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

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

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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

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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 + glutationa reduzida 5 mM; and 8) TGG + 8% LDL + glutationa 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 reduces 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 (H202) 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, H202 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 H202 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 H202 is lower in semen cryopreserved in extenders containing some antioxidants, because these low-molecular weight components may affect sperm quality by removing the H202 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 $1.8x10^9$ sperm/ejaculation, with motility ≥75%, vigor ≥3.0, and ≥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 $100x10^6$ sptz/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%±0.0, a progressive average motility of 85.0%±0.0, a morphologically normal spermatozoa average of 75.8%±4.2, a major defects average of 8.0%±9.6, a minor defects average of 16.5%±9.3, an average of sperm reactive to hypoosmotic test of 26.3%±16.0 and an average of sperm with structurally intact membrane evaluated by fluorescent probes of 73.3%±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	32.2[B]	16.1	12.8[B]	20.8	17.8[B]
	1	21.8	36.0		14.9		20.3	
	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] $\text{arcoseno}(\sqrt{\hat{y}}) = 0.53 - 0.06$; [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 $100x10^6$ sptz/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% ± 6.1 to 18.5 ± 14.6%) or structural integrity of the sperm membranes (average variation between extenders from 10.3% ± 6.9 to 22.8% ± 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.

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