



Semina: Ciências Agrárias

ISSN: 1676-546X

semina.agrarias@uel.br

Universidade Estadual de Londrina
Brasil

Pereira das Neves Snoeck, Paola; Oliveira Moura, Luís Cláudio; Alanis Clemente, Carlos Augusto; Loaiza-Echeverri, Ana Maria; Machado Neves, Mariana; Bezerra Allaman, Ivan; Henry, Marc

Effect of catalase, superoxide dismutase and reduced glutathione in LDL extender on ovine cryopreserved sperm viability

Semina: Ciências Agrárias, vol. 36, núm. 4, julio-agosto, 2015, pp. 2593-2601

Universidade Estadual de Londrina
Londrina, Brasil

Available in: <http://www.redalyc.org/articulo.oa?id=445744150020>

- How to cite
- Complete issue
- More information about this article
- Journal's homepage in redalyc.org

redalyc.org

Scientific Information System

Network of Scientific Journals from Latin America, the Caribbean, Spain and Portugal

Non-profit academic project, developed under the open access initiative

Effect of catalase, superoxide dismutase and reduced glutathione in LDL extender on ovine cryopreserved sperm viability

Efeito da catalase, superóxido dismutase e glutathiona reduzida em diluidor contendo lipoproteínas de baixa densidade na viabilidade de espermatozoides ovino criopreservados

Paola Pereira das Neves Snoeck^{1*}; Luís Cláudio Oliveira Moura²;
Carlos Augusto Alanis Clemente³; Ana Maria Loaiza-Echeverri⁴;
Mariana Machado Neves⁵; Ivan Bezerra Allaman⁶; Marc Henry⁷

Abstract

The aim of this study was to evaluate the motility, kinetics and membrane integrity of ovine sperm cryopreserved in extenders containing 8% LDL with enzymatic antioxidants at different concentrations. Four Santa Inês rams were used to form four pools of semen (each pool containing ejaculates from four ram, totaling four ejaculates per animal). Each seminal pool was divided into eight aliquots for the following treatments: 1) Tris-glucose-glycerol (TGG) + (16%) egg yolk (control 1); 2) TGG + 8% (w/v) LDL (control 2); 3) TGG + 8% LDL + catalase 100 U/mL; 4) TGG + 8% LDL + catalase 200 U/mL; 5) TGG + 8% LDL + superoxide dismutase 100 U/mL; 6) TGG + 8% LDL + superoxide dismutase 200 U/mL; 7) TGG + 8% LDL + reduced glutathione 5 mM; and 8) TGG + 8% LDL + reduced glutathione 10 mM. The samples were packed into 0.25 mL straws, cooled (-0.25 °C/ min), maintained at 5 °C for 2 h and then frozen (-25 °C/ min) using a TK4000®. Immediately after thawing (38 °C/ 30 s), sperm motility and movement characteristics were assessed by computer sperm analysis (CASA). The structural integrity of the plasma and acrosomal membranes was analyzed using fluorescent dyes. The functional integrity of membranes was assessed using a hypoosmotic swelling test. As assessed by ANOVA, significant differences (P<0.05) among treatments were only observed for VCL, VSL and VAP. For the VCL variable, the 2, 3, 4, 5, 6 and 7 extenders were similar and higher than 1 and 8 extenders, the latter being similar to each other. For the VSL variable, the 3, 4, 5, 6 and 7 extenders were similar and higher than 1, 2 and 8 extenders, the latter being similar to each other. For the VAP variable, the 3, 4 and 6 extenders were similar and higher than 1, 2, 5, 7 and 8 extenders, the latter being similar to each other. In conclusion, enzymatic antioxidants as catalase and superoxide dismutase improve the protective activity of extenders containing LDL on frozen ovine sperm.

Key words: Cryopreservation, semen, enzymatic antioxidants, Santa Inês

Resumo

Objetivou-se avaliar a motilidade, cinética e integridade das membranas de espermatozoides ovinos criopreservados em diluidores contendo 8% de LDL com antioxidantes enzimáticos em diferentes

¹ Prof^a, Universidade Estadual de Santa Cruz, UESC, Ilhéus, BA, Brasil. E-mail: paolasnoeck@gmail.com; paolasnoeck@uesc.br

² Discente, UESC, Ilhéus, BA, Brasil. E-mail: lc_@hotmail.com

³ Prof., Universidade Federal da Paraíba, UFPB, Bananeiras, PB, Brasil. E-mail: carlosclemente@cchsa.ufpb.br

⁴ Pesquisadora, Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, MG, Brasil. E-mail: anamvz@hotmail.com

⁵ Prof^a, UFV, Viçosa, MG, Brasil. E-mail: mariana.mneves@ufv.br

⁶ Pesquisador, UESC, Ilhéus, BA, Brasil. E-mail: ivanalaman@gmail.com

⁷ Prof., UFMG, Belo Horizonte, MG, Brasil. E-mail: henrym2601@gmail.com

* Author for corespondence

concentrações. Quatro carneiros da raça Santa Inês foram utilizados para formar quatro pools de sêmen (cada pool contendo ejaculados provenientes dos quatro carneiros, totalizando quatro ejaculados por animal). Cada pool de sêmen foi dividido em oito alíquotas para os seguintes tratamentos: 1) Tris-glicose-glicerol (TGG) + (16%) gema de ovo (controle 1); 2) TGG + 8% (g/L) LDL (controle 2); 3) TGG + 8% LDL + catalase 100 U/mL; 4) TGG + 8% LDL + catalase 200 U/mL; 5) TGG + 8% LDL + superóxido dismutase 100 U/mL; 6) TGG + 8% LDL + superóxido dismutase 200 U/mL; 7) TGG + 8% LDL + glutatona reduzida 5 mM; and 8) TGG + 8% LDL + glutatona reduzida 10 mM. As amostras foram envasadas em palhetas de 0,25 mL, resfriadas (-0,25 °C/min), mantidas a 5 °C por duas horas e em seguida congeladas (-25 °C/min) usando uma máquina de congelar TK4000®. Imediatamente depois da descongelação (38 °C/30 s), as amostras foram submetidas à análise computadorizada (CASA) para avaliação da motilidade e cinética. A integridade estrutural das membranas plasmática e acrossomal foi analisada utilizando corantes fluorescentes. A integridade funcional das membranas foi avaliada utilizando o teste hiposmótico. Como indicado pelo teste ANOVA, diferenças significativas ($P < 0,05$) entre tratamentos só foram observadas para VCL, VSL e VAP. Para a variável VCL, os meios diluidores 2, 3, 4, 5, 6 e 7 foram similares e maiores que os diluidores 1 e 8, sendo os últimos semelhantes entre si. Para a variável VSL, os diluidores 3, 4, 5, 6 e 7 foram similares e maiores que os diluidores 1, 2 e 8, sendo os últimos semelhantes entre si. Para a variável VAP, os diluidores 3, 4 e 6 foram similares e maiores que o 1, 2, 5, 7 e 8, sendo os últimos semelhantes entre si. Em conclusão, os antioxidantes enzimáticos catalase e superóxido dismutase melhoraram a atividade protetora dos diluidores contendo LDL sobre os espermatozoides ovinos.

Palavras-chave: Criopreservação, sêmen, antioxidantes enzimáticos, Santa Inês

Introduction

Cryopreservation is a biotechnique that aims to artificially interrupt the metabolic activities of sperm for prolonged maintenance (WATSON, 1995). However, during the reduction of temperature in cooling and freezing, sperm membrane permeability is affected by changes in its fluidity when passing from liquid to gel (HOLT; NORTH, 1991) that can cause a series of subcellular damage.

The formation of reactive oxygen species (ROS), which occurs during cryopreservation, is detrimental to sperm survival (PEÑA et al., 2003). The generation of ROS is a result of oxidative metabolism, which plays an important role in the normal function of sperm. However, an imbalance between production and degradation of ROS (BALL, 2008), known as oxidative stress, can impair sperm functions such as motility, cause DNA damage (SUDJARWO et al., 2006) and lead to low viability of stored semen of breeders (TREMELLEN, 2008). Exacerbating this situation, the levels of enzymatic antioxidants, present in the semen, are reduced during cryopreservation. Superoxide dismutase suffers more intensely this process in sheep, with

an approximately 65% reduction after freezing (MARTI et al., 2008).

The generation of ROS is significantly increased in the presence of cryodamaged (BALL et al, 2001). Hydrogen peroxide (H₂O₂) appears to be the most important ROS for damage to sperm and is much more membrane permeable than the others free radicals (BAUMBER et al., 2000). However, H₂O₂ is not energetic enough to initiate lipid peroxidation and it is required a transition metal (such as Fe²⁺) to initiate the process (BALL; VO, 2002). The decrease in sperm motility associated with H₂O₂ occurs in the presence of extenders containing egg yolk and glycerol, and this could be due the fact that egg yolk is an important source of iron and other metal ions (BILODEAU et al., 2002). The generation of H₂O₂ is lower in semen cryopreserved in extenders containing some antioxidants, because these low-molecular weight components may affect sperm quality by removing the H₂O₂ and release into the extracellular environment (MAIA et al, 2010).

Câmara et al. (2011) evaluated the supplementation of the antioxidant enzymes catalase (5, 10 and 20 U/mL), superoxide dismutase (5, 10

and 20 U/mL) and reduced glutathione (0.5, 1.0 and 2.0 mM) in Tris-yolk extenders during cooling and freezing of ram semen. They found no positive effect of supplementation on sperm motility, kinetics, plasmatic and acrosomal membrane structural integrity or mitochondrial membrane potential at the concentrations studied. However, the addition of enzymatic antioxidants to diluents containing low-density lipoproteins (LDL) has not yet been tested. It has been shown that extenders containing LDL as a replacement for egg yolk have a cryoprotective effect that is similar or superior to extenders based on egg yolk (ALI AL AHMAD et al., 2008; VARELA JUNIOR et al., 2009; MOUSTACAS et al., 2011; SILVA et al., 2014).

Therefore, the objective was to study the effects of the supplementation of extenders containing low-density lipoprotein with the antioxidant enzymes catalase, superoxide dismutase and reduced glutathione on ram sperm quality after cryopreservation.

Material and Methods

Before starting the experiment, breeders were submitted to andrologic evaluation and depletion of extragonadal reserves. Both the depletion of reserves and the collection of semen were conducted using an appropriate artificial vagina with the aid of a female in natural or induced oestrus. In this study, all breeders used were in good health and nutritional status. All breeder ejaculates used were at concentrations greater than 1.8×10^9 sperm/ejaculation, with motility $\geq 75\%$, vigor ≥ 3.0 , and $\geq 70\%$ of morphologically normal sperm.

Four Santa Inês rams were used as semen donors, and the collected ejaculations were mixed in equal proportions, forming a pool. Each animal was collected four times, totaling four pools (each pool containing ejaculates from four ram, totaling four ejaculates per animal). Each pool of semen was evaluated according to its macroscopic and microscopic characteristics (CBRA, 1998) and

was subsequently diluted and fractionated into the following extenders in order to obtain 100×10^6 spz/mL: 1) Tris-glucose-glycerol (TGG) + (16%) egg yolk (control 1) (SALAMON; VISSER, 1972); 2) TGG + 8% (w/v) LDL (control 2); 3) TGG + 8% LDL + Catalase 100 U/mL; 4) TGG + 8% LDL + Catalase 200 U/mL; 5) TGG + 8% LDL + superoxide dismutase 100 U/mL; 6) TGG + 8% LDL + superoxide dismutase 200 U/mL; 7) TGG + 8% LDL + reduced glutathione 5 mM; and 8) TGG + 8% LDL + reduced glutathione 10 mM. The extraction of LDL used in the extender media was performed using the protocol described by Moussa et al. (2002) with modifications by Neves (2008).

Diluted semen samples were packaged in 0.25 mL straws and cryopreserved. The cooling rate used was -0.25 °C/min up to 5 °C, and the samples were maintained at 5 °C for 2 h until were frozen at a rate of -25 °C/min using a TK4000® (TK – Tecnologia em Congelação LTDA). After freezing, straws were plunged into liquid nitrogen and subsequently stored in cryogenic cylinders.

After thawing straws at 38 °C/30s in a water bath, the motility and kinetic characteristics of the sperm were evaluated by a computer sperm analysis system (CASA, SCA® v.4, *Microptics S.L.*, Barcelona, Spain) after 5 minutes, considered time zero. The samples were maintained in a water bath at 38 °C, and evaluations were repeated 1, 2 and 3 hours after the thaw according to CBRA (1998).

To analyze the functional integrity of the plasmatic membrane, the hypoosmotic test (HOST) was performed according to the technique described by Oberst et al. (2003). The number of sperm reactive to HOST was calculated according to the formula proposed by Melo and Henry (1999).

The structural integrity of the sperm membranes was assessed using the carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) fluorochromes using the protocol proposed by Harrison and Vickers (1990), with the modifications cited by Zúccari (1998).

Statistical analysis was performed according to a randomized experimental design in blocks in an 8x4 factorial arrangement. The ram factor was considered as a block. The assumptions of ANOVA were met, as verified by applying normality tests according to the method of Lilliefors. Homoscedasticity was verified according to the method of Levene. The variables that were not normally distributed underwent arcsine \sqrt{x} angular transformation and boxcox transformation in order to normalize the waste. The Scott-Knott test ($P < 0.05$) was used for multiple comparison of means and polynomial adjustment to quantitative factors.

Results and Discussion

The fresh ram semen used in this experiment had an average motility of $90.0\% \pm 0.0$, a progressive average motility of $85.0\% \pm 0.0$, a morphologically normal spermatozoa average of $75.8\% \pm 4.2$, a major defects average of $8.0\% \pm 9.6$, a minor defects average of $16.5\% \pm 9.3$, an average of sperm reactive to hypoosmotic test of $26.3\% \pm 16.0$ and an average of sperm with structurally intact membrane evaluated by fluorescent probes of $73.3\% \pm 15.0$.

There were no differences ($P > 0.05$) among the extenders for any evaluation time (0, 1, 2 and 3 h) after thawing and incubation in a water bath for any of the variables evaluated (Table 1). Therefore, these factors were studied individually.

There was a decrease in motility and kinetics parameters ($P < 0.01$) during the incubation of the ram sperm after thawing (Table 1). Adjustment of the linear regression ($P < 0.01$) indicates that all studied variables are impacted by incubation time. It is estimated that a one-hour incubation time

after thawing reduces motility, curvilinear velocity (VCL), straight linear velocity (VSL) and average path velocity (VAP) by 0.36%, 3.44%, 2.35% and 2.54%, respectively (Table 1). For the fast velocity, medium velocity, slow velocity, linearity, straightness, wobble coefficient, amplitude of lateral head displacement, frequency of flagellar cross beating and hyperactive variables, we estimate that a one-hour incubation time after thawing causes reductions of 1.49%, 0.86%, 1.84%, 3.29%, 8.46%, 2.50%, 0.36 %, 1.30% and 0.09%, respectively. Furthermore, a reduction of sperm quality is expected as the defrosting time progresses.

When the extenders were evaluated independently of the time of incubation post thawing, there were differences among some kinetic characteristics measured, as VCL, VSL and VAP ($P < 0.05$). The VCL values were similar ($P > 0.05$) for the samples frozen in the control extender with egg yolk and the extender containing LDL with 10 mM reduced glutathione. However, both values were lower ($P < 0.05$) than those obtained with the other extenders. The VSL values for samples frozen in the control extender with egg yolk, the control with LDL and in the extender containing LDL with 10 mM of reduced glutathione were all similar and lower than those found for other extenders ($P < 0.05$). For VAP, no differences ($P > 0.05$) were observed between the samples frozen in control extender with egg yolk, control extender with LDL, extender containing LDL with 100 U/ml of superoxide dismutase and in those containing LDL with either 5 mM or 10 mM of reduced glutathione. Moreover, these groups presented below VAP results ($P < 0.05$) than other extenders groups.

Table 1. Effect of extenders at different evaluation times after thawing (0, 1, 2 and 3 h) in the variables motility, average curvilinear velocity path (VCL), progressive linear velocity (VSL) and average path velocity (VAP).

Extender	Hours	Motility ¹ (%)	VCL ² (µm/s)	General Average*	VSL ³ (µm/s)	General Average*	VAP ⁴ (µm/s)	General Average*
1	0	25.6	37.2		16.1		20.8	
	1	21.8	36.0	32.2 ^B	14.9	12.8 ^B	20.3	17.8 ^B
	2	16.3	30.8		11.7		16.8	
	3	10.4	24.9		8.6		13.2	
2	0	25.8	37.6	38.7 ^A	13.4	12.6 ^B	20.0	19.7 ^B
	1	22.8	45.6		14.7		24.2	
	2	19.0	37.4		12.5		18.5	
	3	15.4	34.1		9.9		16.3	
3	0	21.4	45.0	44.4 ^A	17.9	14.8 ^A	25.6	23.4 ^A
	1	22.3	43.4		13.9		22.2	
	2	22.9	43.6		13.1		21.5	
	3	16.4	45.6		13.8		24.4	
4	0	32.5	42.5	40.4 ^A	20.4	15.4 ^A	26.4	22.5 ^A
	1	20.7	44.5		16.4		24.7	
	2	20.5	40.8		13.3		21.5	
	3	19.6	33.6		11.5		17.3	
5	0	27.3	42.8	38.3 ^A	16.6	13.8 ^A	23.5	20.2 ^B
	1	25.6	42.4		16.0		22.5	
	2	19.7	36.8		12.9		19.4	
	3	11.7	31.2		9.8		15.5	
6	0	18.5	48.7	42.8 ^A	19.7	15.2 ^A	29.4	23.3 ^A
	1	25.7	42.2		13.5		21.2	
	2	18.5	39.6		12.7		19.2	
	3	16.4	40.8		14.9		23.4	
7	0	29.6	47.6	38.1 ^A	17.9	13.4 ^A	25.2	19.9 ^B
	1	27.2	43.8		17.2		24.0	
	2	19.8	31.1		10.2		15.7	
	3	12.2	30.0		8.4		14.4	
8	0	33.3	37.5	31.1 ^B	15.5	10.4 ^B	21.0	15.6 ^B
	1	27.9	37.1		12.0		18.5	
	2	16.4	27.0		7.9		12.5	
	3	12.8	22.9		6.0		10.5	

¹ $\arcseno(\sqrt{\hat{y}}) = 0.53 - 0.06\hat{x}$; ² $\hat{y} = 43.4 - 3.44\hat{x}$; ³ $\hat{y} = 17.06 - 2.35\hat{x}$; ⁴ $\hat{y} = 24.11 - 2.54\hat{x}$

1) Tris-glucose-glycerol (TGG) + (16%) egg yolk (control 1) (SALAMON; VISSER, 1972); 2) TGG + 8% (w/v) LDL (control 2); 3) TGG + 8% LDL + Catalase 100 U/mL; 4) TGG + 8% LDL + Catalase 200 U/mL; 5) TGG + 8% LDL + superoxide dismutase 100 U/mL; 6) TGG + 8% LDL + superoxide dismutase 200 U/mL; 7) TGG + 8% LDL + reduced glutathione 5 mM; and 8) TGG + 8% LDL + reduced glutathione 10 mM in order to obtain 100×10^6 spz/mL.

* Different letters in the column are significantly different according to the Scott-Knott test ($P < 0.05$).

It was observed that reduced glutathione, particularly at a concentration of 10 mM, was not good for preserving sperm motility because there was a reduction in VCL, VSL and VAP values for samples frozen in extenders supplemented with this antioxidant. Silva et al. (2011b) also reported

that reduced glutathione at higher concentrations tested (7 mM) increased the velocity and quality of the ram sperm movement, nevertheless, decreased the viability of the cells (lesser acrosomal and mitochondrial integrity and total motility). On the other hand, Anel-Lopez et al. (2012) reported

improvement of the kinetics and structural parameters of deer sperm after thawing using reduced glutathione at lower concentrations that ranged between 1 and 5 mM. According to Gadea et al. (2004), reduced glutathione would achieve better results if it were added to post-thawed semen, as it could raise the levels of intracellular glutathione to be used by glutathione peroxidase to prevent damage caused by lipid peroxidation.

The addition of antioxidants such as glutathione peroxidase or cysteine to equine semen extenders improves sperm VCL and VAP after thawing (BARROS et al., 2013). In humans, Moubasher et al. (2013) reported increased of the percentage of progressive motility and sperm viability in samples cryopreserved in extenders with catalase. In the same way, Evangelista et al. (2014) reported lesser degree of lipid peroxidation and higher motility in alpaca sperm samples cryopreserved in extender supplemented with superoxide dismutase and catalase. In this study, the same positive effects on VCL, VSL and VAP were observed with ovine semen when catalase (100 and 200U/mL) and superoxide dismutase (200U/mL) were added to extenders containing LDL. In bulls, Farrell et al. (1998) reported that sperm velocity is high correlated with fertility (ranged between 0.63 e 0.67). According to Matos et al. (2008), higher values of VCL, VSL and VAP are viewed in sperm samples that reach more than 50% of oocytes fertilization, because has high capacity of migration and penetration of the cervical mucus in the female reproductive tract. The motility and velocity parameters measured by the CASA reflect indirectly the mitochondrial function of the sperm (PERUMAL et al., 2014). These results could be indicating that the antioxidant catalase in the concentrations used in the present work is useful for improvement the fertility of sperm cryopreserved in extenders with LDL. However, it is not yet possible to determine the influence of these parameters on ram fertility.

The antioxidant activity of the enzymes catalase, superoxide dismutase and glutathione peroxidase

present in seminal plasma or in sperm is affected during the cryopreservation of semen, leading to increased lipid peroxidation in the sperm (PARTYKA et al., 2012). Lipid peroxidation is defined as the oxidative deterioration of polyunsaturated lipids, which are present in large amounts in the membrane of ovine sperm (HOLT; NORTH, 1991; CÂMARA et al., 2011). The supplementation of ovine seminal extenders containing LDL with catalase, superoxide dismutase and reduced glutathione did not result in high improved sperm quality after thawing at any concentration studied. Especially when evaluating the parameters of motility and the structural and functional integrity of sperm membranes, these results confirm the findings of Câmara et al. (2011).

In the present work, the concentration of LDL used was the same in all the extenders (8%) and is probable that this concentration of LDL did not interact well with the antioxidants tested here. There is a need for further studies to assess the optimal concentration of LDL to be used in extenders with the addition of catalase, superoxide dismutase or reduced glutathione. It is possibly that other LDL and antioxidant concentrations improve the sperm quality parameters.

There were no differences ($P>0.05$) among the extenders in preserving the functional integrity (average variation between extenders from $9.0\% \pm 6.1$ to $18.5 \pm 14.6\%$) or structural integrity of the sperm membranes (average variation between extenders from $10.3\% \pm 6.9$ to $22.8\% \pm 23.3$), as assessed with the hypoosmotic tests and fluorescent probes (CFDA/IP), respectively. Câmara et al. (2011) and Anel-López et al. (2012) similarly did not observe any superiority of extenders containing antioxidants for the preservation of membrane integrity. However, the production of free radical in the sperm involved some mechanisms, and more sensitive methods have to be used to detect ROS production and cell damages (AITKEN et al., 2013).

The structural integrity of the sperm membranes in the present work was determined by the use

of PI and CFDA, nevertheless, both does not identify the mitochondrial status of the sperm. The mitochondrial membrane potential mirrors the sperm quality and a better motility pattern of the sperm, and according to Hossain et al. (2011) the integrity of the mitochondrial function have to be measured using specific dyes for these organelles.

There were notable reductions ($P < 0.05$) in motility and functional and structural integrity of sperm membranes after thawing. It is also notable that regardless of the extender used, post-thaw motility did not reach the 30% average recommended by CBRA (1998). Therefore, sperm motility appears to be a sensitive indicator of oxidative stress and may be one of the first parameters affected during oxidative stress (BALL, 2008). During the cryopreservation, the plasma and mitochondrial membranes are equally vulnerable; however, the reduction in the motility is associated with problems in the mitochondrial structure. Catalase improved the motility and viability of human sperm, inhibiting DNA damages and protecting the mitochondrial function, indicating that this antioxidant is useful for decrease the ROS levels (LI et al., 2010). Our results could be indicating that some antioxidants used here (as catalase and superoxide dismutase) were a litter more efficient for the mitochondrial protection than other, as showed by the better values of some kinetics parameters.

It is possible that the freezability of the semen of all breeders used was harmed as a result of the season, given that the freezing of the semen was conducted in early autumn using ejaculations containing sperms produced during the summer. The effect of seasonality on sperm quality for Santa Inês rams is well known, as is the effect on semen freezability (SILVA et al., 2011a).

Conclusions

The enzymatic antioxidants catalase, superoxide dismutase and reduced glutathione in extenders containing LDL showed no high positive effect

on sperm viability during cryopreservation of ovine semen. However, catalase at concentrations of 100 and 200U/mL and superoxide dismutase at a concentration of 200U/mL better preserved the kinetic parameters of VCL, VSL, and VAP. This could be indicating that the addition of these antioxidants to extenders with LDL is useful for protected the mitochondrial structure and function. The addition of antioxidants to extenders with other LDL concentrations should be tested.

Acknowledgements

We thank Prof. Maria Madalena Pessoa Guerra for providing the enzymatic antioxidants used in this experiment, and FAPEMIG and CNPq for the financial support.

References

- AITKEN, R. J.; SMITH, T. B.; LORD, T.; KUCZERA, L.; KOPPERS, A. J.; NAUMOVSKI, N.; CONNAUGHTON, H.; BAKER, M. A.; DE IULIIS, G. N. On methods for the detection of reactive oxygen species generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology*, Malden, v. 1, n. 2, p. 192-205, 2013.
- ALI AL AHMAD, M.; CHATAGNON, G.; AMIRAT-BRIAND, L.; MOUSSA, M.; TAINURIER, D.; ANTON, M.; FENI, F. Use of glutamine and low density lipoproteins isolated from egg yolk to improve buck semen freezing. *Reproduction in Domestic Animals*, Linköping, v. 43, n. 4, p. 429-436, 2008.
- ANEL-LÓPEZ, L.; ALVAREZ-RODRIGUEZ, M.; GARCIA-ALVAREZ, O.; ALVAREZ, M.; MAROTOMORALES, A.; ANEL, L.; PAZ, P.; GARDE, J. J.; MARTINEZ-PASTOR, F. Reduced glutathione and trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. *Animal Reproduction Science*, Amsterdam, v. 135, n. 1-4, p. 37-46, 2012.
- BALL, A. B. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Animal Reproduction Science*, Amsterdam, v. 107, n. 3-4, p. 257-267, 2008.

- BALL, B. A.; VO, A. Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C-11-BODIPY581/591. *Journal of Andrology*, Malden, v. 23, n. 2, p. 259-269, 2002.
- BALL, B. A.; VO, A. T.; BAUMBER, J. Reactive oxygen species generation by equine spermatozoa. *American Journal of Veterinary Research*, Chicago, v. 62, n. 4, p. 5508-5515, 2001.
- BARROS, L. O.; SILVA, S. V.; ALMEIDA, F. C.; SILVA, E. C. B.; GUERRA, M. M. P. Efeito da adição de glutatona peroxidase e cisteína ao diluidor de congelamento de sêmen equino. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 65, n. 2, p. 430-438, 2013.
- BAUMBER, J.; BALL, B. A.; GRAVANCE, C. G.; MEDINA, V.; DAVIES-MOREL, M. C. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology*, Malden, v. 21, n. 6, p. 895-902, 2000.
- BILODEAU, J. F.; BLANCHETTE, F.; CORMIER, N.; SIDARD, M. A. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. *Theriogenology*, California, v. 57, n. 3, p. 1105-1122, 2002.
- CÂMARA, D. R.; SILVA, S. V.; ALMEIDA, F. C.; NUNES, J. F.; GUERRA, M. M. P. Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. *Theriogenology*, California, v. 76, n. 2, p. 342-350, 2011.
- COLÉGIO BRASILEIRO DE REPRODUÇÃO ANIMAL – CBRA. Manual para exame andrológico e avaliação de sêmen animal. Belo Horizonte: CBRA, 1998. p. 31-34.
- EVANGELISTA, S.; MUCHOTRIGO, D.; TRELLES, X.; CHOEZ, K.; SANTIANI, A. Alpaca semen cryopreservation using superoxide dismutase mimics and catalase. *Spermova*, Lima, v. 4, n. 1, p. 4-45, 2014.
- FARRELL, P. B.; PRESICCE, G. A.; BROCKETT, C. C.; FOOTE, R. H. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship with fertility. *Theriogenology*, California, v. 49, n. 4, p. 871-879, 1998.
- GADEA, J.; SELLÉS, E.; MARCO, M. A.; COY, P.; MATÁS, M.; ROMAR, R.; RUIZ, S. Decrease in glutathione content in boar sperm after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology*, California, v. 62, n. 3-4, p. 290-701, 2004.
- HARRISON, R. A. P.; VICKERS, S. E. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of Reproduction and Fertility*, Oxford, v. 88, n. 1, p. 343-352, 1990.
- HOLT, W. V.; NORTH, R. D. Cryopreservation, actin localization and thermotropic phase transitions in ram spermatozoa. *Journal of Reproduction and Fertility*, Oxford, v. 91, n. 2, p. 451-461, 1991.
- HOSSAIN, M. S.; JOHANNISSON, A.; WALLGREN, M.; NAGY, S.; SIQUEIRA, A. P.; RODRIGUEZ-MARTINEZ, H. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian Journal of Andrology*, Shanghai, v. 13, n. 3, p. 406-419, 2011.
- LI, Z.; LIN, Q.; LIU, R.; XIAO, W.; LIU, W. Protective effects of ascorbate and catalase on human spermatozoa during cryopreservation. *Journal of Andrology*, Malden, v. 31, n. 5, p. 437-444, 2010.
- MAIA, M. S.; BICUDO, S. D.; SICHERLE, C. C.; RODELLO, L.; GALLEGU, I. C. S. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Animal Reproduction Science*, Amsterdam, v. 122, n. 1-2, p. 118-123, 2010.
- MARTI, E.; MARTI, J. I.; MUIÑO-BLANCO, T.; CEBRIÁN-PÉREZ, J. A. Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. *Journal of Andrology*, Malden, v. 29, n. 4, p. 459-467, 2008.
- MATOS, D. L.; ARAÚJO, A. A.; ROBERTO, I. G.; TONIOLLI, R. Análise computarizada de espermatozoides: revisão de literatura. *Revista Brasileira de Reprodução Animal*, Belo Horizonte, v. 32, n. 4, p. 225-232, 2008.
- MELO, M. I. V.; HENRY, M. Teste hiposmótico na avaliação do sêmen equino. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 51, n. 1, p. 71-78, 1999.
- MOUBASHER, A. E.; EL DIN, A. M. E.; ALI, M. E.; EL-SHERIF, W. T.; GABER, H. D. Catalase improves motility, vitality and DNA integrity of cryopreserved human spermatozoa. *Andrologia*, Malden, v. 45, n. 2, p. 135-139, 2013.
- MOUSSA, M.; MARTINET, V.; TRIMECHE, A.; TAINURIER, D.; ANTON, M. Low density lipoproteins extracted from hen egg yolk by an easy method: crioprotective effect on frozen-thawed bull semen. *Theriogenology*, California, v. 57, n. 6, p. 1695-1706, 2002.

- MOUSTACAS, V. S.; ZAFFALON, F. G.; LAGARES, M. A.; LOAIZA-ECHEVERRI, A. M.; VARAGO, F. C.; NEVES, M. M.; HENEINE, L. G. D.; ARRUDA, R. P.; HENRY, M. Natural, but not lyophilized, low density lipoproteins were an acceptable alternative to egg yolk for cryopreservation of ram semen. *Theriogenology*, California, v. 75, n. 2, p. 300-307, 2011.
- NEVES, M. M. *Extração das lipoproteínas de baixa densidade da gema do ovo de Gallus domesticus e sua aplicação na criopreservação do sêmen canino*. 2008. Tese (Doutorado em Ciência Animal) – Curso de Pós-graduação em Ciência Animal. Universidade Federal de Minas Gerais, Belo Horizonte.
- OBERST, E. R.; JOBIM, M. I. M.; MATTOS, R. C.; KROTH, E.; LARA, G.; SMIDERIE, W.; BRONZATTO, M. Teste hiposmótico e sua relação com outros métodos da avaliação da integridade da membrana espermática do carneiro. *Revista Brasileira de Reprodução Animal*, Belo Horizonte, v. 27, n. 3, p. 375-376, 2003.
- PARTYKA, A.; LUKASZEWICZ, E.; NIZANSKI, W. Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen. *Theriogenology*, California, v. 77, n. 8, p. 1497-1504, 2012.
- PEÑA, F. J.; JOHANNISSON, A.; WALLGREN, M.; RODRIGUEZ MARTINEZ, H. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. *Animal Reproduction Science*, Amsterdam, v. 78, n. 1-2, p. 85-98, 2003.
- PERUMAL, P.; SRIVASTAVA, S. K.; GHOSH, S. K.; BARUAH, K. K. Computer-assisted sperm analysis of freezable and nonfreezable mithun (*Bos frontalis*) semen. *Journal of Animals*, New York, v. 2014, n. 1, p. 1-6, 2014.
- SALAMON, S.; VISSER, D. Effect of composition of tris-based diluents and of thawing solution on survival of ram spermatozoa frozen by the pellet method. *Australian Journal of Biological Sciences*, Melbourne, v. 25, n. 3, p. 605-618, 1972.
- SILVA, M. C.; MOURA, L. C. O.; MELO, M. I. V.; MAMBRINI, J. V. M.; NEVES, M. M.; HENRY, M.; SNOECK, P. P. N. Prolonged post cooling but not pre-cooling equilibrium length improves the viability of ram sperm cryopreserved in an extender containing low-density lipoproteins. *Small Ruminant Research*, Amsterdam, v. 119, n. 1-3, p. 88-95, 2014.
- SILVA, S. V.; SOARES, A. T.; BATISTA, A. M.; ALMEIDA, F. C.; GUERRA, M. M. P. Interferência da condição climática na integridade de espermatozoides ovinos submetidos à criopreservação. *Arquivos Brasileiros de Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 63, n. 6, p. 1309-1314, 2011a.
- SILVA, S. V.; SOARES, A. T.; BATISTA, A. M.; ALMEIDA, F. C.; NUNES, J. F.; PEIXOTO, C. A.; GUERRA, M. M. P. In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. *Reproduction in Domestic Animals*, Linköping, v. 46, n. 5, p. 874-881, 2011b.
- SUDJARWO, S.; AUUCKY, H.; ZAINI, N. C. The role of oxidative phosphorylation the mechanism of human sperm motility regulation. *Berkala Penelitian Hayati*, Malanj, v. 12, n. 1, p. 87-91, 2006.
- TREMELLEN, K. Oxidative stress and male infertility: a clinical perspective. *Human Reproduction Update*, Oxford, v. 14, n. 3 p. 243-258, 2008.
- VARELA JUNIOR, A. S.; CORCINI, C. D.; ULGUIM, R. R.; ALVARENGA, M. V. F.; BIANCHI, I.; CORRÊA, M. N.; LUCIA J. R. T.; DESCHAMPS, J. C. Effect of low density lipoprotein on the quality of cryopreserved dog semen. *Animal Reproduction Science*, Amsterdam, v. 115, n. 1-4, p. 323-327, 2009.
- WATSON, P. F. Recent developments and concepts in the cryopreservation of spermatozoa and the assesment of their post-thawing function. *Reproduction, Fertility and Development*, Clayton South, v. 7, n. 4, p. 871-891, 1995.
- ZÚCCARI, C. E. S. N. *Efeito da criopreservação sobre a integridade estrutural da célula espermática equina*. 1998. Tese (Doutorado em Medicina Veterinária) – Curso de Pós-Graduação na Faculdade de Medicina Veterinária e Zootecnia. Universidade Estadual Paulista, Botucatu.