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Research

Changes in sperm quality and lipid composition during cryopreservation of boar semen

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The effect of cryopreservation on boar sperm viability, motility, lipid content and antioxidant enzymatic activities was studied. Three classes of semen were determined according to a cluster analysis on the basis of the proportion of live and dead cells after freezing and thawing. The classes identified were: high (H, *n* = 4), average (A, *n* = 12) and low (L, *n* = 3) viability. The concentration of sperm cells decreased from class H to A to L. Fresh semen samples with higher viability and a higher proportion of motile cells also maintained better quality after the freezing and thawing procedure. Sperm viability and motility in both fresh and thawed samples were similar in classes H and A, while significantly lower values were measured in class L. The relative decrease in sperm viability and motility after cryopreservation increased from class H to A to L. The lipid content of spermatozoa (μg per 10^9 cells) increased significantly after freezing and thawing in classes H and A but not in class L. This result indicated that active sperm lipid metabolism might be responsible for the increase in lipid content. Phospholipid and triacylglycerol contents increased whereas free cholesterol content decreased after thawing. The fatty acid composition of fresh spermatozoa was similar in all three classes. The proportion of polyunsaturated fatty acids decreased significantly after freezing and thawing, indicating contamination from the diluent or peroxidation. After freezing and thawing, superoxide dismutase activity in spermatozoa was significantly higher in class L than in classes H and A, which did not differ from each other.

Introduction

Cryopreservation of boar semen is rarely performed in commercial practice. The main reasons for this are the poor survival rate of spermatozoa and, as a consequence, the high concentration required in the insemination dose. Refrigeration at 16– 18°C is the usual method of storing boar semen for a few days and good or acceptable fertility rates are obtained compared with fresh samples. Nevertheless, cryopreservation is a valuable technique, especially for the conservation of genetic resources (that is, through sperm banks), or to ensure a constant commercial supply of semen doses in the case of a temporary epidemiological problem or of impaired semen production as a result of adverse climatic conditions (that is, in hot countries). Cryopreservation affects sperm membrane integrity (for reviews, see Bwanga, 1991; Watson, 1995). Differences in fatty acid composition and lipid class ratios in spermatozoa among species are important factors in the freezability of the male gametes (Parks and Lynch, 1992). Pettitt and Buhr (1998) reported lipid modifications due to freezing and thawing and also indicated that domains of sperm head plasma membrane react differently to cryopreservation. Furthermore, some studies (Neill and Masters, 1972, 1973; Vasquez and Roldan, 1997) investigated the ability of sperm cells to take up lipid components or fatty acids from the surrounding environment during incubation *in vitro*.

The present study focused on the effect of cryopreservation on traditional quality parameters, lipid composition and antioxidant enzymatic changes in boar semen samples classified according to the ability of spermatozoa to survive the freezing and thawing procedure. The aim was to study the effect of the initial sperm quality (that is, before freezing) on freezability, and also on the lipid exchanges between sperm cells and diluent occurring during freezing. Furthermore, the feasibility of using lipid components as markers to predict the ability of a sample to survive the freezing and thawing procedure was investigated.

Materials and Methods

Sperm collection and freezing and thawing procedure

Semen samples were collected by the gloved-hand technique from 19 boars, 1–5 years old, of different genetic lines (Landrace, Large White and commercial hybrids). Animals were housed and bred in an Artificial Insemination

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Centre (ELPZOO Spa, Lodi) in accordance with the Italian and European Union legislation. An aliquot of the sperm-rich fraction was diluted 1:2 (v:v) in Beltsville thawing solution (BTS, 41 g D+ glucose, 1.25 g sodium hydrogen carbonate, 1.25 g EDTA, 6 g trisodium citrate, 0.75 g potassium chloride, 4.4 ml lyncaspectin l^{-1}, all diluted to 1 l in distilled water) and used for assessment of fresh semen quality and sperm lipid analysis. The remainder of the ejaculate was frozen according to the procedure of Westendorf *et al*. (1975) modified by Almlid *et al*. (1987), in 4 ml multi-mini straws (ZM-SYS-411®, ELPZOO Spa, Lodi) at a concentration of 500×10^6 cells ml^{-1} and stored in liquid nitrogen. The straws were thawed in a water bath at 37°C for 2 min; semen was transferred into a glass tube and diluted 1:4 (v:v) in the thawing extender at 20°C.

Sperm quality parameters

Sperm concentration, motility, viability and lipid composition were assessed in fresh and frozen–thawed samples. Sperm concentration was measured by Coulter counter (Coulter® Z1). Sperm motility was assessed subjectively under a microscope (Nikon Diaphot, contrast phase 2, ELWD 0.3, × 10) after 10 min incubation in a water bath at 37°C for fresh samples, and after 1, 4 and 6 h incubation at 37°C for thawed samples. Sperm viability was assessed according to the SYBR 14/propidium iodide (Molecular Probes Inc., Eugene, OR) fluorescent procedure (Garner and Johnson, 1995): live, dead and moribund (dual staining) cells were counted under a fluorescence microscope (Leitz Aristoplan) and the proportion of each category was calculated.

Sperm lipid composition

Washed spermatozoa from fresh and thawed samples were obtained after two centrifugations: the first at 850 ***g*** at 18°C for 20 min and the second at 1900 ***g*** at 5°C for 20 min. Cell pellets were washed with BTS after the first centrifugation. Total lipids were extracted in excess chloroform: methanol (2:1)(v:v) from the washed spermatozoa. A minimum of 1×10^9 cells was used for lipid analysis. Phospholipids, triacylglycerols and free cholesterol were the major lipid classes separated by thin layer chromatography (TLC) on silica gel G60 (Merck, Darmstadt, D) using a solvent system of hexane:diethyl ether:formic acid (80:20:1)(v:v:v) (Sigma Chemical Co., St Louis, MO). After spraying the TLC plate with 0.1% (w/v) 2,7-dichlorofluorescein (Sigma) in methanol, the phospholipid, triacylglycerol and free cholesterol bands, visualized under UV light, were scraped from the plate. Phospholipids were eluted from the silica by washing twice with methanol, and triacylglycerols and free cholesterol were washed with diethyl ether. The fatty acids were *trans*-methylated by refluxing with a mixture of methanol:toluene:sulphuric acid (20:10:1) (v:v:v) in the presence of a pentadecanoic acid standard (Sigma) (Hamilton *et al*., 1992). The resultant fatty acid methyl esters were analysed by injection via a CP9010 autosampler (Chrompack, Speck Analytical, London) onto a 30 mm × 0.25 mm in diameter, 0.25 μm film thickness Carbowax capillary column (Alltech Ltd, Carnforth) fitted to a Chrompack CP9001 gas chromatograph. The data processing system (EZ-Chrom Data Handling System, Speck Analytical, UK) enabled the expression of the fatty acid composition in terms of proportion by weight. The identification of the peak values was confirmed by comparison with the retention times of standard fatty acid methyl ester mixtures (Sigma). The amount of each lipid class was calculated by comparison of the total fatty acid peak value areas to that of the pentadecanoic fatty acid standard (Christie *et al*., 1970). Free cholesterol was determined by standard colorimetric assay (Boehringer Mannheim Spa, Monza). The absolute, μg per 10^9 cells and relative percentage contents of the lipid classes were calculated. The ratio of free cholesterol: phospholipids was calculated on the absolute content in μg per 10^9 cells. The lipid composition of the egg yolk-based freezing extender was carried out following the same procedure.

Superoxide dismutase and glutathione peroxidase assays

Superoxide dismutase (SOD), total glutathione peroxidase (GSH-Px) and selenium-dependent glutathione peroxidase (GSH-Px-Se) activities were measured in both seminal plasma and spermatozoa of fresh samples and in only the spermatozoa of cryopreserved samples owing to the egg yolk contamination from the diluent. Spermatozoa were separated and washed by centrifugation as described above and seminal plasma obtained from the first centrifugation was transferred into a clean tube, centrifuged again at 1900 ***g*** at 5°C for 20 min and the supernatant stored frozen until assayed for enzymatic activity. Washed spermatozoa were suspended in 1 ml BTS, sonicated for 2 min in ice (power 5, duty cycle 10, Branson Sonifier 250), centrifuged at 1900 ***g*** at 5°C for 30 min and the supernatant stored frozen at –20°C for enzymatic assays. Total SOD activity was measured by the colorimetric xanthine–xanthine oxidase method using the Ransod kit (Randox Laboratories Ltd, Crumlin) (Woolliams *et al*., 1983). The GSH-Px activity was measured by colorimetric method, with the total enzyme activity measured using cumene hydroperoxide as a substrate (Ransel kit, Randox Laboratories Ltd) (Ammerman *et al*., 1980) and the selenium-dependent activity using 3% hydrogen peroxide solution as a substrate.

Statistical analysis

Hierarchical cluster analysis (SCAN, 1995) was carried out to classify the boars according to the proportion of live and dead cells measured after freezing and thawing. The proportion of moribund spermatozoa was not included in the analysis to avoid redundant information. General linear model (GLM) procedure (SAS, 1998) was undertaken to assess the difference between the different classes. The following sources of variation were considered in the model: the genetic line of the boar (Landrace, Large White

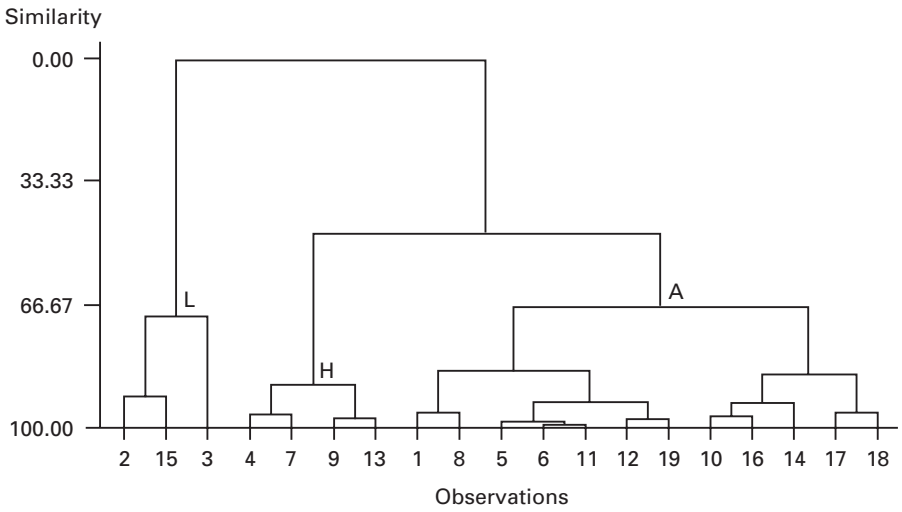

Fig. 1. Dendrogram showing the similarity of boars according to the proportion of live and dead spermatozoa measured after freezing and thawing. The numbers on the horizontal axis represent the 19 boars of the study, the vertical axis shows the proportion of similarity. The low (L), high (H) and average (A) viability classes were determined by cutting the dendrogram at the similarity level of 67.4%.

and commercial hybrids), the freezability classes (H, A and L) and the storage (fresh and frozen–thawed). The age of the boar was also included in the statistical model as covariate. Student's *t* test was used for comparison between least square means.

Results

Semen quality

The dendrogram obtained by hierarchical analysis of semen according to the proportion of live and dead cells after freezing and thawing is represented (Fig. 1). Three classes were obtained by cutting the dendrogram at the similarity level of 67.4%, namely: highest (H), average (A) and lowest (L) proportion of live cells (Table 1). Most boars ($n = 12$) were included in class A, but n was 4 and 3 for classes H and L, respectively. Such a distribution is comparable to a normal distribution.

Sperm concentration decreased progressively from classes H through A to L, with 425.0 ± 98.4, 340.0 ± 40.9 and $303.0 \pm 73.1 \times 10^6$ cells ml^{-1}, respectively.

The freezing and thawing procedure led to a decrease in sperm viability and motility in all classes. Semen samples with the higher proportion of live cells after freezing and thawing also had a higher proportion of live cells before freezing. Sperm viability in fresh samples was significantly higher in classes H and A compared with class L, and the same trend was observed after freezing and thawing (Table 1). As expected, the proportion of dead cells showed a complementary pattern to the proportion of viable cells. The proportion of moribund cells did not differ among the classes either in fresh or frozen semen samples, but it was significantly reduced in stored compared with fresh samples within each class. The relative decreases in the number of live cells owing to the freezing procedure, calculated as the proportions of the difference on the initial value were 45.5, 52.5 and 66.1% in classes H, A and L, respectively.

The proportion of motile cells did not differ among the classes in fresh semen and was significantly decreased, as was viability, in all three classes after freezing and thawing. The loss of motility (fresh versus 1 h after thawing) increased progressively from classes H to L (29.4, 32.2 and 50.9% for classes H, A and L, respectively). However, only the motility of class L differed significantly from classes H and A, which had similar values (Table 1). The relative losses of motility in spermatozoa stored from 1 to 6 h at 37°C after freezing and thawing were 25, 26.6 and 33.2% in classes H, A and L, respectively (Table 1).

Sperm lipid composition

A large variability was found in the total lipid (TL) content of fresh spermatozoa of different boars and the individual values ranged from 236 to 1400 μg per 10^9 cells. The average TL content in class H was lower than it was in classes A and L, but the difference was not significant (Table 2).

All fresh spermatozoa showed a similar lipid class composition, with phospholipids accounting for 75–78% of total lipids (Table 2). Free cholesterol was the second major lipid class, accounting for 24% of total lipid. Triacylglycerols were not detected in fresh cells.

After thawing, the absolute amount of TL increased significantly in classes H, in which the amount almost doubled, and A; TL also increased in class L but the difference was not significant (Table 2), indicating that the viability of spermatozoa before freezing is a determinant

Table 1. Sperm viability (percentage of live, dead and moribund cells) and motility (percentage of motile cells soon after ejaculation or after 1, 4 and 6 h of incubation at 37°C) in boar semen samples classified by high, average and low viability after freezing and thawing

	Fresh semen			Frozen–thawed semen		
	H	A	L	H	A	L
Live	77.5 ± 3.7^{aA}	73.0 ± 1.7^{aA}	58.4 ± 3.7^{aB}	42.2 ± 3.7^{bA}	34.7 ± 1.7^{bA}	19.9 ± 3.7^{bB}
Dead	17.6 ± 3.3^{aA}	23.5 ± 1.6^{aA}	38.3 ± 3.3^{aB}	56.6 ± 3.3^{bA}	64.8 ± 1.6^{bB}	79.3 ± 3.3^{bC}
Moribund	5.0 ± 1.2^{a}	3.9 ± 0.6^{a}	3.2 ± 1.2	1.2 ± 1.2^{b}	0.5 ± 0.6^{b}	0.9 ± 1.2
Motility	63.2 ± 2.2	62.1 ± 1.2	56.4 ± 2.5	–	–	–
Motility 1 h	–	–	–	44.6 ± 4.4^{A}	42.1 ± 2.4^{A}	27.7 ± 5.0^{B}
Motility 4 h	–	–	–	37.1 ± 5.0^{AB}	37.6 ± 2.8^{B}	22.5 ± 5.8^{A}
Motility 6 h	–	–	–	33.5 ± 5.6	30.9 ± 3.1	19.0 ± 6.4

The low (L), high (H) and average (A) viability classes were determined by cutting the dendrogram at the similarity level of 67.4%.
Values are means ± SEM.
^{ab}Within class, values with different superscripts are significantly different ($P < 0.05$); ^{ABC}within type of storage, values with different superscripts are significantly different ($P < 0.05$).

Table 2. Total lipid and lipid classes of washed sperm cells in boar semen samples classified by high, average and low viability after freezing and thawing

	Fresh semen			Frozen–thawed semen		
	H	A	L	H	A	L
µg per 10^9 spermatozoa						
TL	595.7 ± 181.0^{a}	728.5 ± 84.8^{a}	729.8 ± 179.9	1243.9 ± 181.0^{b}	1193.1 ± 84.8^{b}	1084.9 ± 179.9
PL	459.9 ± 106.0	558.9 ± 49.6^{a}	552.3 ± 105.3	690.9 ± 106.0	724.4 ± 49.7^{b}	616.9 ± 105.3
FC	143.3 ± 29.9	168.0 ± 14.0^{a}	172.8 ± 29.7	104.6 ± 29.9	121.1 ± 14.0^{b}	105.8 ± 29.7
TG	nd	nd	nd	440.7 ± 158.2	349.0 ± 72.4	367.9 ± 156.6
FC:PL	0.31 ± 0.07	0.34 ± 0.03^{a}	0.32 ± 0.06	0.15 ± 0.07	0.16 ± 0.03^{b}	0.17 ± 0.06
Percentage of total lipid						
PL	77.7 ± 6.9	75.5 ± 3.2	74.9 ± 6.8	63.9 ± 6.9	63.5 ± 3.2	55.8 ± 6.8
FC	23.6 ± 3.4	24.5 ± 1.6	24.1 ± 3.4	7.4 ± 3.4	10.1 ± 1.6	9.7 ± 3.4
TG	–	–	–	31.0 ± 8.3	23.5 ± 3.8	28.7 ± 8.2

The low (L), high (H) and average (A) viability classes were determined by cutting the dendrogram at the similarity level of 67.4%.
FC: free cholesterol; FC:PL: free cholesterol:phospholipid ratio; PL: phospholipids; TG: triacylglycerols; TL: total lipid.
Values are means ± SEM.
^{ab}Within class, values with different superscripts are significantly different ($P < 0.05$); nd: not detected.

factor for cellular lipid enrichment from the surrounding medium. The increase in the amount of TL was due to a major increase in triacylglycerol content and, to a lesser extent, to an increase in phospholipid content. In contrast, a loss of free cholesterol was measured in all three classes and the decrease was significant in class A when compared with fresh spermatozoa. The proportions of phospholipid and free cholesterol decreased after thawing as a consequence of the increase in triacylglycerol. Triacylglycerols were not detected in fresh samples but accounted for up to 762 µg per 10^9 spermatozoa after freezing. Triacylglycerols are well represented in egg yolk and an interaction between gametes and the egg yolk-based diluent is suggested as the major cause of the described changes in lipid composition. The free cholesterol:phospholipid ratio was similar among classes within fresh and stored samples and was halved after freezing and thawing because of the decrease in the free cholesterol content (Table 2).

The fatty acid composition of the sperm phospholipids did not differ greatly among the three classes, either for fresh or stored semen (Table 3). As expected, palmitic acid (16:0) was the major saturate, and docosapentaenoic (22:5n-6) and docosahexaenoic (22:6n-3) acids were the major polyunsaturates in fresh spermatozoa. The fatty acid compositions of phospholipid and triacylglycerol in the diluent were mainly represented by saturated (16:0) and monounsaturated (18:1n-9) fatty acids (Table 3). The effects of both the freezing procedure and the diluent on sperm phospholipid were to increase significantly the proportion of the most important saturates (16:0 and 18:0), mono-unsaturate (18:1n-9) and linoleic acid (18:2n-6) (Table 3). The relative content of total polyunsaturated fatty acids (PUFA) in frozen–thawed spermatozoa decreased significantly in all classes compared with fresh cells, either as a consequence of the relative increase in the proportions of saturated or of actual loss of PUFA owing to the stress of cryopreservation (resulting in, for example, membrane damage and peroxidation).

Table 3. Relative fatty acid composition of phospholipid in boar spermatozoa and of phospholipid and triacylglycerols in freezing diluent (semen samples are classified by high, average and low viability after freezing and thawing)

	Fresh semen			Frozen–thawed semen			Diluent	
Fatty acid	H	A	L	H	A	L	PL	TG
14:0	4.0 ± 0.5^{a}	3.5 ± 0.3^{a}	4.3 ± 0.6^{a}	1.9 ± 0.5^{b}	2.0 ± 0.3^{b}	2.5 ± 0.6^{b}	< 0.5	< 0.5
16:0	15.2 ± 0.6^{a}	15.1 ± 0.3^{a}	15.1 ± 0.7^{a}	19.1 ± 0.6^{b}	19.4 ± 0.3^{b}	19.4 ± 0.7^{b}	26.9	23.9
18:0	6.7 ± 0.4^{a}	6.4 ± 0.2^{a}	5.9 ± 0.5^{a}	10.3 ± 0.4^{b}	10.2 ± 0.2^{b}	9.9 ± 0.5^{b}	15.1	6.0
18:1n-9	1.5 ± 0.8^{a}	1.9 ± 0.5^{a}	2.1 ± 1.0^{a}	9.5 ± 0.8^{b}	10.1 ± 0.5^{b}	11.0 ± 1.0^{b}	24.0	48.0
18:2n-6	2.5 ± 0.8^{a}	2.4 ± 0.4^{a}	2.7 ± 0.9^{a}	5.0 ± 0.8^{bA}	7.0 ± 0.4^{bB}	7.8 ± 0.9^{bB}	15.1	15.8
20:3n-6	1.5 ± 0.1^{a}	1.5 ± 0.1^{a}	1.2 ± 0.2	0.8 ± 0.1^{b}	1.1 ± 0.1^{b}	0.8 ± 0.2	< 0.5	< 0.5
20:4n-6	3.2 ± 0.2^{a}	3.3 ± 0.1^{a}	3.3 ± 0.2^{a}	4.4 ± 0.2^{b}	4.4 ± 0.1^{b}	4.3 ± 0.2^{b}	6.5	< 0.5
22:4n-6	2.5 ± 0.2^{aAB}	2.4 ± 0.1^{aB}	2.9 ± 0.2^{aA}	1.9 ± 0.2^{b}	1.7 ± 0.1^{b}	1.9 ± 0.2^{b}	nd	< 0.5
22:5n-6	29.1 ± 2.4^{a}	31.0 ± 1.4^{a}	32.3 ± 2.8^{a}	21.1 ± 2.4^{b}	20.8 ± 1.4^{b}	21.5 ± 2.8^{b}	2.5	< 0.5
22:6n-3	29.6 ± 3.1	28.1 ± 1.8^{a}	29.3 ± 3.6	22.0 ± 3.1	19.3 ± 1.8^{b}	17.1 ± 3.6	2.7	< 0.5
SFA	26.5 ± 1.2^{a}	25.7 ± 0.7^{a}	26.0 ± 1.4^{a}	31.9 ± 1.2^{b}	32.2 ± 0.7^{b}	32.4 ± 1.4^{b}	–	–
PUFA	69.3 ± 2.0^{a}	69.7 ± 1.1^{a}	69.7 ± 2.3^{a}	55.9 ± 2.0^{b}	55.1 ± 1.1^{b}	54.2 ± 2.3^{b}	–	–
P:S	2.6 ± 0.2^{a}	2.7 ± 0.1^{a}	2.8 ± 0.2^{a}	1.8 ± 0.2^{b}	1.7 ± 0.1^{b}	1.7 ± 0.2^{b}	–	–

The low (L), high (H) and average (A) viability classes were determined by cutting the dendrogram at the similarity level of 67.4%.
Values are means ± SEM.
[ab]Within class, values with different superscripts are significantly different ($P < 0.05$); [AB]within type of storage, values with different superscripts are significantly different ($P < 0.05$); nd: not detected; SFA: total saturates; PUFA: total polyunsaturates; P:S: polyunsaturates:saturates ratio.

Table 4. Superoxide dismutase (SOD), total glutathione peroxidase (GSH-Px) and proportion of the selenium-dependent glutathione peroxidase (GSH-Px-Se) activities in spermatozoa (spz) and seminal plasma (pl) of boar semen samples classified by high, average and low viability after freezing and thawing

	H	A	L
Fresh semen			
SOD spz (iu mg^{-1} protein)	333.2 ± 135.8	374.4 ± 72.0	283.2 ± 165.6
SOD pl (iu mg^{-1} protein)	5.7 ± 1.3	7.4 ± 0.7	5.8 ± 1.6
GSH-Px pl (iu g^{-1} protein)	6.68 ± 2.8	8.8 ± 1.6	8.2 ± 3.5
GSH-Px-Se pl (%)	90.8 ± 10.0	85.8 ± 4.7	80.7 ± 10.7
Frozen–thawed semen			
SOD spz (iu mg^{-1} protein)	502.2 ± 56.8^{a}	456.7 ± 21.5^{a}	847.6 ± 99.5^{b}

The low (L), high (H) and average (A) viability classes were determined by cutting the dendrogram at the similarity level of 67.4%.
Values are means ± SEM.
[ab]Within type of storage, values with different superscripts are significantly different ($P < 0.05$).

Antioxidant enzyme activities

SOD activity was measured in boar spermatozoa but GSH-Px activity was not detected. In contrast, both enzymatic activities were measured in seminal plasma (Table 4). A large variability in SOD activity was found among the spermatozoa of different boars, with concentrations ranging from 167.7 to 926.6 and from 3.32 to 13.8 iu mg^{-1} protein in spermatozoa and seminal plasma, respectively. Concentrations of GSH-Px ranged from 2.67 to 16.3 iu g^{-1} protein in seminal plasma and the proportion of GSH-Px-Se ranged from 67 to 100% of the total activity. In fresh semen, no significant differences were found among the classes of spermatozoa in terms of antioxidant activities. After freezing and thawing, sperm SOD activity increased in all classes, with a particularly marked difference in class L, which showed a significantly higher activity than those of classes H and A (Table 4).

Discussion

Semen quality in boars is affected markedly by cryopreservation (Almlid and Johnson, 1988; Eriksson and Rodriguez-Martinez, 1996; Rodriguez-Martinez *et al.*, 1996; Woelders *et al.*, 1996). In the present study, the traditional sperm quality parameters, that is, motility and viability, were significantly decreased after the freezing and thawing procedure and an approximate reduction of 50% was observed in sperm viability and of 40% in sperm motility. The magnitude of the loss in sperm quality differed according to the initial quality of the semen: that is, poor quality semen (class L) was more affected, with up to 66 and 51% relative decreases in viability and motility, respectively. Furthermore, the relative loss of motility between 1 and 6 h after thawing increased progressively according to the decrease in semen quality from classes H through A to L. In contrast, Woelders *et al.* (1996) concluded that semen quality before and after

freezing were not related, whereas the breeding line was suggested as a significant source of variation in determining the proportions of motile spermatozoa with normal apical ridge (NAR) in thawed semen.

The fatty acid composition of boar semen was similar to that reported in previous studies (Poulos *et al.*, 1973; Paulenz *et al.*, 1995), with relatively high proportions of 22:5n-6 and 22:6n-3. The total sperm lipid content increased significantly in classes H and A after freezing and thawing compared with the content of fresh cells. The lipids gained during storage were mainly triacylglycerols and, to a lesser extent, phospholipids. If this increase in the lipid content of spermatozoa had been passive (that is, due to contamination from the egg yolk-based diluent or inefficient washing procedure), it would have been expected to occur in a similar fashion in all three classes, which did not prove to be the case. Therefore, we suggest that an active cellular lipid metabolism responsible for lipid transfer or synthesis occurs during incubation of spermatozoa within the egg yolk-based diluent or after the freezing and thawing procedure. Previous studies have reported the ability of egg yolk lipoproteins to bind strongly to the sperm plasma membrane in bulls (Cookson *et al.*, 1984; Vishwanath *et al.*, 1992). Moreover, sperm cells can exchange lipid components with the extracellular environment (Neill and Masters, 1972, 1973; Vasquez and Roldan, 1997) and, furthermore, Buhr *et al.* (1999) suggested a link between successful cryopreservation of boar semen and a given mixture of lipids and fatty acids in the original diluent. Once again, a specific role for the lipids present in the diluent or exchanges with spermatozoa are indicated. The present data also indicate that there was not only an uptake of lipid by sperm cells but also that this uptake was related to the quality of fresh semen. Phospholipase activities may mediate this lipid metabolism, totally or in part (for review, see Roldan, 1998). Buhr *et al.* (1994) reported an increase in the content of phospholipids, and in particular of phosphatidylcholine, during the cryopreservation of boar spermatozoa in the presence of egg yolk. The exact role of yolk components has not yet been clarified. Phosphatidylcholine (also called lecithin) has been proposed as the protective component during freezing (Quinn *et al.,* 1980) since it prevented ultrastructural damage and favoured the maintenance of motility and respiration (Simpson *et al.*, 1987). In contrast, studies have shown that phosphatidylcholine had no effect on boar sperm damage (Pursel *et al.*, 1973) and did not prevent motility loss during cold shock and storage at 5°C (Watson, 1981). Phosphatidylserine has also been proposed as a protective agent in the boar (Butler and Roberts, 1975; Foulkes, 1977). Cationic low density lipoprotein (LDL) of egg yolk, characterized by a specific lipid:protein ratio of 2.7, was found to be the most efficient in protecting bull spermatozoa against cold shock. The cationic protein moiety of the LDL complex bound strongly to the sperm plasma membrane, which is negatively charged, and the lipid moiety was responsible for the protective action (Vishwanath *et al.*, 1992).

In contrast to changes in triacylglycerols and phospholipids, a general loss in the free cholesterol content occurred in spermatozoa after freezing and thawing and a significant difference was measured in class A boars. The loss in sperm cholesterol content is a feature of capacitation (Therien *et al.*, 1998; Visconti *et al.*, 1999). Furthermore, several reports have suggested that cryopreservation increases the proportion of capacitated spermatozoa (Watson, 1995; Gillan and Maxwell, 1999; Maxwell *et al.*, 1999) and that cooling to 4°C induces capacitation-like changes in mouse spermatozoa (Fuller and Whittingham, 1997). The results of the present study support this theory by indicating a loss of cholesterol, which is a characteristic event of capacitation, in cryopreserved spermatozoa.

Alvarez and Storey (1992) demonstrated that cryopreservation enhanced peroxidation in human spermatozoa. A negative correlation between motility and peroxidation was reported during cryopreservation of spermatozoa (Bell *et al.*, 1993). The results of the present study showed that semen samples with the highest viability after freezing and thawing were also characterized by high SOD activity and high proportion of GSH-Px-Se before freezing. However, no significant differences were found among classes in fresh antioxidant enzymatic activities and more data are needed to confirm their importance in successful cryopreservation. There was a general increase in sperm SOD activity after cryopreservation and the difference was particularly marked in spermatozoa with low freezability. Such an increase in SOD activity is unusual as no activity was found in the freezing extender (data not shown). It is possible that the differences in SOD activity were the result of the enzyme extraction procedure having different efficiency on fresh and frozen–thawed cells. In contrast, a 50% decrease in SOD activity was measured after freezing and thawing in bull spermatozoa (Bilodeau *et al.*, 2000). The results of the present study indicate a need for O_2^- and H_2O_2 scavengers. SOD scavenges O_2^- and generates H_2O_2, which is in turn scavenged by GSH-Px-Se. An increased generation of H_2O_2 in class L spermatozoa after thawing may account for the lower quality of this sample (that is, the system might lack H_2O_2 scavengers).

In conclusion, traditional sperm quality parameters are related to the ability of the gametes to survive cryopreservation. High sperm viability before freezing is indicative of both high viability and motility after freezing and thawing. Fresh sperm quality also affects the changes occurring in sperm lipid content after the cryopreservation process, which includes the incubation into the egg yolk-based diluent, and the freezing and the thawing techniques. The best quality gametes after cryopreservation are enriched in lipid, triacylglycerol and phospholipid. More studies are needed to investigate further the mechanism of lipid uptake and its relationship to the freezability of boar spermatozoa.

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