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Motility and viability of ram sperm cryopreserved in a Tris-egg yolk extender supplemented with anti-oxidants

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ABSTRACT

The objective of this study was to evaluate the effects of the addition of the anti-oxidants Trolox and catalase to a ram semen cryopreservation extender, on the basis of post-thaw sperm motility, plasma membrane integrity, acrosomal membrane integrity and mitochondrial membrane potential. Semen from 23 Santa Inês rams was collected using an artificial vagina, and one ejaculate from each ram was evaluated and included in the study. Semen samples were diluted to a concentration of 400×10^6 cells/ml in Tris-egg yolk extender (control) or the same extender supplemented with 50 μM Trolox/10^8 sperm (TRO), 50 μg catalase/ml (CAT) or a combination of Trolox and catalase (TROCAT). The semen was loaded into 0.25 ml straws, cooled and frozen in a programmable freezer and subsequently stored in liquid nitrogen. Frozen straws were thawed in a water bath for sperm evaluation. Post-thaw sperm motility characteristics were determined by CASA, and plasma membrane integrity, acrosome integrity and mitochondrial membrane potential were assessed using PI/FITC-PSA/JC-1 probes. Total sperm motility was significantly higher ($P < 0.05$) in the control (72.0 ± 1.5%) and in the samples in which catalase was included in the extender (CAT = 69.0 ± 1.5%), compared to samples containing only Trolox (TRO = 65.0 ± 1.5%) or Trolox in combination with catalase (TROCAT = 61.0 ± 1.5%). Progressive motility was significantly ($P < 0.05$) higher in the CAT extender (31.0 ± 1.0%) than in the TRO (26.0 ± 1.1%) and TROCAT (27.0 ± 1.1%) supplemented extenders. Catalase supplementation resulted in a significantly ($P < 0.05$) higher percentage of viable sperm (35.5 ± 1.3%) following cryopreservation, than the control (29.9 ± 1.3%) and TROCAT (28.1 ± 1.3%) supplementation. Anti-oxidant supplementation did not improve mitochondrial activity. The total number of sperm showing a high mitochondrial membrane potential was low in all treatments. The percentage of viable sperm was positively related with total sperm motility, progressive motility and rapid movement, while a negative correlation was observed with the percentage of slow and static sperm. Results indicate that supplementation of the extender with the anti-oxidant catalase improves sperm viability and, to a lesser extent, the progressive motility of ram sperm after cryopreservation in a Tris-egg yolk extender.

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1. Introduction

Cervical artificial insemination (AI) using frozen ram semen has limited use due to the low levels of fertility obtained, compared to that obtained using fresh semen. The processes of freezing and thawing of the semen result in ultrastructural, biochemical and functional damage to sperm in several mammalian species (Watson, 2000; Bailey et al., 2000). Some factors that contribute to the reduced viability of cryopreserved ram sperm include extender composition, cryoprotectant concentration and the cooling, freezing and thawing rates, as well as the quality of semen used (Salamon and Maxwell, 1995, 2000). In the last few years, studies carried out on bovine (Chatterjee and Gagnon, 2001), ovine (Sarlós et al., 2002; Kasimanickam et al., 2006; Bucakc et al., 2008) and equine semen (Ball et al., 2001) have shown that reactive oxygen species (ROS) are produced during the freezing and thawing processes. Sperm are very susceptible to this oxidative damage, because of the high concentration of polyunsaturated fatty acids present in the plasma membrane (Aitken, 1995). Oxidative stress caused by the high concentration of ROS in semen may thus affect energy metabolism, motility, viability and DNA integrity in the stallion, bull, ram, buck and human sperm (Armstrong et al., 1999; Krzyzosiak et al., 2000; Baumber et al., 2002; Bilodeau et al., 2002). Therefore, several anti-oxidants have been evaluated regarding their capacity to prevent oxidative stress in semen.

Studies have demonstrated that the addition of antioxidants to the extender improves sperm motility and viability of bovine (Krzyzosiak et al., 2000; Bilodeau et al., 2002), equine (Baumber et al., 2002) and ovine semen (Maxwell and Stojanov, 1996; Maia et al., 2007). This addition has also been shown to enhance the in vitro fertilization capacity of ram sperm (Maxwell and Stojanov, 1996; Sarlós et al., 2002).

It has been observed that catalase can act as an antioxidant to protect mammalian sperm from oxidative stress in equine (Baumber et al., 2002), bovine (Krzyzosiak et al., 2000; Bilodeau et al., 2002) and ovine semen (Maxwell and Stojanov, 1996). Likewise, vitamin E and Trolox have demonstrated that they are effective at inhibiting lipid peroxidation in swine (Breininger et al., 2005), bovine (Beconi et al., 1993) and ovine semen (Jones and Mann, 1976). However, the addition of vitamin E to ram semen does not inhibit lipid peroxidation (Sarlós et al., 2002).

This trial was performed to evaluate the effect of the anti-oxidants Trolox and catalase on sperm motility, membrane integrity and mitochondrial activity in post-thawed ram sperm.

2. Materials and methods

2.1. Semen collection and processing

Semen was collected from 23 Santa Inês rams (Ovis aries) using the artificial vagina. After the evaluation of semen parameters, namely volume, sperm concentration, motility and the morphological characteristics of the sperm, the ejaculate of each ram was processed. Only ejaculates with a sperm progressive motility greater than 70% were used in the study. Each ejaculate was divided into 4 equal aliquots and diluted to a concentration of 400×10^6 sperm/ml with a Tris-based extender composed of 2 fractions. The Fraction 1 solution contained 250.25 mM Tris-hydroxymethyl-aminomethane, 79.71 mM monohydrated citric acid, 9.99 mM glucose, 20.0% (v/v) egg yolk, 1.0% (v/v) Orvus ES Paste, 100 mg/100 ml streptomycin sulfate and 100 000 IU Potassic Penicillin (pH 6.91; osmolarity: 308 mOsm). Fraction 2 solution had the same composition as the Fraction 1 solution with the addition of 14.0% glycerol (v/v). Semen was diluted at 32 °C in a two-step process. The semen was initially diluted in the Fraction 1 solution, and the Fraction 2 solution was subsequently added. Samples of diluted semen were then supplemented with either Trolox (TRO, 50 μM Trolox/10^8 sperm), catalase (CAT, 50 μg catalase/ml), Trolox plus catalase (TROCAT, 50 μM Trolox/10^8 sperm, 50 μg catalase/ml) or no anti-oxidant (control). Diluted semen was then loaded into 0.25 ml French straws, sealed with polyvinyl alcohol, cooled and frozen in a programmable freezer (Tetakon® -TK 3000, TK Tecnologia em Congelação Ltda, Uberaba, MG, Brazil). During cryopreservation, the temperature was decreased from 32 to 5 °C at a rate of 0.25 °C/min. After reaching 5 °C, the samples of semen were stabilized at this temperature for 90 min. Subsequently, the temperature was decreased from 5 to −120 °C at a rate of 20 °C/min. After freezing, the straws were plunged into liquid nitrogen for storage until further evaluation. When further evaluated, the frozen straws were thawed at 42 °C for 20 s in a water bath and immediately microscopically evaluated.

2.2. Evaluation of sperm motility using CASA

For evaluation of sperm motility, one straw from each treatment was thawed, and an aliquot of 33 μl (13.2×10^6 sperm) of the thawed semen was diluted with 250 μl of X-Cell extender (IMV, France), maintained at 37 °C for 5 min and then submitted to evaluation using a computer-assisted sperm analysis (CASA). A sample of 10 μl of diluted semen was placed in a pre-warmed MAKLER chamber (37 °C) and transferred to the CASA analyzer (Hamilton-Thorn Biosciences IVOS 12, Beverly, MA, USA). For each semen sample, three fields were selected at random and evaluated by counting an average of 450 sperm/sample. The following sperm motility parameters were evaluated: total motility (TM; %), progressive motility (PM; %), sperm with rapid movement (RAPID; %), sperm with slow movement (SLOW; %), sperm with no movement (STATIC; %), average path velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s), straight-line velocity (VSL; μm/s), amplitude of lateral head displacement (ALH; μm), beat-cross frequency (BCF; Hz), straightness (STR; %) and linearity (LIN; %) (Moses et al., 1995).

The CASA setup parameters used were frames acquired = 30; frames per second = 60; minimum contrast = 56; minimum cell size = 5; threshold straightness = 80.0; medium VAP cut off = 80.0; low VAP cut off = 20.0; low VSL cut off = 0.0; non-motile head size = 2; non-motile head intensity = 50.

2.3. Evaluation of the membrane integrity and mitochondrial membrane potential

Simultaneous evaluation of the integrity of sperm plasma and acrosomal membranes and mitochondrial function were determined using a stain with the fluorescent probes propidium iodide (PI, SIGMA, São Paulo, SP, Brazil), Fluorescein isothiocyanate conjugated to *Pisum sativum* agglutinin (FITC-PSA, SIGMA, São Paulo, SP, Brazil) and 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetra-ethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes – Invitrogen, São Paulo, SP, Brazil) according to Celeghini's technique (Celeghini et al., 2007), as modified by Azevedo et al. (2007). An aliquot of 60 μl of thawed semen was diluted in X-Cell medium to a final concentration of 25×10^6 sperm/ml. It was then placed into a warmed microcentrifuge tube and 1.5 μl PI (0.5 mg/ml in saline solution), 1.5 μl JC-1 (153 μM in DMSO) and 25 μl FITC-PSA (100 μg/ml in PBS) solutions added. After incubation at 37 °C for 8 min, a drop of the sample was placed on a slide, covered with a coverslip and evaluated immediately by epifluorescent microscopy (Leica DMLB, Leica Microsystems, Leitz, Germany) using a filter I3 (excitation BP 450–490 nm, suppression LP 515 nm) at 1000× magnification.

From each treatment, 100 sperm per slide were evaluated and classified into 8 categories: C1-intact plasma membrane, intact acrosome and high mitochondrial membrane potential; C2-intact plasma membrane, intact acrosome and low mitochondrial membrane potential; C3-intact plasma membrane, damaged acrosome and high mitochondrial membrane potential; C4-intact plasma membrane, damaged acrosome and low mitochondrial membrane potential; C5-damaged plasma membrane, intact acrosome and high mitochondrial membrane potential; C6-damaged plasma membrane, intact acrosome and low mitochondrial membrane potential; C7-damaged plasma membrane, damaged

Table 1
The mean (±SE) effect of extender on post-thaw ram sperm motility parameters as determined by computer-assisted sperm analysis (CASA).

Motility parameter	Extender			
	Control ($n=23$)	TRO ($n=23$)	CAT ($n=23$)	TROCAT ($n=23$)
TM (%)	72.0 ± 1.5[a]	65.0 ± 1.5[b]	69.0 ± 1.5[a]	61.0 ± 1.5[b]
PM (%)	29.0 ± 1.1[ab]	26.0 ± 1.1[c]	31.0 ± 1.1[a]	27.0 ± 1.1[bc]
RAPID (%)	47.0 ± 1.5[a]	41.0 ± 1.5[b]	46.0 ± 1.5[a]	39.0 ± 1.5[b]
SLOW (%)	9.0 ± 0.3[bc]	8.0 ± 0.3[c]	9.5 ± 0.3[ab]	10.0 ± 0.3[a]
STATIC (%)	19.0 ± 1.5[b]	27.0 ± 1.5[a]	21.0 ± 1.5[b]	29.0 ± 1.5[a]
VAP (μm/s)	95.4 ± 1.2[a]	92.6 ± 1.2[b]	96.0 ± 1.2[a]	91.7 ± 1.2[b]
VCL (μm/s)	169.7 ± 1.6[a]	163.7 ± 1.6[b]	168.5 ± 1.6[a]	160.7 ± 1.6[b]
VSL (μm/s)	73.1 ± 1.1	72.3 ± 1.1	75.2 ± 1.1	72.4 ± 1.1
ALH (μm)	7.7 ± 0.1[a]	7.4 ± 0.1[b]	7.5 ± 0.1[b]	7.6 ± 0.1[ab]
BCF (Hz)	36.6 ± 0.4	37.1 ± 0.4	37.1 ± 0.4	36.4 ± 0.4
STR (%)	73.0 ± 0.4[b]	75.0 ± 0.4[a]	75.0 ± 0.4[a]	74.0 ± 0.4[a]
LIN (%)	43.0 ± 0.5[b]	45.0 ± 0.5[a]	45.0 ± 0.5[a]	45.0 ± 0.5[a]

[abc]Values in rows with different superscripts differ significantly ($P<0.05$). TM, total motility; PM, progressive motility; RAPID, sperm with rapid movement; SLOW, sperm with slow movement; STATIC, sperm without movement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; STR, straightness; LIN, linearity.

acrosome and high mitochondrial membrane potential and C8-damaged plasma membrane, damaged acrosome and low mitochondrial membrane potential.

The sperm in categories C1–C4 were all alive (intact plasma membrane), regardless of whether their mitochondrial membrane potential was high or low or their acrosomes were intact or damaged. These results were grouped together in one category, named viable. Sperm classified in categories C5–C8 were all dead (damaged plasma membrane), regardless of their high or low mitochondrial membrane potential or their intact or damaged acrosomes. The sperm in these categories were grouped together in one category, named non-viable. The same was done regarding mitochondrial membrane potential. The results were grouped in high mitochondrial membrane potential (HMP) or low mitochondrial membrane potential (LMP) groups, regardless of whether their plasma membrane and acrosome were intact or not.

2.4. Statistical analysis

One ejaculate from each of the 23 rams was used in the 4 treatments (TRO, CAT, TROCAT and control), totaling 92 samples. The values of sperm motility, membrane integrity and mitochondrial membrane potential in each treatment were expressed as the mean ± S.E.M. Data were analyzed using a one-way ANOVA to determine the effect of the anti-oxidant treatment on the sperm motility parameters, plasma membrane integrity, acrosomal membrane integrity and mitochondrial membrane potential. When statistically significant differences were detected, the Duncan's test was used to compare the means and standard errors. The relationship between the different parameters was analyzed using Pearson's correlation coefficients. A P value < 0.05 was considered to be significant. All analyses were performed using Statgraphics (Statistical Graphics Corporation, Nottingham, UK) software.

3. Results

3.1. Sperm motility

The effects of the addition of the anti-oxidants Trolox and catalase on post-thaw sperm motility and velocity parameters are set out in Table 1. The percentage total motility (TM) was significantly ($P<0.05$) higher in samples cryopreserved with a control extender and in the catalase (CAT)-supplemented samples, than in the samples cryopreserved with Trolox (TRO) or the combination of catalase and Trolox (TROCAT). The percentage progressive motility (PM) was significantly ($P<0.05$) higher in the CAT samples than in the TRO or TROCAT samples and only slightly higher in the CAT samples than the control samples. The percentage rapid sperm movement (RAPID) was significantly ($P<0.05$) higher in the control and CAT samples than in the TRO and TROCAT samples. The percentage slow sperm movement (SLOW) was significantly ($P<0.05$) lower in the TRO samples, compared to the CAT and TROCAT samples and similar to the control group. Lastly the percentage sperm with no movement (STATIC) was higher ($P<0.05$) in the TRO and TROCAT supplemented samples, than in the CAT and control samples.

The addition of Trolox, catalase or the combination of catalase and Trolox to the extender increased ($P<0.05$) the straightness (STR) and linearity (LIN), when compared to the control. Conversely, the addition of TRO or CAT decreased ($P<0.05$) the amplitude of lateral head displacement (ALH) when compared to the control. The path velocity (VAP) and curvilinear velocity (VCL) were lower ($P<0.05$) in the TRO and TROCAT samples than in the other samples. No significant effect was recorded for Trolox and catalase supplementation on the straight-line velocity (VSL) and beat-cross frequency (BCF).

3.2. Membrane integrity and mitochondrial membrane potential

The percentage of viable sperm in the cryopreserved semen differed according to the extender used (Table 2). The addition of catalase to the extender increased ($P<0.05$) the percentage of post-thaw viable sperm, compared to the control group and the percentage of non-viable sperm (damaged plasma membranes) was higher ($P<0.05$) in the control samples, compared to the CAT samples.

The percentage of sperm with low mitochondrial membrane potential was high in all extenders, however, anti-oxidant supplementation did not result in an increase in the number of sperm showing high mitochondrial activity. The percentage of sperm with high mitochondrial membrane potential was significantly ($P<0.05$) higher in the control samples, compared to the CAT or TRO samples (Table 2).

As shown in Table 3, the percentage of viable sperm was positively correlated with the sperm motility parameters, TM, PM and RAPID, and inversely correlated with SLOW

Table 2
The mean (±SE) effect of extender on plasma membrane integrity and mitochondrial membrane potential of frozen–thawed ram semen.

Fluorescence classification	Extender			
	Control ($n = 23$)	TRO ($n = 23$)	CAT ($n = 23$)	TROCAT ($n = 23$)
Viable (%)	29.9 ± 1.3[b]	31.6 ± 1.3[ab]	35.5 ± 1.3[a]	28.1 ± 1.3[b]
Non-viable (%)	70.1 ± 2.0[a]	68.4 ± 2.0[ab]	64.5 ± 2.0[b]	71.9 ± 2.0[a]
LMP (%)	97.7 ± 0.3 [b]	98.8 ± 0.3[a]	98.7 ± 0.3[a]	98.3 ± 0.3[ab]
HMP (%)	2.3 ± 0.3[a]	1.2 ± 0.3[b]	1.3 ± 0.3[b]	1.7 ± 0.3[ab]

[ab]Values in rows with different superscripts differ significantly ($P < 0.05$). Viable, intact plasma membrane; non-viable, damaged plasma membrane; LMP, low mitochondrial membrane potential; HMP, high mitochondrial membrane potential.

and STATIC. The percentage of non-viable sperm was positively correlated to SLOW and STATIC sperm and inversely correlated to the TM, PM and RAPID sperm. The percentage of high mitochondrial membrane potential (HMP) sperm was inversely correlated with STATIC sperm. No correlations were recorded between TM, PM, RAPID and SLOW parameters.

4. Discussion

The addition of anti-oxidants to cryopreservation medium was performed to protect the sperm against damages caused by ROS to motility, viability, energy production and DNA integrity (Armstrong et al., 1999; Krzyzosiak et al., 2000; Bilodeau et al., 2002). In this study, the addition of catalase or Trolox to the Tris extender resulted in certain improvements in the quality of the frozen–thawed sperm.

The addition of catalase increased the viability (as revealed by the intactness of the plasma membrane) of the sperm, but did not improve the sperm motility, when compared to the control. Catalase as such works by removing the hydrogen peroxide from the medium, thus preventing the generation of hydroxyl radicals, which are powerful oxidants, by the Fenton reaction (Halliwell and Gutteridge, 1999). As hydrogen peroxide is involved in sperm capacitation and the acrosome reaction (O'Flaherty et al., 2003), the freezing of semen in an extender containing catalase possibly avoids premature capacitation and subsequent cell death. This effect may explain the current findings of higher viability when catalase was added to the extender.

The reason for the lack of an effect on sperm motility when catalase is added to the semen, is unclear. One reason may be a low level of endogenous catalase activity in frozen–thawed ram semen, as reported by Bucak et al. (2008). Therefore, even if semen is supplemented with catalase, its activity could not reach the necessary level to promote an improvement in sperm motility. Conversely, it is well know that cryopreservation and thawing induce membrane damage and thus impair sperm motility. A positive correlation between viability and both total and progressive sperm motility was observed in the present study. Nevertheless, the higher percentage of viable sperm in the CAT samples was not associated with higher total sperm motility. Surprisingly, the total sperm motility was higher in the control samples, which showed a higher percentage of non-viable sperm. Garner et al. (1997), utilizing dual staining (PI plus JC-1), identified a population of moribund sperm that exhibited red heads (non-viable) with green mitochondria (signifying a low mitochondrial membrane potential). Ollero et al. (1998) also reported that many sperm that exhibit damaged membranes are motile after thawing. Thus, the results indicate that a percentage of non-viable sperm that continued to exhibit slower motility were included in the population of motile sperm, increasing the percentage of total sperm motility in the control sample. This is evidenced by the positive correlation between non-viable sperm and SLOW and the lower percentage of STATIC sperm found in the control sample.

The plasma membrane of ram sperm is rich in polyunsaturated fatty acids, and this makes it susceptible to peroxidative damage (Jones and Mann, 1976). Lipid peroxidation induces membrane damage (Aitken, 1995), and this has been negatively correlated with sperm motility (Beorlegui et al., 1997; Breininger et al., 2005; Kasimanickam et al., 2006). Trolox, a water-soluble analog of vitamin E, is a chain-breaking anti-oxidant that acts as scavenger of lipid peroxyl radicals (Barclay et al., 1995), thus preventing lipid peroxidation. Its protective effect against oxidative damage has been demonstrated in human cells (Wu et al., 1990) and bovine embryos (Feugang et al., 2004). In the current study, sperm treated with Trolox did not exhibit improved motility and only showed slightly increased viability, when compared to control sperm. However, a previous study (Maia et al., 2007) showed that the Trolox effect on post-thaw motility of ram sperm was dose-dependent and varied with

Table 3
Correlation coefficients (r) between sperm viability (viable, non-viable), mitochondrial membrane potential and ram sperm motility parameters.

	Viable		Non-viable		HMP	
	r	P	r	P	r	P
Total motility	0.68	<0.0001	−0.68	<0.0001	0.14	NS
Progressive motility	0.62	<0.0001	−0.62	<0.0001	0.11	NS
RAPID	0.65	<0.0001	−0.66	<0.0001	0.08	NS
SLOW	−0.39	<0.0001	0.41	<0.0001	0.04	NS
STATIC	−0.65	<0.0001	0.64	<0.0001	−0.15	<0.0441

HMP, high mitochondrial membrane potential; RAPID, sperm with rapid movement; SLOW, sperm with slow movement; STATIC, sperm without movement.

the freezability of semen. Trolox concentrations from 50 to 100 μM/10^8 sperm increased the sperm motility, while concentrations above 100 μM/10^8 sperm induced a reduction in sperm motility parameters. In boar semen, Trolox also resulted in significant increases in sperm motility, with the effect depending on the concentration of the antioxidant and on the fraction of the ejaculate (Peña et al., 2003). In stallion semen, Trolox failed to improve sperm motility (Ball et al., 2001). From these studies in different species, it is clear that the addition of Trolox can be beneficial to sperm. The lack of a marked effect of Trolox on sperm motility and viability in the current work may be due to the dose used or to differences in the freezability of sperm between rams, causing the sperm to be more or less vulnerable to Trolox, as has been previously reported (Maia et al., 2007).

The evaluation of mitochondrial membrane potential has been used as a tool to measure mitochondrial function and its relation to ATP synthesis. It has also been reported that mitochondrial activity is directly related to sperm motility (Gravance et al., 2001; Martinez-Pastor et al., 2004; Espinoza et al., 2009). In the present study, the addition of anti-oxidants to the extender did not improve mitochondrial function, when compared to the control extender. After cryopreservation, the percentage of sperm with high mitochondrial membrane potential was extremely low for all treatments, varying between 1.22% and 2.26%. No correlation was found between mitochondrial activity and sperm motility. Garner and Thomas (1999) reported that the proportion of sperm exhibiting J-aggregates (high mitochondrial membrane potential) was markedly reduced after cryopreservation. Thus, it is possible that the anti-oxidants Trolox and catalase do not protect the mitochondrial membrane against cryodamage. Additionally, as the generation of ATP inside mitochondria is a membrane-dependent process, any damage to the cell membrane could negatively affect mitochondrial ATP generation and consequently the mitochondrial membrane potential. Without sufficient energy, sperm are not progressively motile. Nevertheless, the lack of a correlation between the mitochondrial membrane potential and sperm motility (TM and PM) does not reflect this. Therefore, it can be concluded that the measurement of mitochondrial membrane potential with JC-1, as assessed in this study, does not reflect the real mitochondrial activity of ram sperm. This is in agreement with Meseguer et al. (2004), who stated that mitochondrial activity is not a good indicator of sperm metabolism.

5. Conclusion

The addition of catalase and Trolox to Tris-egg yolk extender as tested in this trial improved the viability and, to a lesser extent, the motility of ram sperm after thawing. However, the ideal concentration of Trolox still needs to be determined, as the concentration used did not have a significant effect, when compared to the effect of catalase. Conversely, when a combination of catalase and Trolox was added to the extender, a deleterious effect on the motility and viability of the sperm was demonstrated.

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