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Ascorbic acid or pyruvate counteracts peroxidative damage in boar sperm cryopreserved with or without α-tocopherol*

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Ejaculates of four crossbred boars were frozen with or without 200 µg/mL of α-tocopherol. Frozen samples were then thawed in the extender containing either ascorbic acid (2.5, 5 or 10 mM) or pyruvate (1 or 5 mM) to obtain the samples in which sperm quality parameters and lipid peroxidation level were evaluated. Ascorbic acid decreased lipid peroxidation in a dose-dependent manner and increased post-thaw sperm motility. The addition of pyruvate decreased lipid peroxidation level without improving sperm quality parameters. Although the addition of ascorbic acid or pyruvate did not show a synergic effect with α-tocopherol, the decrease of lipid peroxidation observed when the antioxidants were added to the thawing extender could be indicating a protective effect on the sperm plasma membrane from peroxidative damage. Since α-tocopherol protects from the lipid peroxidation and improves the sperm motility, it would be considered as the recommended antioxidant to use in boar sperm cryopreservation to extend the time of storage for the use in commercial applications.

KEY WORDS: α-tocopherol / ascorbic acid / boar / lipid peroxidation / pyruvate / sperm

Semen cryopreservation has attained a stage of being an indispensable and integral component of assisted reproductive technologies as artificial insemination, *in vitro* fertilization and embryo transfer. However, the biotechnology of reproduction in pigs

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has not shown much progress mainly due to the limitation in obtaining frozen-thawed sperm samples of high quality. Inseminations with frozen-thawed boar sperm result in lower farrowing rates and smaller litter sizes than the ones obtained with fresh semen [Johnson *et al.* 2000, Watson 2000, Eriksson *et al.* 2002]. Even though intrauterine insemination with fixed-time insemination have accomplished a satisfactory fertility outcome [Kaeoket *et al.* 2010], its utilization clearly limits the application of this biotechnology in commercial development.

Cryopreservation is associated with an increase of reactive oxygen species (ROS) generation (oxidative stress) that leads to an impaired function of thawed sperm [Chatterjee and Gagnon 2001]. Boar sperm is very susceptible to peroxidative damage due to the high content of unsaturated fatty acids in plasma membrane phospholipids [Cerolini *et al.* 2000]. Recently, the research on the application of antioxidants to improve frozen-thawed boar sperm quality has been extensively revised by Zhang *et al.* [2012]. However, the effect of each antioxidant depends on the molecule type and concentration used for each species [Cabrita *et al.* 2011]. Alpha-tocopherol is a natural antioxidant of the group of chain-breaking antioxidants, which prevents chain propagation reactions [Niki 1987]. The addition of α-tocopherol to the freezing extender produces an improvement in post-thaw semen quality preventing oxidative damage [Breininger *et al.* 2005], diminishing capacitation-like changes [Satorre *et al.* 2007] and lowering the expression of apoptotic genes [Jeong *et al.* 2009].

Sperm thawing alters the sperm plasma membrane [Parks and Graham 1992]. Boar sperm is sensitive to the damage produced during the thawing process due to warm shock [Bamba and Cran 1985] and rapid warming [Bamba and Cran 1988]. Ascorbic acid is a water-soluble antioxidant that acts as scavenger of ROS [Madhavi *et al.* 1996]. Several studies indicate that this molecule reduces H_2O_2-induced DNA damages, recycles inactive vitamin E and reduces lipid peroxidation [Sierens *et al.* 2002]. The addition of ascorbic acid to the cryoprotective medium reduces oxidative stress and increases sperm motility [Hua *et al.* 2010]. Pyruvate acts as hydrogen peroxide scavenger and it can be used as antioxidant in semen extenders [Upreti *et al.* 1998] to avoid peroxidative damage [Salahudeen *et al.* 1991]. The addition of pyruvate to the cryopreservation extender in bovine semen leads to a higher level of intracellular ATP and post-thaw motility [Bilodeau *et al.* 2002].

Previous studies of our group have proved that α-tocopherol supplementation has a positive effect on post-thawed sperm survivability. The addition of 200 µg/ml of α-tocopherol to the freezing extender protects the sperm membrane from oxidative damage, improves sperm motility [Breininger *et al.* 2005] and reduces both tyrosine phosphorylation and capacitation-like status [Satorre *et al.* 2007]. We hypothesized that the addition of antioxidants to the thawing extender may improve the post-thaw quality of cryopreserved boar sperm acting by its own or synergically with the α-tocopherol present in cryopreserved samples.

The aim of this study was to evaluate the effect of the addition of selected antioxidants as pyruvate or ascorbic acid to the thawing extender on post-thaw sperm quality of samples cryopreserved with or without α-tocopherol.

Material and methods

Reagents

Chemicals were obtained from SIGMA Chemical Company (St. Louis, MO, USA). Orvus ES Paste (Equex-Paste) was purchased from MINITÚB (Tiefenbach b. Landshut, Germany).

Sperm freezing

Samples from four crossbred boars (Pietrain x Yorkshire) of proven fertility, 1-1.5 year of age were used. Each ejaculate was split into two portions. One was frozen without antioxidant (control samples) and the other with 200 μg/ml of α-tocopherol acetate included in the Beltsville F5 extender (BF5, Tab. 1). Sperm suspensions were cooled to 5°C for 120 min and mixed with an equal volume of BF5 with 2% glycerol to obtain a final concentration of $1x10^8$ sperm/ml. The content was mixed by inversion and frozen into pellets of 0.1 ml in dry ice at -76°C and stored in liquid nitrogen until utilization [Breininger *et al*., 2005].

Sperm thawing

Pellets were placed into a conical centrifuge tube containing Beltsville thawing solution (BTS, Tab. 1, 1:1 pellet:mL). All samples were centrifuged for 5 min at 600 g to separate the freezing extender. Then sperm samples were suspended in BTS (final concentration of 3 x 10^7 sperm/ml) with or without sodium pyruvate (1, 5 mM) and with or without ascorbic acid (2.5, 5, 10 mM) to evaluate sperm quality parameters and lipid peroxidation level. All determinations were performed after 10 min of equilibration time.

Table 1. Composition of BF5 and BTS extenders

Ingredient (g/ml)	BF5	BTS
Tes-N-TRIS	1.2	
TRIS	0.2	
Orvus ES Paste	0.5	
Egg yolk	20	
Dextrose	1.2	3.7
Sodium citrate		0.6
Sodium bicarbonate		0.125
EDTA		0.125
Potassium chloride		0.075

Evaluation of sperm motility, viability and acrosome integrity

Sperm motility was evaluated three times by the same observer, using light microscopy (400x magnification) and a thermal stage at 37°C. Viability was determined by the eosin-nigrosin technique. The percentage of intact acrosomes was evaluated by differential-interferential contrast (DIC) microscopy combined with the supravital stain Trypan blue [Breininger *et al.* 2005]. Two hundred sperms were evaluated to determine viability and acrosome integrity.

Determination of lipid peroxidation

Lipid peroxidation was evaluated fluorometrically by production of thiobarbituric acid reactive substances (TBARS) with a Shimadzu F1501 spectrofluorometer (SHIMADZU CORPORATION, Kyoto, Japan) – Breininger *et al.* [2005]. Results are expressed as nmol TBARS/10^8 sperm.

Thermoresistance test

The capability of sperm to resist incubation at 37°C for 3 h was evaluated in each treatment assessing sperm motility every hour.

Statistical

Data of sperm quality parameters and lipid peroxidation level were analysed by one-way ANOVA and post-hoc Bonferroni comparison according to a totally random experimental design. The thermoresistance test was evaluated by a repeated measure two-way ANOVA (treatment, incubation time) and posterior contrast analysis. P value < 0.05 was considered significant.

Results and discussion

Sperm quality parameters were determined in samples thawed in various concentrations of sodium pyruvate BTS-solution (1 or 5 mM) or ascorbic acid BTS-solution (2.5, 5 or 10 mM) in order to determine the optimum concentration of these antioxidants that would improve post-thaw sperm functionality. Post-thaw motility was increased compared with control samples ($P<0.05$) with 5 mM of ascorbic acid. A significant decrease of lipid peroxidation level was observed being the lowest levels obtained with 5 or 10 mM of ascorbic acid (Tab. 2). However, no significant differences were observed in viability and acrosome integrity parameters. The addition of 5 mM sodium pyruvate to the thawing medium decreased the lipid peroxidation level but failed to modify sperm quality parameters (Tab. 3).

Cryopreservation, which includes thawing, alters sperm membranes [Parks and Graham 1992] and produces oxidative stress [Chatterjee and Gagnon 2001], being boar sperm especially susceptible to this process [Cerolini *et al.* 2000]. *In vivo*, seminal plasma provides the main defense against ROS due to the low content of cytoplasm in sperm [Ben Abdallah *et al.* 2009]. The protocols of boar sperm cryopreservation

Table 2. Parameters of frozen boar sperm thawed with different concentrations of ascorbic acid (means±SEM)

Ascorbic acid concentration of samples	Motility (%)	Viability (%)	Acrosome integrity (%)	nmol TBARS/10^8 sp/h
Control	29^{a}±2	57^{a}±1	45^{a}±2	23^{a}±1
2.5 mM	32^{ab}±1	53^{a}±0	46^{a}±1	18^{b}±1
5 mM	35^{b}±1	55^{a}±2	50^{a}±3	10^{c}±0
10 mM	29^{a}±1	53^{a}±1	48^{a}±2	11^{c}±1

^{abc}Within columns means bearing different superscripts differ significantly at P<0.05.

Table 3. Parameters of frozen boar sperm thawed with different concentrations of sodium pyruvate (means±SEM)

Sodium pyruvate concentration of samples	Motility (%)	Viability (%)	Acrosome integrity (%)	nmol TBARS/10^8 sp/h
Control	29^{a}±1	55^{a}±1	45^{a}±2	24^{a}±1
5 mM	31^{a}±4	54^{a}±0	44^{a}±1	26^{a}±1
10 mM	32^{a}±2	53^{a}±1	43^{a}±2	16^{b}±1

^{abc}Within columns means bearing different superscripts differ significantly at P<0.05.

include removal of seminal plasma and dilution before cryopreservation. As a consequence, cryopreservation reduces the concentration of antioxidant components and diminishes the protection of sperm [Martínez-Páramo *et al.* 2009].

Ascorbic acid is a water-soluble antioxidant that prevents chain propagation reactions and regenerates α-tocopherol [Niki 1987]. In the present study the addition of ascorbic acid to the thawing extender decreased lipid peroxidation and increased sperm motility (P<0.05, Tab. 2). Pyruvate is a scavenger of hydrogen peroxide. In several cellular systems, its protective effect against peroxidative damage has been demonstrated [Salahudeen *et al.* 1991]. The addition of 5 mM sodium pyruvate increased sperm motility in cryopreserved bovine sperm [Bilodeau *et al.* 2002]. In boar sperm, the same concentration diminished TBARS levels (P<0.05) but failed to modify sperm motility (Tab. 3). The results observed may be attributed to the different composition of the plasma membrane, being boar sperm more susceptible to oxidative damage than bull sperm. The differences observed in the decrease of lipid peroxidation levels in samples thawed with ascorbic acid (56%) or sodium pyruvate (33%) compared with control samples may be due to the mechanisms of ROS scavenging of each antioxidant.

The synergic effect of the addition of antioxidants to the freezing and thawing extenders was evaluated in two ways: (1) evaluating the post-thaw viability, acrosome integrity and lipid peroxidation level (Tab. 4) and (2): measuring the changes in sperm

Table 4. Parameters of boar sperm frozen with or without α-tocopherol and thawed with sodium pyruvate or ascorbic acid (means±SEM)

Sample	Viability(%)	Acrosome integrity (%)	nmol TBARS/10^8 sp/h
Control samples	57^{a}±2	47^{a}±2	24^{a}±1
α-tocopherol samples	57^{a}±1	45^{ab}±1	11^{b}±0
Pyruvate samples	53^{a}±1	42^{b}±1	12^{b}±1
Ascorbic acid samples	53^{a}±1	51^{a}±2	12^{b}±1

^{abc}Within columns means bearing different superscripts differ significantly at P<0.05.
Samples frozen without α-tocopherol (controls); samples frozen with α-tocopherol and thawed in BTS (α-tocopherol samples); samples frozen with α-tocopherol and thawed in sodium pyruvate BTS-solution (pyruvate samples), samples frozen with α-tocopherol and thawed in ascorbic acid BTS-solution (ascorbic acid samples).

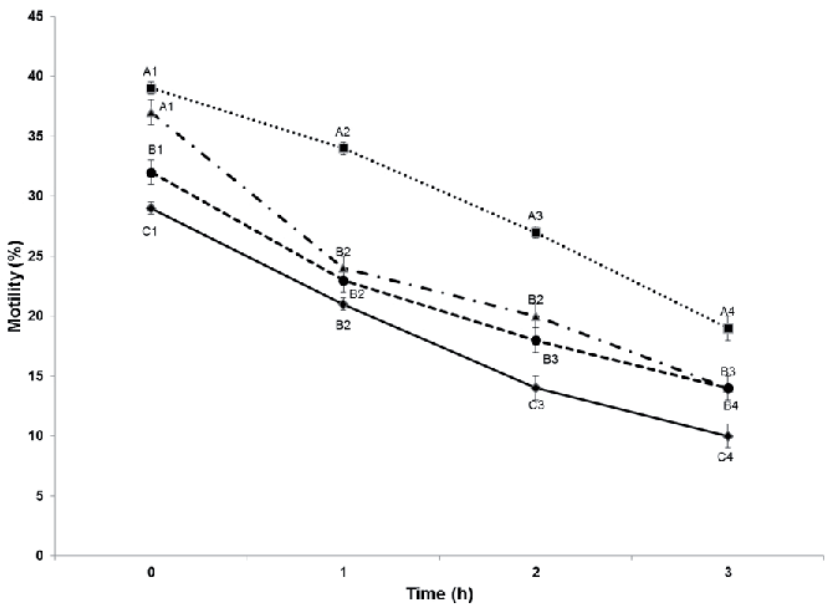

Fig. 1. Changes in motility of frozen-thawed boar sperm during incubation (means). "—" - frozen samples without α-tocopherol and thawed in BTS; "·····" - frozen samples with α-tocopherol and thawed in BTS; "- · - · -" - frozen samples with α-tocopherol and thawed in sodium pyruvate BTS-solution; "----" - frozen samples with α-tocopherol and thawed in ascorbic acid BTS-solution. A, B, C – within time means bearing different letters differ signisicantly at P<0.05. 1, 2, 3, 4 – between times means bearing different numbers differ significantly at P<0.05.

motility during the thermoresistance test (Fig. 1). The evaluation was performed in samples frozen with 200 µl/mL of α-tocopherol and thawed with 5 mM of sodium pyruvate or ascorbic acid. The sodium pyruvate or ascorbic acid concentration was ascertained according to the results obtained in the evaluation of sperm parameters (Tab. 2 and 3). The addition of sodium pyruvate or ascorbic acid to the thawing extender of α-tocopherol frozen samples did not enhance the effect observed when only α-tocopherol was used. No significant differences were observed in the viability level. The acrosome integrity level was significantly lower in samples thawed in sodium pyruvate BTS-solution compared to control samples. The lipid peroxidation level diminished in the presence of antioxidants, without significant differences between treatments (Tab. 4). The presence of pyruvate or ascorbic acid increased motility at time 0 of incubation time compared to control samples, but the highest motility level was observed in α-tocopherol frozen samples (Fig. 1).

Several authors have demonstrated that the addition of molecules with antioxidant capacity to the cryopreservation extender reduces oxidative damage [Peńa *et al.* 2003, Michael *et al.* 2007, Bansal and Bilaspuri 2009, Cabrita *et al.* 2011]. However, contradictory results were obtained in some studies, as the right balance among oxidation and reduction is not easily achieved. For example, the presence of ascorbic acid in the *in vitro* fertilization medium lowered the *in vitro* fertilization rates in samples of bull sperm frozen with α-tocopherol [Dalvit *et al.* 1998]. Moreover, the capacitation level was lower in samples frozen with α-tocopherol when α-tocopherol or ascorbic acid were added to the *in vitro* capacitation medium [O´Flaherty *et al.* 1997]. These studies are in accordance with our results in the thermoresistance test, as the combined addition of antioxidants to the freezing and thawing extenders did not produce any synergic effect. However, the presence of pyruvate or ascorbic acid in the thawing extender improved sperm motility compared with control samples (Fig. 1). Also, these antioxidants diminished lipid peroxidation levels compared with control samples (Tab. 4). The levels of lipid peroxidation increase during incubation [Breininger *et al.* 2005], altering sperm motility due to the ROS-induced damage in ATP utilization as proposed by Guthrie and Welch [2012]. We suggest that the possible increase of ROS due to the decrease of the antioxidant capacity of pyruvate or ascorbic acid may explain the results observed in the thermoresistance test. Pyruvate is consumed in the reaction: $CH_3COCOOH + H_2O_2 \rightarrow CH_3COOH + CO_2 + H_2O$ [Melzer and Smith 1988]. Ascorbic acid is unstable when exposed to highly oxidative environments, being rapidly oxidized into inactive dehydroascorbate [Linster and Van Schaftingen 2007]. The results observed after an hour of incubation may be due to the need to replenish the sodium pyruvate or the acid ascorbic present in the extender. Although it was not possible to observe the synergic effect of ascorbic acid or pyruvate with α-tocopherol in the parameters of sperm quality (Tab. 4), the decrease of lipid peroxidation observed may indicate the protection of the sperm plasma membrane from peroxidative damage. The use of these antioxidants would depend on the technique used in the fertilization process, being useful in those procedures that are compatible

with a limited lifespan of the sperm as intrauterine insemination. However, since α-tocopherol protects from lipid peroxidation and improves sperm motility, it would be considered as the recommended antioxidant to use in boar sperm cryopreservation to extend the time of storage after thawing for the use in commercial applications.

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