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Original Article

Effect of packaging method on quality and functional parameters in cryopreserved porcine spermatozoa with alpha tocopherol

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ABSTRACT

Objectives: Evaluate the effect of the packaging method in porcine semen cryopreserved with α-tocopherol on quality and functional sperm parameters. In porcine production, although the use of frozen semen is very limited, there are obvious advantages to use this technology.

Material and Methods: Sperm samples were cryopreserved in pellets or straws with or without α-tocopherol and quality and functional parameters were determined in all groups.

Results: As regards quality parameters, a significant individual effect was observed, with a similar behavior despite the packaging system evaluated. The same results were obtained in functional tests. Both packaging systems, pellets and straws, showed a similar behavior with respect to the effect of the antioxidant α-tocopherol on the quality and functional sperm parameters. Interestingly, the better results were obtained in pellets than in straws.

Conclusion: The results obtained allow us to suppose that this efficient, economical and simple method, with little expensive equipment or supplies, can be used to cryopreserve boar spermatozoa for research. In fact, since the results have been better in tablets, if identification and storage problems of pellets were solved, this freezing method could be used for production purposes.

Keywords: Pellet, Straw, Boar-frozen spermatozoa

INTRODUCTION

Beyond the obvious advantages of using frozen semen for artificial insemination, for use in commercial production, the results obtained must be comparable to those accomplished by natural mating. In cattle, this objective has been achieved, but for sheep, pigs, and horses the situation is very different.[1] Boar spermatozoa are very much affected by the freeze-thawing processes and these are the principal responsibility in the limited use of frozen semen in commercial pig production as the low reproductive performance achieved with frozen semen is well documented.[2,3] The relatively long shelf life of extended boar semen at ambient temperatures and the results obtained with refrigerated semen, have solved the problems involved in collecting and distributing semen for insemination without the need for cryopreservation.[1]

The original protocols for boar spermatozoa cryopreservation were developed with the establishment of two methods: the American or Beltsville method designed by Pursel and Johnson[4] and the German or Hülsenberger method, set by Westendorf *et al.*,[5] developed in pellets or straws, respectively. The volume/surface ratio in pellets facilitates the temperature transmission and can

provide acceptable post-thawed sperm quality, however, pellets present some disadvantages, including difficulty to identify and that it is necessary to use them in large amounts per inseminating dose. These factors have discouraged its commercial use in porcine production.[6] The introduction of technological changes such as the control of the cooling curve with programmable freezing equipment and the use of "packages" with a greater surface/volume ratio and small cross-section, allowed obtaining improvements in the post-thawing seminal quality.[7] However, even under these conditions, the fertility results, farrowing rate, and litter size, are still lower than those obtained with fresh semen[2] and despite the results obtained <1% of all artificial inseminations (AI) worldwide use frozen–thawed boar spermatozoa.[8]

Cryopreservation increases the generation of reactive oxygen species (ROS) and it is associated with oxidative stress.[9] Boar spermatozoa are particularly sensitive to peroxidative damage because of the high content of unsaturated fatty acids in the phospholipids of the plasma membrane.[10] Most of the works published in the last few years have evaluated the effects of supplementing freezing and/or thawing media with antioxidants.[11] Our research group has extensively been working with α-tocopherol, a primary antioxidant, which acts at the end of the lipid peroxidation chain.[12] We have observed that the use of alpha-tocopherol improves the functionality of cryopreserved boar spermatozoa.[13,14,15] The facility for cryopreservation and subsequent manipulation of the frozen samples for experimental purposes prompted us to use frozen samples in pellets are not routinely used in porcine production. In this regard, the objective of this study was to evaluate the effect of the packaging method in porcine semen cryopreserved with α-tocopherol on quality and functional sperm parameters.

MATERIAL AND METHODS

Materials

Reagents were provided by Sigma Chemical Company (St. Louis, MO, USA). Orvus ES Paste (Equex-Paste) was from Minitub (Tiefenbach b. Landshut, Germany). Eosin yellowish and nigrosine were obtained from Mallinckrodt (St. Louis, MO, USA). Dextrose, sodium citrate, sodium bicarbonate, sodium chloride, EDTA, potassium chloride, disodium hydrogen phosphate, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Semen samples, from four crossbred boars (Pietrain x Yorkshire) of proven fertility, 1–1.5 year of age, were provided by the Porcine Productive Unit of the School of Veterinary Sciences (University of Buenos Aires). This Productive Unit replicates a small porcine production system in uniform and controlled feeding and handling conditions. All animal experiments were performed in accordance with the guidelines of the Institutional Committee for Care and Use of Experimental Animals of the School of Veterinary Sciences, University of Buenos Aires. Only samples with a minimum of 70% motile and 80% membrane intact spermatozoa were included in the study.

Sample processing

The ejaculates obtained by the gloved hand technique were divided into two fractions: pellets or straws. These fractions in turn were sub-fractionated in two different freezing media: with or without the addition of 200 μg/mL alpha-tocopherol to the freezing extender (alpha-tocopherol (αT) samples or control (C), respectively). Pellets were cryopreserved according to Pursel and Johnson's protocol, as described by Breininger *et al*.[13] and straws fraction were cryopreserved according to a modification of Westendorf protocol.[16] Pellets were thawed at 37°C in Beltsville thawing solution (BTS, 2 pellets: 2 ml), straws were thawed at 37°C for 1 min and then suspended in BTS (1 straw: 1 mL). Thawed spermatozoa were kept in a water bath for 10 min for equilibration.

Evaluation of parameters of sperm quality

In order to compare the different cryopreservation methods, motility (MOT), plasma membrane integrity (PMI), acrosomal integrity in live spermatozoa (AI), and the post-thawing cryocapacitated level were evaluated.

Total sperm motility was evaluated three times by the same observer using an optical microscope (400x magnification) equipped with a thermal stage at 38°C to obtain a mean value of this parameter. The percentage of sperm membrane integrity was determined by the supravital eosin-nigrosin stain. At least 200 spermatozoa were counted in each sample using an optical microscope (Binocular microscope XSZ 100 BN, Arcano, China) at 400x magnification.[14] The percentage of intact acrosomes in live cells was determined by evaluating spermatozoa stained with Trypan blue by Differential-Interferential Contrast optical microscopy (DIC). An aliquot of the sperm suspension was incubated with an equal volume of Trypan blue (0.25%) for 15 min at 38°C, centrifuged at 600 g for 10 min to remove excess stain and fixed with 2% [v/v] glutaraldehyde in saline. Spermatozoa (200/sample) were assessed at 1000x in a Jenamed-2-microscope (Carl Zeiss, Jena, Germany).[13] Cryocapacitation levels were evaluated through fluorescence modifications in chlortetracycline epifluorescence stain (CTC) patterns. The level of cryocapacitation was measured by determining the B pattern of CTC (CTC-B) according to Wang *et al.* descriptions.[17] Samples were mixed with an equal volume of CTC solution (CTC 500 μM, NaCl 130 mM, DL-cysteine 5 mM, Tris–HCl 20 mM, pH 7.8). Glutaraldehyde (0.1%) was then added to the mixture. Slides were examined at 400x magnification under epifluorescence at 410 nm excitation filter using a Jenamed-2-fluorescence microscope (Carl Zeiss, Jena, Germany), 200 spermatozoa per sample were evaluated.

Functional tests

In vitro sperm capacitation and acrosome reaction induction.

Thawed samples were centrifuged at 300 g for 5 min to separate the freezing extender. Then spermatozoa were resuspended in capacitating medium (modified Tris buffered medium, mTCM: 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 20 mM TRIS, 11 mM Glucose, and 5 mM sodium pyruvate) supplemented with bicarbonate 46 mmol to a final concentration of 3×10^7 spermatozoa/mL. Samples were incubated during 30 min at 38°C under 5% CO_2 in humidified air. *In vitro* capacitation level was evaluated by CTC technique and the percentages of capacitated spermatozoa (CTC-B) were obtained by subtracting the values obtained at zero time of incubation, in order to rule out cells destabilized during the freezing-thawing process.[15] The ability of capacitated spermatozoa to undergo acrosome reaction was assessed as follows: capacitated samples were incubated with follicular fluid (FF) (30% v/v) at 38°C under 5% CO_2 in humidified air for 15 min. True acrosome reaction (TAR) was evaluated using the combined technique of DIC and Trypan Blue stain as described previously. TAR value at zero time was subtracted in order to rule out spontaneous acrosome reaction evaluating 200 spermatozoa per sample.[18]

Statistical analysis

Percentages of MOT, PMI, AI, CTC-B and TAR are given as mean ± SD. Three ejaculates of each boar were obtained so 12 experimental units were analyzed. The quantitative data collected were analyzed for assumption of normality by Shapiro–Wilk test and homogeneity of variances using the Levene test. When necessary, percentages were normalized by arcsine transformation. Data were analyzed by two-way ANOVA (cryopreservation treatment, type of packaging, and their interactions) in a completely randomized block design, taking the boar as the block. A value of $p < 0.05$ was considered statistically significant. All statistical tests were performed using InfoStat (Universidad de Córdoba, Córdoba, Argentina, see http://www.infostat.com.ar/).

RESULTS

Our results shows an individual effect, when comparing the packaging methods within individuals, a significant difference can be observed in all the parameters, being always lower the values obtained in straws, except for the boar 4 where no significant differences were observed. In samples cryopreserved with alpha-tocopherol (αT), the behavior was similar to Control samples (C), and significantly, higher values were found when it was cryopreserved in pellets, except for the CTC-B percentages of boar 2 that did not show significant differences between the packaging methods. The protective effect of alpha-tocopherol was observed in both packaging methods. Cryopreservation with alpha-tocopherol increased the values of motility and plasma membrane integrity [Table 1], acrosomal integrity and decreased the cryocapacitation levels [Table 2] both in samples cryopreserved in pellets and straws, with a similar behavior for both methods of packaging.

All samples incubated in capacitation medium (mTCM with bicarbonate) showed significant differences with their respective controls (incubated in mTCM without bicarbonate, data not shown). Similarly, to quality parameters results, differences between boars were found, so the results are presented for each one. However in all samples, both in pellets and in straws, level of capacitation in C samples were significant lower than αT ones. The percentage of live spermatozoa with reacted acrosome was significantly higher in the capacitated samples incubated with follicular fluid, compared to their controls (incubated without follicular fluid, data not shown). Samples cryopreserved in the presence of α-tocopherol produced the highest response to *in vitro* capacitation induction, with similar responses in both packaging methods [Table 3].

DISCUSSION

Semen packaging is important for practical reasons since it determines both the means of identification of each dose of

Table 1: Effect of packaging method on motility and plasma membrane integrity in cryopreserved boar spermatozoa with or without α-tocopherol.

	Pellets				Straws			
	Control		α-tocopherol		Control		α-tocopherol	
Boar	MOT	PMI	MOT	PMI	MOT	PMI	MOT	PMI
1	31 ± 4^{a}	34 ± 3^{a}	$42 \pm 2^{a+}$	$43 \pm 4^{a+}$	$19 \pm 1^{a*}$	$30 \pm 3^{a*}$	$29 \pm 1^{a*+}$	$37 \pm 2^{a*+}$
2	27 ± 2^{ab}	32 ± 2^{a}	$36 \pm 4^{b+}$	36 ± 2^{a}	$28 \pm 2^{b*}$	$24 \pm 3^{ab*}$	$12 \pm 1^{b*+}$	$24 \pm 3^{b*}$
3	23 ± 2^{b}	26 ± 3^{b}	$34 \pm 3^{b+}$	$33 \pm 2^{a+}$	$7 \pm 3^{bc*}$	$23 \pm 1^{b*}$	$12 \pm 2^{b*}$	$26 \pm 1^{b*+}$
4	8 ± 2^{c}	24 ± 1^{b}	8 ± 2^{c}	24 ± 1^{b}	3 ± 2^{c}	20 ± 3^{b}	7 ± 1^{c}	25 ± 4^{b}

Data are expressed as means ± SD of 4 replicates. MOT: motility, PMI: plasma membrane integrity.
[a-c]Significant differences between boars.
[*]Significant differences between packaging methods within same freezing extender.
[+]Significant differences between freezing extender within same packaging method, $p < 0.05$.

Table 2: Effect of packaging method on acrosome integrity and cryocapacitation level in cryopreserved boar spermatozoa with or without α-tocopherol.

	Pellets				Straws			
	Control		α-tocopherol		Control		α-tocopherol	
Boar	AI	CTCB	AI	CTCB	AI	CTCB	AI	CTCB
1	32 ± 2^{a}	23 ± 2^{a}	$39 \pm 3^{a+}$	$18 \pm 2^{a+}$	$26 \pm 3^{a*}$	$20 \pm 2^{a*}$	$32 \pm 2^{a*+}$	$22 \pm 2^{a*}$
2	26 ± 3^{a}	25 ± 1^{a}	$35 \pm 3^{a+}$	$19 \pm 2^{a+}$	$21 \pm 2^{b*}$	$29 \pm 3^{b*}$	$21 \pm 2^{b*}$	$20 \pm 2^{a*+}$
3	26 ± 3^{b}	28 ± 2^{b}	$35 \pm 4^{a+}$	$18 \pm 3^{a+}$	$20 \pm 1^{b*}$	$32 \pm 1^{b*}$	$23 \pm 1^{b*+}$	$27 \pm 2^{b*+}$
4	25 ± 3^{b}	26 ± 1^{b}	25 ± 3^{b}	26 ± 1^{b}	3 ± 2^{c}	20 ± 3^{b}	7 ± 1^{c}	25 ± 4^{b}

Data are expressed as means ± SD of 4 replicates. AI: acrosomal integrity in live spermatozoa, CTCB: cryocapacitated level.
[a-c]Significant differences between boars.
[*]Significant differences between packaging methods within same freezing extender.
[+]Significant differences between freezing extender within same packaging method, $p < 0.05$.

Table 3: Effect of packaging method on sperm functional parameters of cryopreserved boar spermatozoa with or without α-tocopherol

	Pellets				Straws			
	Control		α-tocopherol		Control		α-tocopherol	
Boar	IVC	TAR	IVC	TAR	IVC	TAR	IVC	TAR
1	21 ± 1^{a}	20 ± 3^{a}	$35 \pm 2^{a+}$	$30 \pm 1^{a+}$	$16 \pm 1^{a*}$	16 ± 2^{a}	$27 \pm 3^{a*+}$	$26 \pm 2^{a*+}$
2	18 ± 1^{a}	17 ± 2^{a}	$29 \pm 3^{a+}$	$29 \pm 2^{a+}$	16 ± 2^{a}	14 ± 1^{a}	$22 \pm 1^{b*+}$	$24 \pm 2^{a*+}$
3	19 ± 1^{a}	18 ± 1^{a}	$29 \pm 3^{a+}$	$25 \pm 1^{b+}$	$14 \pm 1^{a*}$	$13 \pm 2^{a*}$	$22 \pm 1^{b*+}$	$22 \pm 2^{a+}$
4	15 ± 3^{b}	12 ± 3^{b}	$22 \pm 2^{b+}$	$20 \pm 2^{c+}$	$9 \pm 2^{b*}$	7 ± 3^{b}	$17 \pm 3^{c+}$	$15 \pm 1^{b*+}$

Data are expressed as means ± SD of 4 replicates. IVC: level of in vitro capacitation, TAR: level of true acrosome reaction
[a-c]Significant differences between boars.
[*]Significant differences between packaging methods within same freezing extender.
[+]Significant differences between freezing extender within same packaging method, $p < 0.05$.

semen and how it may be arranged for storage in the liquid nitrogen container. The most common packages for frozen storage of boar semen are pellets and PVC straws. Pellet freezing generally produces the best results, but there are commercial pressures to use other methods of packaging.[19] In our laboratory, the facility for cryopreservation and subsequent manipulation of the frozen samples for metabolic determinations raised the possibility of using frozen samples in pellets. In this regard, we evaluated the effect of the packaging method in porcine semen cryopreserved with α-tocopherol on quality and functional sperm parameters.

Motility, structural integrity of the plasma membrane and live spermatozoa with intact acrosome were lower in straws cryopreserved samples than pellets. These results could be due to a response to suboptimal freezing curves that happens when freezing is performed in static liquid nitrogen vapor as freezing speed varies with the sample size.[20] Only with the use of programmable freezers that allows reaching uniform freezing speeds and better control during the freezing process under standardized conditions[21] good results of post-thaw sperm quality that can be obtained for commercial AI.[8] Our results show that the quality parameters of spermatozoa after freezing in pellets are comparable to that in straws. Moreover, results from straw freezing packages were inferior to pellets. In different species, a different behavior was observed when semen was cryopreserved in pellets or straws. Some authors have found that straws were better than pellets[22] while others have found that semen frozen in straws was inferior to that in pellets.[23] Ram spermatozoa survive cryopreservation more efficiently when frozen in the pellet form, while bull spermatozoa survive more efficiently when frozen in straws.[24]

In cryopreserved boar semen, supplementation with α-tocopherol has a positive effect on sperm motility, mitochondrial membrane potential and membrane integrity,[25,26] and acrosome membrane integrity[27] in coincidence with our results. Moreover, the addition of α-tocopherol to the freezing extender allowed to increase the values of motility and to reduce the level of lipid peroxidation[13] and the levels of cryocapacitation, demonstrated by CTC and tyrosine phosphorylation proteins, in boar semen samples cryopreserved in pellets.[14] Similarly, to that occurs in pellets, samples cryopreserved in straws showed a significant decrease in the state of cryocapacitation, when α-tocopherol was added to the freezing diluent. The protection of the plasma membrane against destabilization, caused by cryopreservation, would be a way to improve the fertilizing capacity of frozen-thawed spermatozoa. Therefore, the addition of

α-tocopherol to the freezing diluent allows increasing the post-thawing quality of the cryopreserved samples.

During sperm capacitation, there is a restructuring of membrane components intimately related to sperm function, and in order to fertilize, the capacitated spermatozoa must undergo an exocytotic process called acrosome reaction.[28] Bicarbonate, a well-known in vitro capacitation inducer in porcine,[29] effectively induced *in vitro* capacitation in cryopreserved samples in straws as occurs in pellets. The presence of α-tocopherol in the freezing extender significantly increased the response to the capacitation inducer in both packaging methods, being the results higher in pellets. This level of capacitation was confirmed by the induction of the true acrosomal reaction, where the better results were observed in the samples cryopreserved in pellets too.

Cryopreservation produces changes in the sperm membranes[30] these alterations can affect sperm fertility. The samples, cryopreserved with α-tocopherol, have a greater fertilizing potential, due to the better characteristics of motility, plasma and acrosomal membrane integrity and functionality of the membranes. Regardless the packaging system, individual differences were observed. It is well known that variability in sperm freezability exists, and boars are usually classified as good or bad freezers according to their cryopreservation results.[31] Our results support this idea, showing similar results in the same despite the packaging system and the cryopreservation treatment.

CONCLUSION

Both packaging systems, pellets and straws, showed a similar behavior with respect to the effect of the antioxidant α-tocopherol on the quality and functional sperm parameters. Interestingly, better results were obtained in pellets than in straws. Thus, the results obtained allow us to suppose that this efficient, economical, and simple method, with little expensive equipment or supplies, can be used to cryopreserve boar spermatozoa for research. In fact, since the results have been better in pellets, if identification and storage problems of pellets were solved, this freezing method could be used for production purposes.

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Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Conflicts of interest

Dr. Elizabeth Breininger is on the editorial board.

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