.72	22.75 ± 6.09
POPE	62.33 ± 12.68	60.00 ± 13.93	63.33 ± 7.59	63.33 ± 8.95
DAB I	30.33 ± 16.44	28.00 ± 17.86	30.75 ± 14.78	31.91 ± 18.79
DAB II	22.41 ± 6.89	30.08 ± 12.66	28.66 ± 10.77	24.75 ± 10.73
DAB III	11.50 ± 10.78	8.91 ± 5.61	8.33 ± 5.80	8.50 ± 9.19
DAB IV	35.75 ± 20.71	33.00 ± 19.10	32.25 ± 22.67	34.83 ± 22.31

Acrosome integrity (POPE), mitochondrial activity (DAB) and plasma membrane integrity (E/N). Treatment with 50 g of macadamia industrial by-product (MAC 50); treatment with addition of 150 g of macadamia industrial by-product (MAC 150); and treatment with 50 g of protected fat (ME). Different letters in the row indicate significant differences (SNK test; $p < 0.05$).

freezing-thawing, but when flax seed oil and vitamin E were co-supplemented, the studied parameters was significantly higher. These suggest that membranes of sperm cell have a high concentration of polyunsaturated fatty acids, and especially during the freezing and thawing process it is extremely susceptible to oxidative stress.

In terms of cryopreserved semen (Table 4), the treatments had no significant impacts on acrosome integrity (POPE), mitochondrial activity (DAB) and plasma membrane integrity (E/N). The functionality and integrity of the sperm plasma membrane is determined by the fatty acid profile, which makes the composition of fatty acids extremely important for male fertility. Besides that, fatty acids acts as a key of testosterone biosynthesis of leyding cells and has a straight relation with the hypothalamic-pituitary-testicular axis, promoting high efficiency and sensitivity of FSH on sertoli cells, as well as improving polyunsaturated fatty acid uptake, since ruminants do not perform bio hydrogenation (VAN TRAN et al., 2017). According to Gholami et al. (2011), polyunsaturated fatty acids of the omega-3 family have a highly flexible chain and can be easily extended in the phospholipid bilayer, thus guaranteeing membrane quality by increasing the resistance and recovery thereof against the compressive forces of the membrane and the lateral plane of the bilayer. In relation to

Table 5 - Mean and standard deviation of the sperm kinetic parameters evaluated in the computer-assisted sperm analysis (CASA) in cryopreserved semen from Morada Nova sheep, Sao Paulo, Brazil.

Parameters	Control	MAC 50	MAC 150	ME
VAP (μm/s)	89.3 ± 37.2[a]	87.7 ± 37.2[a]	77.2 ± 20.7[a]	81.3 ± 13.5[a]
VSL (μm/s)	73.8 ± 37.3[a]	73.3 ± 34.5[a]	57.6 ± 18.2[a]	64.5 ± 13.2[a]
VCL (μm/s)	146.4 ± 42.2[a]	145.8 ± 52.7	147.5 ± 39.3	144.8 ± 26.4
ALH (μm)	5.5 ± 1.9[a]	6.4 ± 2.8[a]	6.7 ± 2.2[a]	6.8 ± 1.5[a]
BCF (Hz)	41.3 ± 2.3[a]	35.3 ± 12.6[a]	38.3 ± 3.8[a]	40.2 ± 3.6[a]
STR (%)	76.1 ± 8.1[a]	70.4 ± 23.4[a]	71.9 ± 10.6[a]	74.3 ± 7.2[a]
LIN (%)	47.9 ± 11.7[a]	44.7 ± 17.2[a]	41.1 ± 9.2[a]	45.5 ± 7.5[a]

VCL, Actual sperm displacement velocity; VAP, Curvilinear velocity on a uniform path, neglecting the lateral displacement of the sperm cell; VSL, Rectilinear velocity considering the spermatic trajectory a straight line; ALH, lateral displacement of head; BCF, Beat frequency; LIN, Linearity; STR, Stratigraphic index of spermatic movement. Treatment with 50 g (MAC 50) or 150 g (MAC 150) of macadamia industrial by-product and treatment with 50 g of protected fat (ME). Different letters in the row indicate significant differences (SNK test; $p < 0.05$).

acronym integrity (POPE), plasma membrane integrity (E/N) and mitochondrial activity (DAB), the treatments did not exert an effect (p>0.05) (Table 4). The damages occurring in acrosomes and membranes may be the result of the action of reactive oxygen species (ROS) and even the formation of ice crystals during the cryopreservation process, which would cause mechanical damage to the spermatic structures (SARAGUSTY et al., 2009). Cryopreservation exposes semen to cold shock and atmospheric air, chemically and physically damaging cell membranes, increasing lipid peroxidation through ROS and causing mechanical stress on membranes due to osmotic stress and changes in temperature (CÂMARA et al., 2011).

The ovine spermatozoon has a higher ratio of polyunsaturated and saturated fatty acids and a lower ratio of cholesterol and molar phospholipid compared to that of other species, making the cell considerably more vulnerable to oxidative damage caused by ROS (MOTLAGH et al., 2014). There is a greater predisposition of polyunsaturated fatty acids to undergo lipo peroxidation (LOSANO et al., 2018). According to Moraes et al. (2010), animals on diets supplemented with salmon oil showed a decrease in the total antioxidants in the semen, since this food is rich in PUFAs, resulting in a greater predisposition to lipid peroxidation.

Mitochondria participate in many crucial processes in eukaryotic cells, as the production of ATP via oxidative phosphorylation preceded by generation of reduced electron carriers in the cytoplasm and mitochondrial matrix, in addition to contributing for the conversion of cholesterol to pregnenolone and generate ATP required for steroid biosynthesis in Leydig cells. This process generates reactive oxygen species that can have pathological effect on the sperms if in excess, or if the balance with available antioxidant defences compromised, resulting in a decrease In viability, motility and increases in DNA damage, morphology defects as well as lipid peroxidation (AMARAL et al., 2013). According to Sampaio et al. (2015), there were no differences between treatments in which different concentrations of lipids (oleic-linoleic acid and beta-sitosterol) were used in the freezing dilution of semen from bulls and stallions in terms of mitochondrial activity. Studies of the role of vitamin E in the dilution of epididymis semen have shown that the addition of docosahexaenoic acid (DHA), a component of the omega-3 family associated with vitamin E, causes a decrease in mitochondrial activity, showing the combined activity of vitamin E with polyunsaturated fatty acids.

Samadian et al. (2010) reported increased sperm concentration, percentage of motility and progressive motility in sheep supplemented with fish oil as a source of polyunsaturated fatty acids. Similarly, Alizadeh et al. (2014) observed improvements in sperm concentration and total sperm production in sheep supplemented with fish oil.

There was no difference (p>0.05) in terms of sperm kinetics (VAP, VSL, VCL, ALH, BCF, STR and LIN) in cryopreserved semen (Table 5), perhaps because of the low resistance of the spermatozoids to freezing. O_2-, H_20_2 and OH generation in cryopreserved sheep semen in the TRIS-egg diluent, with or without the addition of Trolox-C and catalase and with or without antioxidants, indicates the generation of such ROS in the cryopreservation process (SICHERLE et al., 2011). According with Silva et al. (2011), the addition of superoxide dismutase and reduced glutathione in Tris-egg semen extender, provides the integrity of the acrosome preservation when submitted in cryopreservation, moreover, the superoxide dismutase addition also offers protection to sperm cell membrane, showing a relation between cryopreservation and the antioxidants enzymes action in the semen.

CONCLUSION

The inclusion of macadamia by-product and protected fat in the diet of Morada Nova sheep did not improve the quantitative and qualitative aspects of fresh and post-thawed semen.

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