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Effect of trolox added to freezing extenders over goat and ram spermatozoa

Efeito da adição de trolox em diluentes de congelação sobre o sêmen caprino e ovino

Efecto de la adición de trolox en los diluyentes de congelación en el semen de cabra y oveja

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Abstract
Semen cryopreservation is responsible for decrease the gamete fertility, due to structural and functional damages. Among the various causes, oxidative stress, resulting from the higher generation of reactive oxygen species (ROS), has been attributed to affect semen quality. Thus, it was objectified evaluate the effect of Trolox on ram and goat sperm, subjected to freezing. Semen pools of goat (n=5) and ram (n=6) were diluted in skimmed milk (7% glycerol) or Tris-egg yolk (5% glycerol) extender, respectively, added or not of Trolox (0, 20 or 40 μM/ml) and frozen. After thawing (37 °C/30 s), aliquots of semen were evaluated for lipid peroxidation by high performance liquid chromatography, coupled with a photodiode array detector (HPLC-DAD), and flow cytometry (C11-BODIPY581/591), besides of plasma membrane and acrosome integrity by fluorescence microscopy, and sperm kinetics by computerized sperm analysis (CASA). The antioxidant treatment with Trolox did not determine significant effects ($p>0.05$) on lipid peroxidation, plasma membrane integrity, acrosomal integrity and on the kinetic parameters evaluated. Thus, it is concluded that Trolox (20 or 40 μM) did not have a protective or deleterious effect on goats and ram sperm, submitted to freezing.
Keywords: Antioxidants; Lipoperoxidation; Ruminant; Semen; Vitamin E.

Resumo
O processo de criopreservação do sêmen ocasiona a diminuição da fertilidade dos gametas, devido a danos estruturais e funcionais. Entre as várias causas, o estresse oxidativo, resultante da maior geração de espécies reativas de oxigênio (ROS), tem sido atribuído por afetar a qualidade seminal. Assim, objetivou-se avaliar o efeito do Trolox sobre os espermatozoides caprinos e ovinos submetidos à congelação. Pools de sêmen caprino (n=5) e ovino (n=6) foram diluídos em diluidores a base de leite desnatado (Glicerol 7%) ou Tris-gema de ovo (Glicerol 5%), respectivamente, adicionados ou não de Trolox (0, 20 ou 40 μM/mL), e congelados. Após descongelação (37 °C/30 s), alíquotas foram avaliadas quanto à peroxidação lipídica, por cromatografia líquida de alta performance acoplada a detector de arranjo

de diodos (HPLC-DAD) e por citometria de fluxo (C11-BODIPY581/591), à integridade de membrana plasmática e acrossomal, por microscopia fluorescente, e à cinética espermática, pelo sistema computadorizado de análise espermáticas (CASA). O tratamento antioxidante com Trolox não determinou efeitos significativos (p>0,05) sobre a peroxidação lipídica, integridade de membranas plasmática e acrossomal e parâmetros cinéticos avaliados. Assim, conclui-se que o Trolox (20 ou 40 μM) não apresenta efeito protetor ou nocivo sobre os espermatozoides caprinos e ovinos submetidos à congelação.
Palavras-chave: Antioxidantes; Lipoperoxidação; Ruminante; Sêmen; Vitamina E.

Resumen
El proceso de criopreservación del semen provoca una disminución de la fertilidad de los gametos debido a daños estructurales y funcionales. Entre las diversas causas, el estrés oxidativo, resultante de la mayor generación de especies reactivas de oxígeno (ROS), ha sido atribuido por afectar a la calidad seminal. Así, se pretendía evaluar el efecto de Trolox en los espermatozoides de cabra y oveja sometidos a congelación. Se diluyeron pooles de semen de cabras (n=5) y ovejas (n=6) en diluyentes a base de leche desnatada (Glicerol 7%) o de Tris-gema de huevo (Glicerol 5%), respectivamente, añadidos o no de Trolox (0, 20 o 40 μM/mL), y sometido a congelación. Tras la descongelación (37 °C/30 s), se evaluó la peroxidación lipídica de las alícuotas, mediante cromatografía líquida de alto rendimiento acoplada a un detector de matriz de diodos (HPLC-DAD) y también mediante citometría de flujo (C11-BODIPY581/591), la integridad de las membranas plasmáticas y acrosómicas, por médio de microscopía de fluorescencia, y la cinética de los espermatozoides, mediante el sistema computarizado de análisis de esperma (CASA). El tratamiento antioxidante con Trolox no determinó efectos significativos (p>0,05) sobre la peroxidación lipídica, la integridad de las membranas plasmáticas y acrosomales y los parámetros cinéticos evaluados. Así pues, se concluye que Trolox (20 o 40 μM) no presenta un efecto protector o perjudicial sobre los espermatozoides de cabra y oveja sometidos a congelación.
Palabras clave: Antioxidantes; Lipoperoxidación; Rumiantes; Semen; Vitamina E.

1. Introduction

The semen cryopreservation process determines cellular changes that contribute to the fertility reduction, when compared to fresh semen (Bicudo et al., 2007), being the plasma membrane one of the most affected structures (Castro et al., 2016). These changes in sperm cells are associated with the biochemical, osmotic, thermal and mechanical stresses, which are seen at different stages of freezing process (Gangawar et al., 2016). Additionally, reactive oxygen species (ROS) can play a negative impact on sperm integrity during the cryopreservation, which have as main substrate the polyunsaturated fatty acids, mainly present in the cell membranes (Colagar et al., 2013; Castro et al., 2016).

The ROS reaction with cellular polyunsaturated fatty acids (PUFA), start a chain process known as lipid peroxidation (LPO) (Bollwein & Bittner, 2018). Because of the sperm damages, caused by ROS, occur a reduction in semen quality, that includes abnormalities in the intermediate piece, motility decrease, loss of the sperm capacity to perform the acrosome reaction, apoptosis acceleration and fertility impairment (Aitken et al., 1993). Thus, the evaluation and control of the oxidative status and antioxidant defenses system is important as an indicator of the male fertility, especially (Colagar et al., 2013).

Trying to minimize the sperm damage, caused by LPO, antioxidants have been tested (Solihati et al., 2018; Ugur et al., 2019). Among these, vitamin "E" and its hydro-soluble analog, Trolox, stands out. It is indicated as an excellent protector against lipid peroxidation and has been successfully tested in ram sperm (Maia et al., 2010; Sicherle et al., 2011; Silva et al, 2013). Sperm from humans (Taylor et al., 2009; Minaei et al., 2012), ram (Silva et al., 2013) and boar (Pena et al., 2003; Zanella et al., 2016), cryopreserved with Trolox supplementation, present better motility, that do of this agent an important ally to the semen conservation.

Based on the exposed above, was objected in this study evaluated the Trolox effect on goat and ram cryopreserved sperm, through the lipid peroxidation, membranes integrity and kinematics evaluation.

2. Methodology

All reagents used to the scientific research were purchased from Sigma-Aldrich (St. Louis, USA), with the exception of 2-tiobarbituric acid (MERCK, Darmstadt, Germany), potassium hydroxide (VETEC, Rio de Janeiro, Brazil), sodium hydroxide (NUCLEAR, São Paulo, Brazil) and methanol (CARLOS ERBA, Val de Reuil, France). Moreover, national and institutional guidelines for the care and use of animals were followed, having been the animal procedures approved by the Ethics Committee on the Use of Animals (UFRPE Process Number CEUA / UFRPE 014/2012).

To perform the study around the Trolox effect on goat and ram cryopreserved sperm, were used five goats and six ram sexually mature, with a fertility history and handled intensively. The animals were feed with Tifton hay and 400 g/day of commercial concentrated, besides water and mineral salt *ad libitum*. From these, ejaculated were obtained by artificial vagina, in presence of a female as a dummy (Oliveira et al., 2013), in alternate days, totaling five ejaculated per goats and six per ram (25 and 36 ejaculated, respectively). The semen samples were subjectively evaluated macroscopic and microscopically, under a phase contrast microscope (Olympus, Japan; 100x), and only ejaculated with motility ≥ 70% (CBRA, 2013) were approved and destined to form the semen *pools*.

Each experimental repetition corresponded to a semen *pool*, formed by 5 goats or 6 ram ejaculated, totaling 5 and 6 *pools* per experiment, respectively. It was done to eliminate the individual variation (Bucak et al., 2008). The minimum requirements to freeze the seminal *pool* were: volume ≥ 3.0 mL, motility ≥ 70% and sperm concentration ≥ $2x10^9$ sperm/mL. Spermatic motility was subjectively analyzed using a phase contrast microscope (Olympus, Japan; 100x) and spermatic concentration in a Neubauer chamber (400x), after dilution of semen aliquots in saline formalin solution (1:400) (CBRA, 2013).

For cryopreservation, was used the protocol described by Silva et al. (2019), where the goat semen *pools* were diluted (1:9) in Tris buffer solution and centrifuged twice (3000 rpm; 10 min). Then, the spermatozoa *pellets* were diluted in skimmed-milk based extender, to a concentration of $200x10^6$ sperm/mL, fractionated and treated, according to the experimental groups (G1=control, G2=20 µM Trolox and G3=40 µM Trolox).

The diluted semen was packaged in straws (0.25 mL) and frozen in an automated system (TK 3000® - TK Tecnologia em Congelação Ltda., Brazil). The cooling curve used showed a drop of -0.25 °C/min in temperature until reaching 5 °C, at which the material remained for 120 min (stabilization time). Then the freezing curve was initiated, with a drop of -20 °C/min, until reaching -120 °C, when the straws were immersed and stored in liquid nitrogen (-196 °C).

Regarding the ram semen *pools*, they were diluted in Tris-egg yolk extender (Silva et al., 2012), for the final concentration of 200 $x10^6$ sperm/mL, fractionated and treated according to the experimental groups (G1=control, G2=20 µM and G3=40 µM of Trolox). Subsequently, the samples were stored in straws (0.25 mL) and frozen in an automated system, as previously described for goat semen.

The evaluation of the lipid peroxidation, normally associated with sperm damages during cryopreservation (Aitken et al., 1993; Bollwein & Bittner, 2018) and acting as an indicator of the male fertility (Colagar et al., 2013), was did dosing MDA (malondialdehyde) by HPLC according Candan & Tuzmen (2008), with modifications. For this, work solutions were prepared and, previously to the lipoperoxidation analysis in semen samples, a standard curve was prepared with TEP. The TEP working solution (40 nmol/mL; 2 mL TEP stock solution, 50 mL sulfuric acid) was diluted in series using water to standard concentrations of 10, 5, 2.5, 1.25 and 0.625 nmol/mL, and incubated for 2 h at room temperature.

To a volume of 500 µL of each standard sample, 750 µL H_3PO_4 (440 mM) and 50 µL TBA (40 mM) were added, and the resulting mixtures heated for 1 h at 100 °C to promote the derivatization reaction. At room temperature, an aliquot (500 µL) of these standards was mixed with 500 µL of the precipitation reagent [MeOH:1 M NaOH (91:9)], filtered through a membrane filter (0.22 µm) and evaluated.

For the analysis of lipid peroxidation by HPLC in frozen semen, four semen straws of each experimental group and species were thawed in a water bath (37 °C/30 s) and processed as described by Candan & Tuzmen (2008), with modifications. In a cover glass tube, mixtures of 200 µL semen, 750 µL H_3PO_4 (440 mM) and 50 µL TBA (40 mM) were heated for 1 h at 100 °C. After reaching room temperature, 500 µL was transferred to a microcentrifuge tube and added of 500 µL precipitation reagent [MeOH: 1 M NaOH (91:9)]. The samples were centrifuged (13,000 rpm for 5 min) and the supernatant was removed and filtered (0.22 µm membrane filter).

The chromatographic separations were performed using Rexchorm ODS column (150 mm x 4.6 mm x 5 µm) and Security Guard Catridge Holder guard column at 30 °C, after injection of 400 µL of each standard in the HPLC equipment (Shimadzu Prominence, model LC-20AT). Methanol (A) and potassium phosphate buffer solution (50 mM, pH=6.8, B) were used as a mobile phase (40:60), with a flow of 0.6 mL/min. The chromatograms were recorded at wavelength of 532 nm for 8 min, and the sample injection volume was 10 µL. All samples were prepared and analyzed in triplicate and the adduct concentrations expressed in nmol/mL.

The modified methodology of Partyka et al. (2011) was used to lipid peroxidation analysis, employing C11-BODIPY$^{581/591}$ fluorescent probe [4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; Invitrogen^{TM}, Eugene, Oregon, USA]. Thawed semen samples were centrifuged (1500 rpm for 5 min) twice in PBS (Buffered Saline Phosphate), resuspended in this with 1 µL C11-BODIPY$^{581/591}$ (2 mM) and incubated (37 °C for 30 min) in the dark. Subsequently, the samples were washed in PBS to remove the disconnect probe and analyzed by flow cytometry (flow cytometer BDFACSAria II; Becton Dickinson, EUA). The data were collected from BD FACSDivaTM Software (Becton Dickinson), where 10000 cells were evaluated and classified as oxidized, when green, or as non-oxidized, when red.

Regarding the study of plasma membrane integrity (PMi), parameter positively correlated with fertility (Lee et al., 2009) and most affected by semen cryopreservation (Castro et al., 2016), it was performed by double staining with carboxifluorescein diacetate (CFD) and propidium iodide (PI) (Silva et al., 2019). A total of 200 spermatozoa were evaluated under an epifluorescence microscope (Carl Zeiss, Germany; 400x) and classified as carriers of intact membrane, when stained in green, or damaged membrane, when stained in red.

The acrosome integrity, essential to the fertility (Fernandes & Pimentel, 1997), was evaluated through fluoresceine isothiocyanate-conjugated peanut agglutinin (FITC-PNA) (Silva et al., 2019). A total of 200 spermatozoa per slide were analyzed under a fluorescence microscope (Carl Zeiss, Germany) and classified as having intact acrosome (Aci), when stained in green, or reacted acrosome (Acr), when stained in mixed green, without staining or stained only in the equatorial region of the sperm head.

For the study of sperm kinematics, significantly compromised with the cryopreservation process (Anand & Yadav, 2016), an aliquot of semen was diluted in Tris solution (1:3) to reduce the cell concentration and the medium density, avoiding the cell superposition and facilitating the images capture. As Silva et al. (2019), the samples were evaluated under a phase contrast microscope (Eclipse 50i; Nikon, Japan; 100x), coupled with a Basler A312FC digital camera (Basler Vision Technologies, Germany), and the kinetic parameters analysis by the SCA software ™ v 5.1 (Microptics, Spain). Were considered the total motility (TM, %), progressive motility (PM, %), linearity (LIN, %), straight linearity (STR, %) and oscillation index (WOB, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s) and mean trajectory velocity (VAP, µm/s).

The statistical analyses were performed using GraphPad InStat (version 3.10, 2009). The data obtained to lipid peroxidation, plasma membrane integrity, acrosome integrity and sperm kinematic were tested for normality and homogeneity of variance by Kolmogorov-Smirnov method and Bartlett´s test. Subsequently, these were submitted to analysis of variance (ANOVA), according to each species and experimental group, to determine the effects of the treatments and their interactions,

considering significance of 5%. When presented significance, Tukey-Kramer multiple comparison test was realized for mean comparisons. The results were expressed as means and standard deviation (means ± SD).

3. Results

The results demonstrated that the Trolox addition, at 20 or 40 µM concentrations, to the goat and ram semen cryopreservation extender does not determine significant differences ($p>0.05$) in the levels of MDA or in the percentage of cells marked with C11-BODIPY$^{581/591}$, when compared to the control group (Table 1).

Table 1 - Malonaldehyde concentration (nmol/mL) and percentage (%) of spermatozoa marked to peroxidation by C11-BODIPY581/591 fluorescent probe (mean ± SD) in samples of goat and ram cryopreserved semen, with or without the addition of Trolox.

Parameters	Experimental Groups		
	Control	**Trolox 20 µM**	**Trolox 40 µM**
Goat			
MDA (nmol/mL)	0.2±0.1	0.2±0.0	0.2±0.1
Cells with LPO (%)	2.1±0.1	2.1±0.0	2.2±0.0
Ram			
MDA (nmol/mL)	2.9±0.7	2.9±0.8	2.8±0.6
Cells with LPO (%)	2.5±0.0	2.4±0.0	2.6±0.3

MDA: malondialdehyde, LPO: lipoperoxidation. Source: Authors.

Similarly, to the above exposed, no differences ($p>0.05$) were observed between the experimental groups for sperm kinematics, plasma membrane integrity, and acrosome integrity of goat and ram semen, after freezing-thawing, in presence or absence of Trolox (Table 2).

Table 2 - Kinetics parameters, plasma membrane integrity and acrosome integrity (mean ± SD) of goat and ram cryopreserved sperm, with or without Trolox addition.

Spermatic Parameters	**Experimental Groups**		
	Control	**Trolox 20 µM**	**Trolox 40 µM**
Goat			
TM (%)	70.0±12.9	70.4±5.6	62.7±10.7
PM (%)	26.0±2.8	29.4±5.9	29.0±7.2
VCL (µm/s)	87.0±8.4	80.4±5.6	88.1±13.1
VSL (µm/s)	46.5±7.2	45.0±4.9	51.6±8.4
VAP (µm/s)	61.2±7.8	56.6±3.4	63.9±10.8
LIN (%)	53.6±8.7	56.2±7.1	58.6±5.5
STR (%)	75.8±7.4	79.4±6.0	80.8±3.9
WBO (%)	70.3±5.4	70.5±3.7	72.4±4.0
PMi (%)	51.3±8.4	58.3±9.0	51.6±10.5
Aci (%)	86.7±3.7	92.1±3.6	90.6±1.4
Ram			
TM (%)	62.7±7.2	66.0±10.6	69.7±7.8
PM (%)	32.8±4.6	36.4±9.2	33.1±6.2
VCL (µm/s)	88.0±18.4	85.3±12.6	87.0±9.9
VSL (µm/s)	63.4±14.2	62.1±11.0	58.7±9.2
VAP (µm/s)	76.8±17.5	74.7±12.5	74.4±10.3
LIN (%)	71.9±3.9	72.6±3.8	67.3±4.7
STR (%)	82.6±3.2	83.0±2.3	78.7±3.3
WBO (%)	86.9±2.2	87.4±2.4	85.3±2.9
PMi (%)	50.3±14.0	58.2±6.9	56.0±7.6
Aci (%)	61.5±7.0	64.5±6.5	64.9±8.8

TM: total motility, PM: progressive motility, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity, STR: straightness, WBO: oscillating index, PMi: plasma membrane integrity, Aci: acrosome integrity. Source: Authors.

4. Discussion

High ROS levels in semen lead to lipid peroxidation, that is a process harmful to spermatozoa, which may contribute to low motility, morphology and sperm count (Colagar et al., 2013). In this sense, a negative correlation between oxidative stress and sperm motility has been described in ram (Peris et al., 2007). However, in the present study, there was no decline in motility, as well as in any other kinetic parameter, plasma membrane and acrosome integrity after cryopreservation, regardless of antioxidant addition (0, 20 or 40 µM of Trolox), being the total motility higher than 70% for goats and 60% for ram.

Based on the above exposed, is evident the absence of a protective effect of Trolox, in the concentrations studied, on the sperm of goats and ram. These findings corroborate with Sicherle et al. (2011) that did not observe a positive action of this agent at 100 µM, on ram semen, after thawing, except when lipid peroxidation was induced. Similarly, Cabrita et al. (2011) when using 0.1 and 0.5 mM of α-tocopherol for freezing fish sperm, did not find a better preservation of gametes kinetics, viability or DNA integrity.

In opposition to the previous reports, Silva et al. (2013) showed that the Trolox addition at 60 or 120 µM to the egg yolk medium improved the parameters of PM, VSL, VAP, LIN, WOB, STR and PMi. Similarly, Azawi & Hussein, (2013) also saw improvement to plasma membrane integrity, but not to acrosome, for up to 120 h of refrigeration (5 °C) of ram sperm, added with 1 mg/mL of vitamin E.

The absence of protective effect in relation to the antioxidant studied can be attributed to factors such as the dose-dependent action (Aitken, 1995) once that, despite Silva et al. (2013) having used the same cryopreservation methodology of the present report, the antioxidant concentrations applied were higher. In addition, it is worth highlighting the specie-specific variation, and the composition of the medium to which the antioxidant is added (Guthrie & Welch, 2007; Lecewicz et al., 2018; Silva et al., 2019).

The use of 20 and 40 µM of Trolox did not reduce the concentrations of malonaldehyde present in cryopreserved semen of goats and ram, when compared to the control group. This fact also was observed by Sicherle et al. (2011) after the use of Trolox (100 µM/10^8 spermatozoa), during the process of ram semen frozen and thawed. On the other hand, inducing the lipid peroxidation with 0.24 nmol $FeSO_4$, these authors verified that the antioxidant became efficient in reduce this harmful process (Sicherle et al., 2011). The same was observed by Maia et al. (2010), when using Trolox (50 µM Trolox/10^8 spermatozoa) for ram semen freezing, as well as by Cerolini et al. (2000), when added α-tocopherol for boar semen refrigeration.

According to the above exposed, it is possible that the protective antioxidant effect of Trolox, beyond to be a dose-dependent way, is evidenced only in situations of extreme stress, as is the case of lipoperoxidation induction (Partyka et al., 2011). The variability of results obtained after Trolox therapy may be justified by factors as the influence caused by the iron concentration in semen samples over the ROS production (Peris et al., 2007), the quantity and associations of antioxidants added and present in the semen, as well as the variations in the sperm membrane lipid composition (Guthrie & Welch, 2007; Lecewicz et al., 2018).

5. Conclusion

It is concluded that Trolox (20 or 40 µM) does not favor the preservation of kinematic parameters, plasma membrane and acrosome integrity, as well as the reduction of lipid peroxidation degree in goat and ram sperm submitted to freezing. However, is important realize new investigations that consider a greater variety of antioxidant concentrations and correlated it with the specie and extender employed, as well as the induction or not of the lipid peroxidation.

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