REPRODUCTION
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Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs

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Abstract

This study evaluated the effects of cooling, freezing and thawing on the plasma membrane integrity, kinetics and expression of two sugar transporters glucose transporter-3 and -5 (GLUT-3 and GLUT-5) in spermatozoa from Iberian boars. Semen samples were collected twice weekly from eight young, fertile Iberian boars of the 'Entrepelado' and 'Lampiño' breeds. The samples were suspended in a commercial extender and refrigerated to 17 °C for transport to the laboratory (step A), where they were further extended with a lactose–egg yolk-based extender and chilled to 5 °C (step B) prior to freezing in the presence of glycerol (3%). Spermatozoa were assessed for plasma membrane integrity and sperm motility at each of the steps, including post-thaw (step C). Aliquots were also prepared for immunocytochemical localisation of the sugar transporters (fixed and thin smears for transmission and scanning electron microscopy levels respectively) and for SDS–PAGE electrophoresis and subsequent western blotting, using the same antibodies (rabbit anti-GLUT-3 and anti-GLUT-5 polyclonal antibodies). The results showed lower percentages of progressively motile spermatozoa at step C in both breeds, while the percentage of live spermatozoa was significantly lower only in the 'Entrepelado' breed. The results obtained from electron microscopy clearly showed that Iberian boar spermatozoa expressed the hexose transporters, GLUT-3 and GLUT-5. The pattern of expression, in terms of location and concentration, was characteristic in each case but, in the case of isoform GLUT-5, it remained constant during the different steps of freezing–thawing protocol. These results indicate that cryopreservation affects the status of sperm cells of Iberian boars by altering the distribution of some membrane receptors and decreasing the percentage values of parameters linked to sperm quality.
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Introduction

In recent years, the development of freezing techniques for semen cryopreservation has become a major resource for the preservation of genetic material in most domestic species (Songsasen & Leibo 1997*a*,1997*b*, Ollero *et al.* 1998, Colenbrander *et al.* 2003, Cremades *et al.* 2005). Although the use of cryopreserved semen for artificial insemination (AI) has spread worldwide on a commercial basis for cattle (Curry *et al.* 2000), this has not been the case for pigs, where extended semen in liquid form still dominates and <1% of frozen semen is used mostly in genetic nuclei or for export (Eriksson & Rodriguez-Martinez 2000). Among the reasons for this is the better survival rate, with fertilising capacity intact, of liquid semen that, under farm or farmer AI conditions, provides almost the same prolificacy (number of piglets born) as natural mating. This has enormous sanitary and economic advantages over the more expensive and less fertile frozen–thawed semen.

Moreover, boar spermatozoa suffer extensive membrane and tail damage during cooling and thawing (Watson *et al.* 1981, Parks & Graham 1992), and those spermatozoa that survive suffer from a shortened life-span, requiring AI to be carried out with large numbers of spermatozoa and closely timed to the moment of ovulation (see, for example, Wongtawan *et al.* 2006). The sensitivity of the boar sperm membranes to cooling

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seems to be strongly related to the characteristic lipid composition of these membranes (Bwanga 1991, Parks & Lynch 1992, Watson 1995). Focussing on this point, it is worth noting that Pettit & Buhr (1998) reported that lipid modifications also occur during freezing and thawing, indicating that different domains of the sperm head plasma membrane react differently to cryopreservation. Furthermore, the structural changes produced in the sperm membrane of thawed cells are linked to altered abilities for energy sourcing, which would alter both the housekeeping metabolism of the cell and sperm motility (De Leeuw *et al.* 1990, Watson 1995, Cerolini *et al.* 2001). Another sperm alteration linked to cooling/freezing is related to the transfer of proteins through the cell, which is modulated by the distribution of lipids along the membrane (Parks & Graham 1992), altering the response to induction of capacitation and the acrosome reaction of frozen/thawed spermatozoa during fertilisation (Guthrie & Welch 2005).

The characteristic lability of the boar spermatozoon during cooling (Mazur 1963, Pursel *et al.* 1973, Watson *et al.* 1981) and the specific lipid composition of its membrane (Holt & North 1984, Bwanga 1991, Maxwell & Johnson 1997, Johnson *et al.* 2000, He *et al.* 2001) affect its survival post-thaw that, even when proven protocols are used (Westendorf *et al.* 1975, Bwanga 1991, Eriksson & Rodríguez-Martínez 2000, Johnson *et al.* 2000, He *et al.* 2001, Carvajal *et al.* 2004, Saravia *et al.* 2005), still remains sub-optimal. Many attempts have been made to reduce the alterations produced in sperm cells during this process (Breininger *et al.* 2005, Peña *et al.* 2005, Saravia *et al.* 2005, Bathgate *et al.* 2006), but the results are still far from optimal. Success in the cryopreservation of boar sperm cells might be aided by testing this technique in Iberian pigs, an older breed, which has not been selected for freezability and for which no studies have been reported. Semen conservation of the Iberian boar offers the possibility of preserving genetic variability through biotechnological reproduction programmes. The bottom line is that more insight into the mechanisms underlying cryopreservation-related boar sperm membrane alterations is still needed.

Another major problem related to cryopreservation is the dislocation of proteins in the plasma membrane, for example those belonging to the glucose transporter (GLUT) family. These GLUT proteins, as a whole, are mainly responsible for the transport of hexose across mammalian sperm membranes (Burant *et al.* 1992, Farooqui *et al.* 1997, Angulo *et al.* 1998, Kokk *et al.* 2005). GLUT proteins have been localised in the sperm plasmalemma of dog (Rigau *et al.* 2002), rat (Farooqui *et al.* 1997), human (Kokk *et al.* 2005) and boar (Medrano *et al.* 2006), highlighting their very important role in the regulation of sperm glucose and fructose metabolism.

The aim of the present study was to determine variations in the temporal localisation of the hexose-specific transporters, GLUT-3 and GLUT-5, during cooling and thawing of boar spermatozoa from Iberian pigs with respect to changes in plasma membrane integrity and motility, both of which are indicators of sperm viability and metabolic intactness.

Material and Methods

Animals and sample collection

Eight young (8–10 months old) Iberian boars of the 'Entrepelado' breed ($n=3$, Semen Cardona, SL, Cardona, Spain) and the 'Lampiño' breed ($n=5$, Agropecuaria de Guissona, Guissona, Spain) were used in this study. All the boars had proven fertility following AI with extended liquid semen. The experiment was carried out over a period of 3 months, with the sperm-rich fraction of the ejaculate being manually collected twice weekly, using the gloved-hand method, and analysed to ensure the quality and the homogeneity of the ejaculates. Three ejaculates were evaluated per boar (one per month), so that a total of 24 ejaculates were processed for the analysis of sperm kinematics and membrane integrity and for immunoassays. Immediately after collection, the ejaculated spermatozoa were suspended (1:2; v/v) in Beltsville thawing solution (BTS; IMV, L'Aigle, France). The extended semen samples were cooled and maintained at 17 °C for shipment within 24 h of collection to the Division of Comparative Reproduction, Obstetrics and Udder Health, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden for further processing and analyses. The experimental protocol was designed in accordance with the guidelines established by the Animal Welfare Directive of the Government of Catalonia (Spain) and the Local Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Semen cryopreservation

Immediately after the shipped semen samples were received, they were assessed for sperm motility and morphology, and only those samples with a minimum of 70% progressive motility and 80% morphologically normal spermatozoa were further processed, using a proven protocol (Eriksson & Rodriguez-Martinez 2000). The BTS-extended semen was centrifuged in a programmable refrigerated centrifuge (Centra MP4R, IEC, Needham Heights MN, USA) set at 17 °C at 800 ***g*** for 10 min. After centrifugation, the supernatant was discarded. The remaining pellets were re-extended with a lactose–egg yolk (LEY) extender (80 ml (80% v/v, 310 mM) β-lactose + 20 ml egg yolk), at a ratio that led to a final concentration of 1.5×10^9 spermatozoa/ml. The sperm concentration was manually assessed in a Bürker haemocytometer. After thorough mixing, the semen was further cooled to +5 °C for 2 h in the

centrifuge. At this temperature, the semen was slowly mixed with a third extender consisting of 89.5 ml LEY extender, 9 ml glycerol and 1.5 ml Equex STM (Nova Chemicals Sales Inc., Scituate, MA, USA), which is equivalent to Orvus Es Paste (Graham *et al.* 1971), at a ratio of two parts of semen to one part of extender, yielding a final concentration of glycerol of 3% and a concentration of 1×10^9 spermatozoa/ml at 5 °C. This was verified by manual counting (Bürker haemocytometer). Spermatozoa were packaged at 5 °C in a cool cabinet (IMV, L'Aigle, France) in 0.5 ml polyvinyl chloride (PVC) plastic straws (Minitüb, Tiefenbach, Germany), which were sealed with PVC powder and placed on racks for freezing (Saravia *et al.* 2005). The racks were transferred to the chamber of a programmable freezer (Mini Digitcool 1400; IMV) set at 5 °C. The cooling/freezing rate used was: 3 °C/min from 5 to -5 °C, 1 min for crystallisation and thereafter 50 °C/min from −5 to −140 °C. The samples were then plunged into liquid N_2 (−196 °C) for storage (Saravia *et al.* 2005).

Experimental design

Sperm kinematics, plasma membrane integrity and expression of GLUT-3 and GLUT-5 were assessed at three specific stages: A) after being extended in BTS and kept chilled at 17 °C for ∼24 h, B) after being re-extended in extender-II and cooled to 5 °C for 2 h and C) post-thawing. For each semen processing stage A–C), aliquots of spermatozoa were taken and examined for sperm kinematics using computer-assisted sperm analysis (CASA) and for membrane integrity using SYBR-14/ethidium homodimer (EthD-1). Samples were analysed after 20 min of incubation at 38 °C. From each aliquot, samples were fixed by resuspension in 0.5% (w/v) paraformaldehyde, and smears were made for immunocytochemistry of GLUT-3 and GLUT-5, for later examination in transmission and scanning electron microscopy respectively. Finally, samples were taken for SDS electrophoresis and further western blot analysis of GLUT-3 and GLUT-5.

Sperm plasma membrane integrity and motility

Sperm plasma membrane integrity

Spermatozoa were loaded with SYBR-14 and EthD-1 fluorophores (Molecular Probes Europe BV, Leiden, The Netherlands; Garner & Johnson 1995). Semen sample (0.5 ml) was added to 2.7 µl of a 1 mM SYBR-14 solution in dimethylsulphoxide (suspension A) and 20 µl of this suspension A were mixed with 20 µl solution B, containing 4 µl EthD-1 in 1 ml PBS (pH 7.2–7.4). Loaded spermatozoa were incubated in the dark at 34–37 °C for 30 min before the preparation of wet smears for fluorescence microscope observation (Leitz Aristoplan, 600×). Spermatozoa were screened and classified into three categories: live (green), dead (red) or damaged (dual staining), and the proportion of each category was counted by two trained operators over a total of 200 spermatozoa.

Sperm motility

Twenty microlitres of each sperm sample was placed in a prewarmed (38 °C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and immediately analysed with a CASA instrument (SM-CMA; MTM Medical Technologies, Montreux, Switzerland) on eight predetermined optical fields around the central reticulum of the chamber. A minimum number of 200 spermatozoa were accounted for per sample. The percentage of progressively motile spermatozoa and the straight linear velocity (VSL; µm/s) were determined.

Sperm motility was assessed in a microscope equipped with 38 °C microscope stage and phase contrast optics (200×; Optiphot-2; Nikon, Japan), using the CASA instrument (Microptic, SL, 2002).

Western blot analysis

For western blot analysis, sperm samples were washed twice in PBS and centrifuged twice at 600 ***g*** for 10 min. The pellet was then resuspended and homogenised in mixer buffer by sonication in ice-cold 10 mM Tris–HCl buffer ('Western buffer', pH 7.4) containing 1% (w/v) SDS; (homogenation proportion 1:5, v/v) +15 mM EDTA+150 mM KF +0.6 M sacarose +14 mM β-mercaptoethanol +10.1 g leupeptin +1 mM benzamidine +1 mM phenyl methyl sulphonyl fluoride.

The homogenised suspension was briefly boiled and then centrifuged at 10 000 ***g*** for 15 min at 4 °C. Western blotting was carried out on the resultant supernatants. The total protein content of these supernatants was calculated using the Bradford method (Bradford 1976), after applying a commercial kit (Bio-Rad Laboratories).

After this procedure, which assured a homogeneous loading of protein, a total protein content of 20 µg was loaded into each lane in all the experiments. The immunological analysis was based on SDS gel electrophoresis (Laemmli 1970), followed by transfer to nitrocellulose membranes (Burnette 1981). Since the freezing extender holds a significant amount of non-sperm proteins, the transfer of an equal quantity of sperm proteins to each electrophoretic lane was tested by previously staining each transferred membrane with Poinceau Red (Bio-Rad). Only those membranes holding nearly equal amounts of sperm protein per lane were subsequently subjected to western blotting. The transferred membranes were tested with the primary antibodies at a dilution (v/v) of 1:500 for GLUT-3 and of 1:1000 (v/v) for GLUT-5 (rabbit anti-GLUT-3 (MYK antibody), an affinity-purified polyclonal antibody

(16 amino acid synthetic peptide), which recognises the facilitative GLUT-3 in mammalian tissues, including sperm cells and rabbit anti-GLUT-5 polyclonal antibody (12 amino acid peptide), which reacts with GLUT-5 and does not cross-react with GLUT-1, -2, -3 or -4 (Chemicon Europe Ltd, Hampshire, UK)). Immunoreactive proteins were tested using a peroxidase-conjugated anti-rabbit secondary antibody (Amersham) and the reaction was developed with an ECL-Plus detection system (Amersham). The protein amount was calculated using the Bradford method (Bradford 1976), after applying a commercial kit (Bio-Rad Laboratories).

Immunocytochemistry and electron microscopy

Scanning electron microscopy

Smears were prepared by spreading sperm suspensions of each sampling point (A–C) on to superfrost polylysine-coated slides, which, immediately after being shortly air-dried, were fixed in buffered paraformaldehyde (0.5%) for 15 min at room temperature and further washed in PBS (pH 7.2–7.4). For each slide, two or three optimal areas where spermatozoa were gathered were selected under light microscopy for immunolabelling. Non-specific binding was blocked by incubating the smears for 1 h at room temperature with 2% BSA in PBS. The smears were then rinsed in PBS and incubated for 2 h at room temperature with the same western blot analysis antibodies GLUT-3 and GLUT-5, at a dilution of 1:500 (v/v) in PBS for both cases. The spermatozoa were then washed in PBS and incubated with goat anti-rabbit IgG–15 nm gold (1:25; Auroprobe; Amersham) for 1 h 50 min at room temperature. After this, the samples were washed in PBS and distilled water and then subjected to silver enhancement (IntenSE silver enhancement system for microscopy) for 10 min and air-dried for 24 h in the dark. The smears were coated with platinum/palladium (High Resolution Sputter Coater; Agar Scientific, Accessories for Microscopy, Essex, England) for 10 min and selected regions of the glass were cut and mounted on to stubs for observation under an electron scanning microscope (Jeol SEM 1230 Electron Microscope, at 20 kV of filament intensity).

Transmission electron microscopy

Spermatozoa suspension fixed in 0.5% (w/v) paraformaldehyde in 0.1 M phosphate (Sorensen's) buffer (pH 7.3) for 1 h at 4 °C was centrifuged at 800 ***g*** for 10 min. The resulting pellet was washed thrice in PBS and pre-embedded in 2% (w/v) Agar in PBS to obtain working blocks, which were conventionally dehydrated in graded alcohol solutions (70°, 96° and absolute alcohol, 15 min per step). The dehydrated samples were progressively embedded at 4 °C in LR-White acrylic resin (Acrylic Resin; TAAB Essentials for Microscopy, Berkshire, England), transferred to capsules (1 block/capsule) and polymerised for 24 h at 50 °C. Semithin (1–3 μm) and ultrathin (60–80 nm) sections were obtained, and the latter mounted on 300 mesh nickel grids (Amersham) for immunocytochemical labelling. Non-specific binding was blocked by incubating the sections for 1 h at room temperature with 2% normal goat serum in 1% BSA/PBS. The sections were then washed in PBS and incubated with a 1:500 dilution of GLUT-3 or GLUT-5 antibodies in 0.1% BSA/PBS for 2 h at room temperature. The grids were coated with 1% (w/v) BSA in PBS (buffered BSA) for 20 min, followed by incubation with either GLUT-3 or GLUT-5 antibodies (1:500, v/v in PBS) for 2 h at room temperature. Preimmune rabbit serum and the specific antibody, preabsorbed with saturating concentrations of the corresponding peptide, were used in the primary incubation step as negative stained controls. After extensive washing in a buffer composed of 0.1% (w/v) BSA, 0.15 M NaCl and 0.01 M phosphate buffer (PBB buffer; pH 7.4), the sections were incubated with A-protein–gold (AuroProbe EM G 15 nm; Amersham Corporation) at a dilution of 1:100 (w/v) in PBB buffer for 1 h at room temperature. After being washed thrice in 0.15 M NaCl for 3 min, the cells were finally counterstained with 2% (w/v) uranyl acetate in distilled water for 30 min and 3% (w/v) lead citrate in distilled water for 10 min (Reynolds 1963).

Statistical analysis

For the evaluation of sperm integrity and motility, statistical comparisons of samples were performed by multi-ANOVA (MANOVA). Student's *t*-test was used to compare least square means, and a general linear model was included to assess the differences among the different classes. The level of significance was set at $P<0.05$.

Results

Sperm functional parameters

Sperm plasma membrane integrity

The percentage of live spermatozoa after thawing (step C) was significantly lower in the Entrepelado breed ($P<0.05$), but not in the Lampiño breed. No such difference between the breeds was noted for steps A and B (Table 1).

Sperm motility

Percentages of both motility and VSL showed no significant differences during cryopreservation between the Entrepelado and Lampiño breeds. Comparisons among the three stages of the cryopreservation process showed significant lower values ($P<0.05$) of motility in post-thawed semen (step C) in both varieties, but VSL remained similar (Table 2). Moreover, percentages of motile spermatozoa were similar between 0- and 30-min post-thawing (step C),

Table 1 Membrane integrity in three stages of cryopreservation process (A, B and C). Results are expressed as mean ± s.d. of 24 separate experiments.

	***Entrepelado* boars**			***Lampiño* boars**		
	17 °C (A)	5 °C (B)	Post-thawing (C)	17 °C (A)	5 °C (B)	Post-thawing (C)
Live (%)	65.05±8.10	69.75±7.44	44.76*±12.05	62.72±11.98	64.44±9.76	51.37±7.45
Dead (%)	20.93±6.81	30.70±9.31	46.22*±11.29	28.69±7.49	28.25±6.49	40.06±6.23
Damaged (%)	7.07±0.24	4.32±0.06	9.02*±0.71	6.87±0.77	8.56±0.48	8.56±1.12

*Values statistically different from refrigerated semen ($P<0.05$).

but VSL was significantly lower ($P< 0.05$) after more than 30-min post-thawing in both varieties (Table 3).

Immunocytochemistry of GLUT-3 and GLUT-5

The analyses of the SEM and TEM immunolabelling clearly showed that the boar spermatozoa expressed the hexose transporters, GLUT-3 and GLUT-5, in both the outer and inner plasmalemma. The expression of the hexose transporter isoform GLUT-3, however, showed a different distribution, in terms of both location and concentration among spermatozoa as well as during the various steps of the freezing–thawing protocol used (steps A–C; Figs 1 and 2).

Strong GLUT-3 immunoreactivity was observed in the sperm acrosome membrane of 17 °C-semen (step A), especially in the anterior half of the head clearly delimited by the equatorial segment. The post-acrosomal region showed moderate immunoreactivity with the anti-GLUT-3, while the connecting piece, the midpiece and the principal piece of the sperm tail presented weak immunoreactivity with this antibody (Fig. 1). This pattern of reactivity was maintained in these cells at 5 °C (step B) after adding glycerol during the cryopreservation process, but, post-thaw (step C), the immunolabelling became less clear in terms of both intensity and distribution (Fig. 1).

The immunolabelling of the hexose isoform GLUT-5 in 17 °C-spermatozoa (step A) was mainly located in the apical and principal segments of the acrosomal region, with a moderate labelling in the post-acrosomal region (Fig. 2). However, strong immunoreactivity was also detected along the connecting piece, the midpiece and the principal piece of the sperm tail (Fig. 2). By contrast with what was seen for GLUT-3, the immunolabelling pattern was maintained at 5 °C (step B) and post-thaw (step C; Fig. 2).

Western blot analysis of GLUT-3 and GLUT-5 expression

The presence of hexose transporters, GLUT-3 and GLUT-5, was confirmed by western blotting of the boar spermatozoa using the same specific antibodies for each hexose transporter. For GLUT-3 and GLUT-5, results showed the presence of specific bands of about 50 kDa, which were maintained during cooling (steps A and B) and post-thaw (step C). The band for GLUT-3 corresponded well to that already described in boar spermatozoa (Medrano *et al.* 2006). However, the intensity of this specific GLUT-3 band in supernatants from sperm extracts decreased, in almost all cases, during the cooling and thawing steps (A–C) when compared with equivalent fresh sperm samples (Fig. 3), while that of GLUT-5 increased during steps A–C when compared with equivalent freshly collected spermatozoa (Fig. 3).

Discussion

Cryopreservation, as per the protocol used in the present study, caused dramatic deterioration in sperm viability in Iberian boars, with very low post-thaw survival. Cooling to 5 °C does not seem to be the problem, since no significant changes were detected at either 17 or 5 °C. This suggests that an understanding of the physiological status achieved by boars at temperatures below 5 °C would be crucial to explain boar sperm survival during the cooling–freezing process. These results agree with those of Maldjian *et al.* (2005), who pointed out that the temperature which produces the greatest effect during cryopreservation is the cooling–freezing step below 5 °C.

In our study, the sperm motility percentages obtained are lower than the standard values for post-thawed semen in non-Iberian boars (45–50%; Bwanga *et al.* 1990). However, no real data exist for this parameter in Iberian boars and differences in experimental design, package systems, breeds or individual male responses to

Table 2 Total sperm motility and straight linear velocity (VSL) in three stages of cryopreservation process (A–C). Results are expressed as mean ± s.d. of 24 separate experiments.

	***Entrepelado* boars**		***Lampiño* boars**	
	Motility (%)	VSL (μm/s)	Motility (%)	VSL (μm/s)
Refrigerated semen (17 °C; A)	79.9±11.7	62.6±16.1	73.3±39.6	47.3±21.6†
Semen at 5 °C (B)	80.1±9.8	79.4±14.5	77.0±13.9	82.5±18.5
Post-thawed semen (C)	37.6±13.3*†	79.9±10.9	35.6±9.0*†	65.0±8.3†

*Values statistically different from refrigerated semen ($P<0.05$). †Values statistically different from semen at 5 °C ($P<0.05$).

Table 3 Total sperm motility and straight linear velocity (VSL) post-thawing (C). Results are expressed as mean±s.d. for 24 separate experiments.

	***Entrepelado* boars**		***Lampiño* boars** (μm/s)	
Post-thawing (time after)	Motility (%)	VSL (μm/s)	Motility (%)	VSL
0 min	37.6±13.3	79.9±10.9	35.6±16.3	61.0±11.0*
30 min	35.6±9.0	65.0±8.3	36.3±9.4	52.2±7.3*

*Values statistically different between 0- and 30-min post-thawing (C; $P<0.05$).

cryopreservation must also obviously be taken into account.

Our results showed a VSL increase at 5 °C, but values between refrigerated and post-thawed semen remain similar. Other authors have reported a slight increase in sperm non-progressive hyperactivity movement at 5 °C, although the proportion differs among boars (Cremades *et al.* 2005). The VSL represents the progressive linear motility in a sperm sample, with the result that many kinds of movement are included in the total sperm motility category.

Our study showed that the sperm membrane integrity in Iberian boars was severely affected post-thaw, and that at 5 °C, the most important effect is at acrosomal level. Several other authors have also observed an increase of 50–60% altered acrosomes after thawing (Bwanga *et al.* 1990, Fiser & Fairfull 1990, Hofmo & Almlid 1991, Verheyen *et al.* 1993).

One of the major findings in this study, besides the presence of both GLUT-3 and GLUT-5 on the outer and inner plasma membrane of boar spermatozoa, is the fact that the protein distribution changed during the process, particularly for GLUT-3, whose labelling decreased dramatically post-thaw. These concomitant changes in the membrane would result in a reduction in its ability to use energetic nutrients (De Leeuw *et al.* 1990, Watson 1995), resulting in compromised motility and/or membrane integrity.

Our results indicate that boar spermatozoa express the family members of the facilitative hexose transporters, GLUT-3 and GLUT-5. All these proteins are localised on specific cellular compartments at the level of the sperm head and tail, and their distribution is characteristic. This is logical, since the uptake of essential sugars, such as glucose and fructose, to maintain energy metabolism is mediated for both transporters. Thus, GLUT-3 is a very effective glucose transporter, as has been already reported not only for boar spermatozoa (Medrano *et al.* 2006), but also for the sperm membrane of other mammals, such as rat (Farooqui *et al.* 1997), bull (Angulo *et al.* 1998), dog (Rigau *et al.* 2002) and human (Haber *et al.* 1993). On the other hand, GLUT-5 is a fructose-specific transporter, which has been reported in dog (Rigau *et al.* 2002), bull (Angulo *et al.* 1998) and human (Burant *et al.* 1992) spermatozoa. Our results show that the GLUT-3 molecules in the head of boar spermatozoa are located only at the acrosome region in 17 °C-semen. This location is maintained during further cooling and after thawing. The expression along the midpiece and the principal piece of the tail was moderate in intensity in 17 °C-semen and 5 °C-semen, and poor post-thaw. This carrier is essential for the entry of substrates (in this case glucose) into the spermatozoon, and the uneven distribution in the different sperm domains might link its presence to different metabolic pathways in the spermatozoon, pathways that are not necessarily linked to sperm motility but rather to housekeeping metabolism (membrane activity, etc). This would account for it being located at the highest intensity in the rostral region. Fraser & Quinn (1981) suggested a direct relationship between the expression of GLUT-3 in the acrosome region and the accommodation to a medium with low levels of glucose. Regarding this point, it has been said that boar spermatozoa show a very different affinity to utilise separate monosaccharides, such as glucose, fructose, sorbitol and mannose, and one of the key points in regulating this is the presence and location of separate hexose transporters such as GLUT-3 (Ballester *et al.* 2004, Medrano *et al.* 2006). This implies that the exact location of a specific hexose transporter would be the first regulatory step in the utilisation of a particular carbohydrate to obtain energy. During cryopreservation, the lipids of the sperm membrane are modified in terms of relocation, dispersion and exit, all provoking different alterations in sperm morphology and, in particular, function. Obviously, the changes in the fluidity of the lipid bilayer are accompanied by relocation of the structural proteins of the membrane, including the changes in the localisation and distribution of hexose transporters during cryopreservation. This hypothesis is supported by our results, which show that post-thawed spermatozoa lost the characteristic display of immunolabelling for GLUT-3, perhaps as a result of the loss of these carriers from the membrane. This phenomenon can be related to the decrease of GLUT-3 in the sperm supernatants during cooling, freezing and thawing. Obviously, maintaining the structure of the plasma membrane is perhaps the most challenging task when preserving boar spermatozoa, since alterations to the plasmalemma conspire against the integrity of the cell, its survival and, even for the survivors, the ability to maintain metabolism and fertilising capacity. GLUT-3 has recently been co-localised at various stages of differentiation in lipid membrane domains of spermatogenic cells also harbouring caveolin-1 (Rauch *et al.*

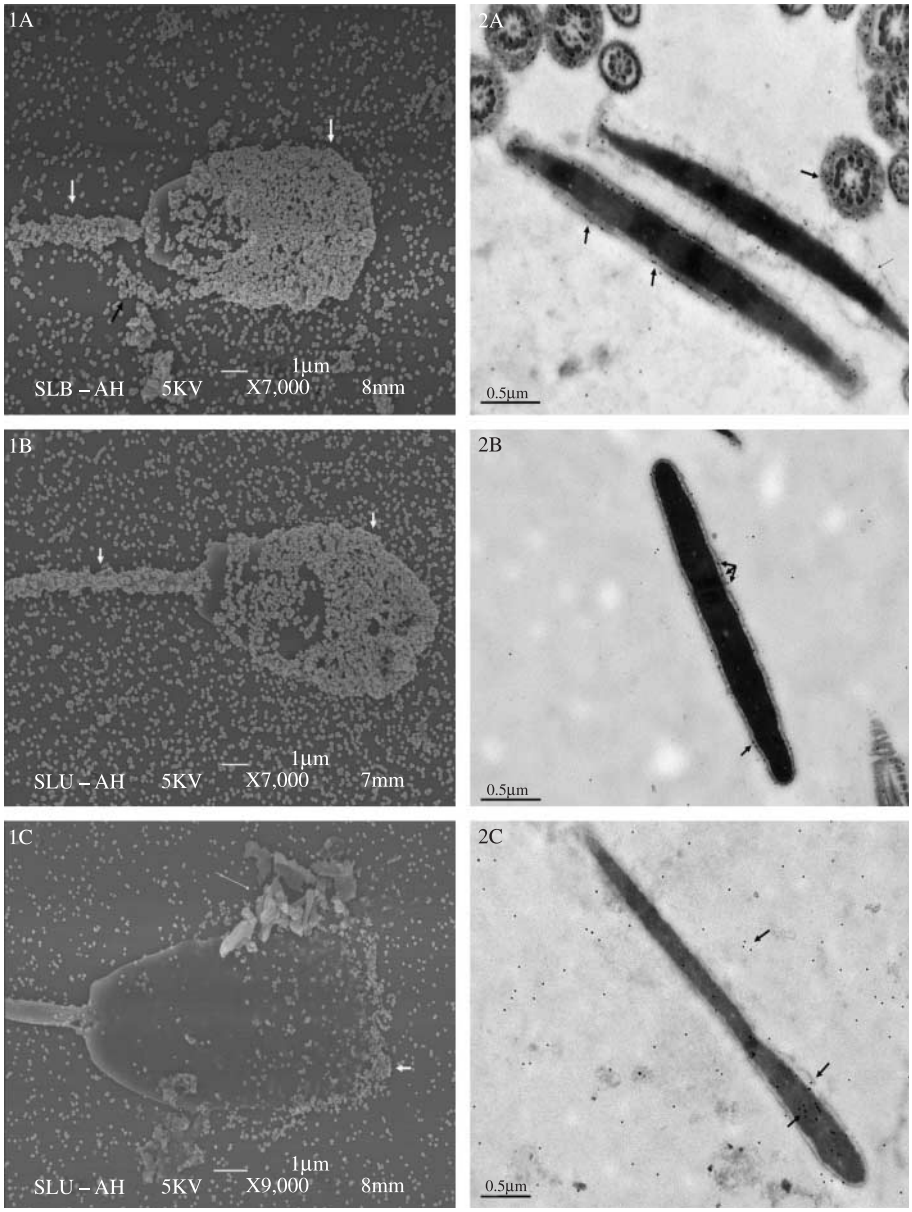

Figure 1 Immunolocalisation of the hexose transporter, GLUT-3, in Iberian boar spermatozoa. Spermatozoa were spread onto coated slides (1) or embedded and cut in ultrathin sections (2) and probed with the GLUT-3 antibody followed by incubation with a secondary antibody coupled to colloidal gold and counter-stained with silver enhancement. Images came from three steps of the freezing–thawing protocol. A: in refrigerated semen at 17 °C; B: at 5 °C, after addition of glycerol; C: post-thaw.

2006). This suggests that the lipid disorder linked to cooling/freezing/thawing would lead to a concomitant alteration in the caveolar structure of the sperm membrane, which, in turn, would greatly alter the location and the presence of caveolae-linked membrane proteins such as GLUT-3. The maintenance of the GLUT-3 location in 5 °C-spermatozoa suggests that the problem is not present there, but it appears during either the sub-zero handling or the thawing process.

The expression of GLUT-5 followed a different pattern, being centred in the post-acrosomal region and along the midpiece and the principal piece of the tail and undergoing no changes in location, distribution or intensity of immunolabelling, despite the spermatozoa being damaged by the cryopreservation process. This distribution in boar spermatozoa differs from that seen in human spermatozoa, where GLUT-5 was only moderately expressed in the post-acrosomal region but strongly expressed along the tail (Angulo *et al.* 1998). In mammals, fructose is mainly internalised from the seminal plasma by the GLUT-5 sperm membrane carrier, directly affecting the glycolytic activity of the midpiece of the tail and, consequently, sperm motility (Grootegoed & Den Boer 1990). Fructose is produced and excreted by seminal vesicles and, in boars, represents an important substrate to store for special situations (Setchell *et al.* 1994, Garner & Hafez 1996, Catt *et al.* 1997, Jones & Bubb 2000, Jones & Connor 2000, Sancho *et al.* 2004, 2006), although the affinity for and the rate of use of fructose in pig spermatozoa are lower than that of

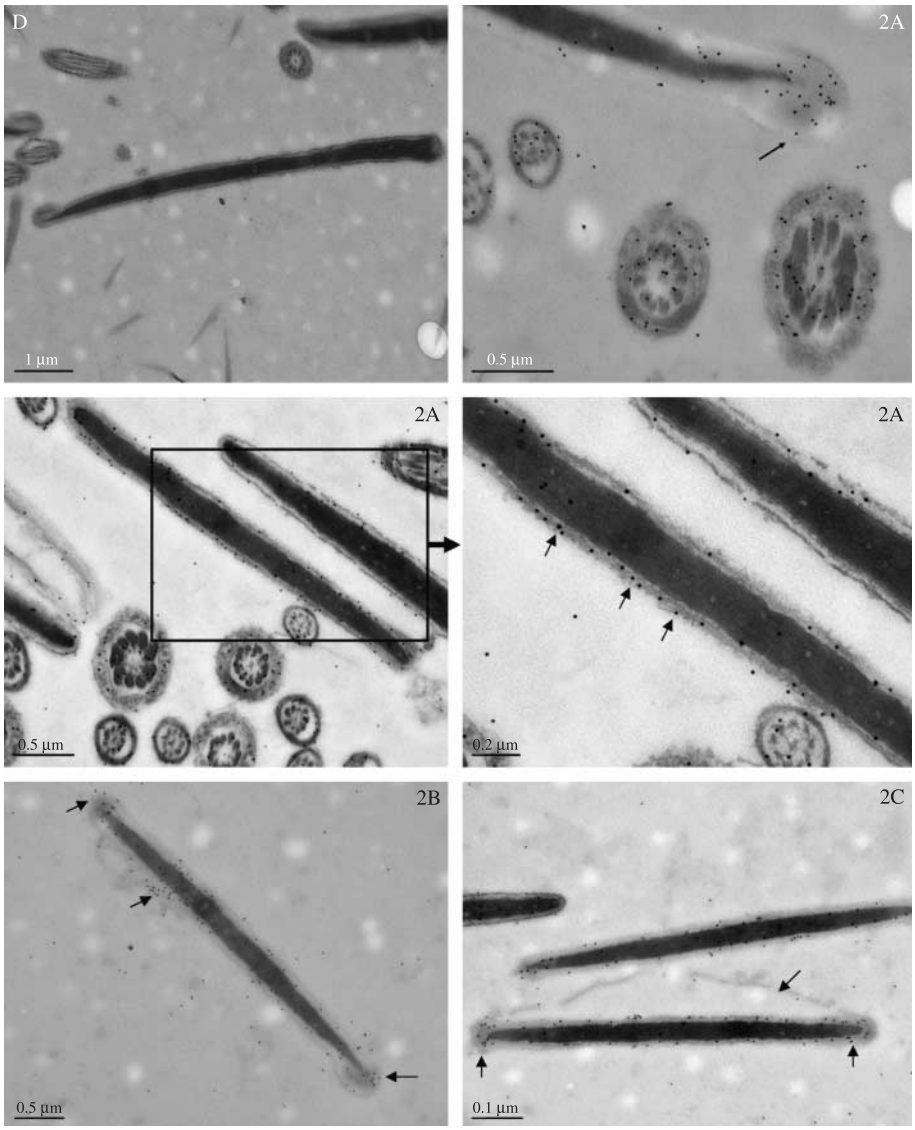

Figure 2 Immunolocalisation of the hexose transporter, GLUT-5, in Iberian boar spermatozoa. Spermatozoa were spread onto coated slides (1) or embedded and cut in ultrathin sections (2) and probed with the GLUT-5 antibody followed by incubation with a secondary antibody coupled to colloidal gold and counter-stained with silver enhancement. Images came from three steps of the freezing–thawing protocol. A: in refrigerated semen at 17 °C; B: at 5 °C, after addition of glycerol; C: post-thaw; D: control for the technique.

glucose (Jones & Connor 2000, Medrano *et al.* 2006). These differences in the affinity of both glucose and fructose might be due to several factors, one of which is the different affinity that the total hexokinase activity of boar sperm shows for each of them (Medrano *et al.* 2006). However, it is certain that the first step marking the different utilisation of glucose and fructose would be related to their specific transporters. The differences observed in the specific location of GLUT-3 and GLUT-5 could be related to these different uses. Our results suggest that both GLUT-3 and GLUT-5 are differently associated with other sperm cell structures. Thus, whereas cooling/freezing caused a decrease in the presence of GLUT-3 in the soluble membrane-linked sperm fraction, the same process caused an increase in the presence of GLUT-5. This difference could be related to GLUT-5 being associated with non-soluble sperm structures, such as dense fibres or mitochondria, and thus primarily linked to motility. There are various possible pathways for the use of these energetic substrates, for example glycolytic versus aerobic pathways, to maintain both the housekeeping needs and the active physiology of the cell. The flagellar function, related to sperm motility and the ATP consuming process, is today a changing concept, since flagellar movement is related to the local ability to produce ATP anaerobically by glycolysis, while the aerobic (e.g. mitochondrial) producing ATP is used for housekeeping metabolism in the midpiece and head domains (Miki *et al.* 2004, Silva & Gadella 2006).

The overall structural alteration caused by the cooling/freezing process would, in turn, induce an alteration in the association of GLUT-5 with these non-soluble structures, and the result would be an increased presence of GLUT-5 in the sperm soluble fraction. On the other hand, GLUT-3, which seems to be mainly linked to the cell

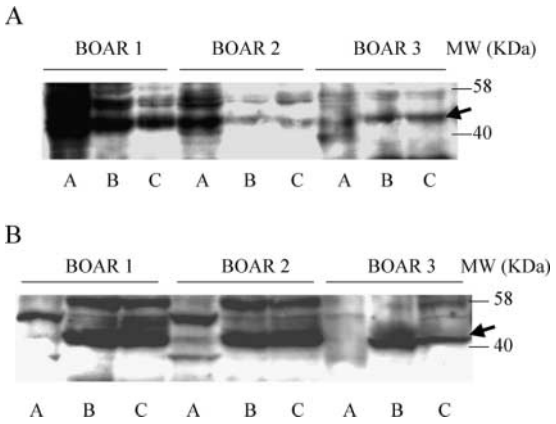

Figure 3 Specific expressions of hexose transporters, GLUT-3 and GLUT-5, in supernatants obtained from Iberian boar sperm extracts. The Figure shows a representative image of western blot analysis for GLUT-3 (a) and GLUT-5 (b) in supernatants from Iberian boar sperm extracts subjected to separate steps of cryopreservation (A–C) run in three experiments (BOAR 1, BOAR 2 and BOAR 3). The obtainment of supernatants from boar sperm extracts has been described in the Materials and Methods section. A: extracts from fresh samples before freezing/thawing. B: extracts from samples after the cooling phase included into the freezing/thawing process (final of the step 3 of the freezing/thawing protocol; see Materials and Methods). C: extracts from thawed samples (final of the step 8 of the freezing/thawing protocol; see Materials and Methods). MW: molecular weight markers. Arrows indicate locations of the specific bands of both GLUT-3 and GLUT-5 (number of replicates = 6).

membrane, will simply be lost after cooling/freezing/thawing, explaining the clearly visible decrease in the presence of GLUT-3 in the supernatants of boar sperm extracts post-thaw. We can only speculate as to how these differences in both localisation and association with other sperm structures modulate the activity of hexose transporters. In any case, the cooling/freezing-associated alterations would induce changes in the ability of boar sperm to manage their energy levels, thus altering overall sperm function after thawing.

In conclusion, the present study confirms the reduction of sperm quality in cryopreserved Iberian semen and reveals that temperatures under 5 °C seem to be a major factor in explaining the failure of sperm to survive the freezing–thawing process. In addition, the effects of cooling–freezing temperatures on the sperm boar membranes, the specific liability of this sperm cell and a wide variation among individual males might contribute to the poor successful response to the cryopreservation of this gamete.

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