Effects of freezing-thawing on the nuclear structure, the mitochondrial function and the sperm subpopulational structure on porcine ejaculates

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Efectes de la congelació-descongelació sobre l'estructura nuclear, la funció mitocondrial i l'estructura subpoblacional espermàtica en ejaculats porcins

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Certifica:

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I perquè consti a tots els efectes que correspongui, signo la present a Bellaterra a 21 de setembre de 2009

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Resum

L'objectiu d'aquest treball és l'avaluació dels efectes que té el procés de congelació-descongelació en diferents estructures de l'espermatozoide porcí, com ara l'estructura nuclear, la funció mitocondrial i l'estructura subpoblacional espermàtica.

En el primer estudi es va comprovar l'efecte de la congelació-descongelació sobre l'estructura nuclear de l'espermatozoide porcí. El procés de congelació-descongelació no va causar trencament de l'ADN però sí que va provocar una desestabilització dels complexes protamina-1–ADN. Aquesta desestabilització ja es va començar a fer evident en les mostres refrigerades a 5 ºC, demostrant que és la fase de refrigeració fins a 5 ºC una de les més letals durant la congelació d'espermatozoides porcins.

En el segon treball es van avaluar els canvis que pateix l'estructura subpoblacional mòtil del semen de porc durant el procés de congelació-descongelació, tenint en compte la congelabilitat dels ejaculats, a més de l'activitat mitocondrial i la formació d'espècies reactives de l'oxigen (ROS) d'origen mitocondrial. Així, es van observar 4 subpoblacions mòtils en tots els tractaments (fresc i congelat-descongelat) i totes les congelabilitats (bons, moderats i mal congeladors) estudiades. La resposta de molts dels paràmetres de motilitat estudiats després de la congelació-descongelació, va ser molt diferent en el semen dels mals congeladors en comparació amb els bons congeladors. A més, en l'estudi de l'activitat mitocondrial es va observar que les mostres dels mals congeladors eren les que presentaven una activitat mitocondrial més baixa.

Finalment, en el tercer treball es va estudiar l'efecte de la criopreservació sobre la funció mitocondrial de l'espermatozoide porcí. Es va avaluar l'activitat mitocondrial juntament amb el ritme de formació de ROS d'origen mitocondrial, així com l'expressió i la localització de dues proteïnes importants en la funció mitocondrial, la mitofusina-2 (Mfn-2) i l'actina. Es va observar que tant l'activitat mitocondrial com la formació de ROS disminuïen amb la congelació amb un descens inicial en la refrigeració. A més, també es van observar canvis en l'expressió i localització tant de la Mfn-2 com de l'actina, que ja es van fer aparents en la refrigeració.

En conclusió, aquests estudis han demostrat que la majoria dels danys provocats pel procés de congelació-descongelació s'inicien durant la refrigeració a 5 ºC, sent aquest pas molt important per a la supervivència espermàtica. El procés de congelació-descongelació provoca desestructuració nuclear, sense arribar a provocar el trencament nuclear, així com canvis en l'estructura de subpoblacions espermàtiques mòtils, sent aquests canvis més evidents en ejaculats amb baixa qualitat en la congelació. Finalment, la congelació-descongelació provoca una disminució de l'activitat mitocondrial deguda a canvis en l'expressió i localització de proteïnes que regulen la funció mitocondrial.

Resumen

El objetivo de este trabajo es el de evaluar los efectos que tiene el proceso de congelación-descongelación en diferentes estructuras del espermatozoide porcino, como la estructura nuclear, la función mitocondrial y la estructura subpoblacional espermática.

En el primer estudio se comprobó el efecto de la congelación-descongelación sobre la estructura nuclear del espermatozoide porcino. El proceso de congelación-descongelación no causó fraccionamiento del ADN, pero sí que provocó una desestabilización de los complejos protamina-1–ADN. Esta desestabilización ya se empezó a hacer evidente en las muestras refrigeradas a 5 ºC, demostrándose que es la fase de refrigeración hasta 5 ºC una de las más letales durante la congelación de espermatozoides porcinos.

En el segundo trabajo se evaluaron los cambios que sufre la estructura subpoblacional mótil del semen de cerdo durante el proceso de congelación-descongelación, teniendo en cuenta la congelabilidad de los eyaculados, además de la actividad mitocondrial y la formación de especies reactivas del oxigeno (ROS) de origen mitocondrial. Así, se observaron 4 subpoblaciones mótiles en todos los tratamientos (fresco y congelado-descongelado) y todas las congelabilidades (buenos, moderados y malos congeladores) estudiadas. La respuesta de la mayoría de los parámetros de motilidad estudiados después de la congelación-descongelación, fue muy diferente en el semen de los malos congeladores en comparación con los buenos congeladores. Además, en el estudio de la actividad mitocondrial se observó que las muestras de los malos congeladores eran las que presentaban una actividad mitocondrial

más baja.

Finalmente, en el tercer trabajo se estudió el efecto de la criopreservación sobre la función mitocondrial del espermatozoide porcino. Se evaluó la actividad mitocondrial juntamente con el ritmo de formación de ROS de origen mitocondrial, así como la expresión y la localización de dos proteínas importantes en la función mitocondrial, la mitofusina-2 (Mfn-2) y la actina. Se observó que tanto la actividad mitocondrial como la formación de ROS disminuían con la congelación con un descenso inicial en la refrigeración. Además también se observaron cambios en la expresión y localización tanto de la Mfn-2 como de la actina, que ya se hicieron aparentes en la fase de refrigeración.

En conclusión, estos estudios han demostrado que la mayoría de los daños provocados por el proceso de congelación-descongelación se inician durante la refrigeración a 5 ºC, siendo este paso muy importante para la supervivencia espermática. El proceso de congelación-descongelación provoca desestructuración nuclear, sin llegar a provocar el fraccionamiento nuclear, así como cambios en la estructura de subpoblaciones espermáticas mótiles, siendo estos cambios más evidentes en eyaculados con baja calidad en la congelación. Finalmente, la congelación-descongelación provoca una disminución de la actividad mitocondrial debida a cambios en la expresión y localización de proteínas que regulan la función mitocondrial.

Abstract

The aim of this work is to evaluate the effects of freezing-thawing process on different structures of porcine spermatozoa, such as nuclear structure, mitochondrial function and spermatic subpopulational structure.

In the first study we tested the effect of freezing-thawing on the nuclear structure of porcine spermatozoa. Freezing-thawing did not cause DNA fragmentation, although a clear destabilization of the protamine-1–DNA complexes was apparent. This destabilization started after cooling phase to 5 ºC included in the freezing-thawing procedure. This indicates that this cooling phase is one of the most lethal phases during the freezing-thawing process of porcine spermatozoa.

In the second study we evaluated the changes that the boar sperm motile subpopulational structure suffered during the freezing-thawing procedure taking into account the freezability of the ejaculates, as well as the mitochondrial activity and the mitochondrial formation of reactive oxygen species (ROS). We observed 4 motile subpopulations in all the treatments (fresh and frozen-thawed) and all the freezabilities (good, average and bad freezers) studied. The response of the majority of the motility parameters studied after the freezing-thawing was very different in the bad freezers semen compared with the good freezers. Furthermore, in the study of the mitochondrial activity we observed that the bad freezers samples presented the lowest mitochondrial activity.

Finally, in the third work we studied the effect of the cryopreservation on the mitochondrial function of porcine spermatozoa. We evaluated the mitochondrial activity together with the rhythm of mitochondrial ROS formation, as well as the expression and

localization of two important proteins on the mitochondrial function, mitofusin-2 (Mfn-2) and actin. We observed that mitochondrial activity as well as mitochondrial ROS formation decreased during the freezing with an initial drop in the cooling phase. Furthermore we also observed changes on the expression and localization of Mfn-2 and actin, which became apparent on the cooling phase.

In conclusion, this study has demonstrated that the majority of the damage caused by the freezing-thawing process began during the cooling phase to 5 ºC, being this step very important for sperm survival. The freezing-thawing process caused a nuclear destructuration, without causing nuclear fragmentation, as well as changes on the motile sperm subpopulational structure, being these changes more evident on ejaculates with the poorest freezing quality. Finally, freezing-thawing caused a decrease on the mitochondrial activity due to changes on the expression and localization of proteins which regulate mitochondrial function.

Als meus pares,

A la meva germana,

A en Marc

Força
Valor
Equilibri
Seny

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Introducció / Introduction

1. Història i importància de la criopreservació espermàtica

1.1. Història de la criopreservació

El punt de partida de la criopreservació espermàtica va ser el descobriment de les propietats crioprotectores del glicerol per part de l'equip d'investigació de Polge l'any 1949 (Polge *et al*, 1949). Degut al gran èxit obtingut amb la congelació de semen boví, es va creure que amb el semen porcí també s'aconseguirien els mateixos resultats, però aviat es va veure que seria una tasca difícil que encara avui no està resolta del tot (Polge, 1965). Els primers godalls nascuts a partir d'espermatozoides criopreservats van néixer al 1970 gràcies també al grup de Polge, que van inseminar truges per laparotomia amb espermatozoides criopreservats (Polge *et al*, 1970). Un any més tard, tres equips d'investigació dels Estats Units van obtenir els primers godalls a partir d'espermatozoides criopreservats després d'inseminació artificial tradicional (Crabo i Einarsson, 1971; Graham *et al*, 1971; Pursel i Johnson, 1971). Els primers protocols de criopreservació específics per espermatozoides porcins van arribar el 1975 a Alemanya (Westendorf *et al*, 1975) i a Estats Units (Pursel i Johnson, 1975). Actualment els mètodes més utilitzats són modificacions d'aquests protocols. El 1985 es va celebrar a Uppsala (Suècia) el primer Congrés Internacional sobre criopreservació espermàtica porcina. A la dècada dels 90 es van millorar les condicions del procés de congelació amb l'estudi de nous envasos, corbes de congelació, etc. En aquest període i fins ara, la

Facultat de Veterinària d'Uppsala (Suècia) va ser un referent mundial, amb els treballs dirigits pels Drs. Einarsson i Rodríguez-Martínez. En aquesta mateixa dècada es van realitzar importants estudis sobre les bases de la criopreservació i les particularitats del procés en l'espècie porcina, on destaquen els treballs dels britànics Watson i Holt i del grup espanyol del Dr. Emilio Martínez de la Universitat de Múrcia.

1.2. Importància de la criopreservació

La criopreservació de semen té molts avantatges:

- Permet transportar mostres seminals a grans distàncies sense comprometre la viabilitat espermàtica, eliminant a més la dificultat associada al transport d'animals o de semen fresc a distàncies llargues o per períodes de temps perllongats.
- Permet conservar material genètic d'elevada importància, creant bancs de germoplasma per preservar mostres seminals d'animals de races en perill d'extinció o d'animals genèticament valuosos.
- Pot ser una eina important per a millorar la producció de carn porcina a nivell internacional fent els aparellaments adients per a obtenir un progrés òptim.
- Ofereix una seguretat extra en el cas que s'hagin de prendre mesures per al control d'una malaltia o plaga per tal de no perdre la diversitat genètica existent.

Tot i les aplicacions potencials del semen congelat-descongelat en la indústria porcina, la seva utilització per inseminació artificial és limitada, ja que menys de l'1 %

de les inseminacions artificials fetes a tot el món en l'actualitat són amb semen congelat-descongelat (Saravia *et al*, 2005). Aquest fet és degut a que l'ús de semen congelat en els programes d'inseminació artificial en les granges porcines actualment és poc rentable comparat amb la utilització de semen fresc o refrigerat a 17 ºC. Els principals factors que expliquen aquesta baixa rendibilitat són:

- Cost elevat de la congelació tant pel que fa a equipament de laboratori com a temps requerit.
- Baixa supervivència dels espermatozoides congelats, que fa que es necessitin un gran nombre d'espermatozoides per dosi d'inseminació per tal que aquesta tingui èxit.
- Baixes taxes de fecunditat i baix nombre de godalls nascuts vius (Watson, 2000; Guthrie i Welch, 2005; Bailey *et al*, 2008), si bé aquest punt pot minimitzar-se aplicant-ne tècniques d'inseminació més eficients, com ara la d'inseminació intrauterina profunda (Eriksson *et al*, 2002; Roca *et al*, 2003).
- Gran variabilitat entre mascles en l'èxit de la congelació.
- Temps crític per a la inseminació degut a un temps de vida curt dels espermatozoides congelats (Johnson *et al*, 2000).
- Manca de tests de laboratori fiables per a una determinació acurada de la qualitat seminal post-congelació.

A més, les dosis seminals refrigerades presenten una vida relativament llarga, entre 7 i 14 dies, cosa que permet solucionar molts dels problemes de recol·lecció i distribució del semen sense la necessitat de la criopreservació.

Tenint en compte aquesta situació, les vies per a millorar la criopreservació espermàtica porcina han anat dirigides, entre d'altres punts, a optimitzar els sistemes de

congelació per obtenir una qualitat seminal acceptable després de la congelació. Des dels primers protocols de congelació desenvolupats fa més de 30 anys (Pursel i Johnson, 1975; Westendorf *et al*, 1975), s'han publicat nombrosos treballs en els quals s'han avaluat diferents passos del procés de congelació-descongelació, com les taxes de refredament i descongelació, els agents crioprotectors i els sistemes d'empaquetament, entre d'altres (revisat per Holt, 2000; Johnson *et al*, 2000; Watson, 2000). Gràcies a aquests estudis i a les noves tècniques d'inseminació introduïdes, la congelació de semen porcí està arribant a nivells força satisfactoris (Eriksson *et al*, 2002; Roca *et al*, 2003). No obstant, encara no s'han assolit els límits òptims per l'aplicació pràctica d'aquesta tècnica. Per tant, cal un estudi més acurat dels factors que influencien la supervivència espermàtica després del procés de congelació-descongelació per tal de conèixer els danys que pateixen els espermatozoides porcins durant aquest procés.

2. Protocols de criopreservació espermàtica porcina

L'evolució dels protocols de criopreservació espermàtica en els últims 10-15 anys ha donat lloc a mètodes que permeten una millora substancial de la qualitat espermàtica post-descongelació. Els canvis més significatius s'han donat en els mètodes d'envasat de les dosis seminals substituint les macropalletes de 5 mL i les píndoles utilitzades al principi per palletes de 0,5 mL o els FlatPack®, que permeten una congelació i descongelació homogènia degut a una major relació de superfície/volum. També s'han produït canvis en la velocitat de congelació i descongelació gràcies al coneixement més acurat dels canvis físics que es donen durant aquest procés. Aquests canvis han permès desenvolupar corbes de congelació i velocitats de descongelació

específiques per als espermatozoides porcins. Finalment, la incorporació de biocongeladors automatitzats ha permès una congelació precisa i homogènia d'un gran nombre de palletes simultàniament.

Els protocols actuals de congelació i descongelació de semen porcí consten així de les següents etapes:

- Recollida i manipulació de l'ejaculat
- Preparació dels diluents de congelació i dilució espermàtica
- Envasat de les dosis seminals
- Congelació
- Descongelació

2.1. Recollida i manipulació de l'ejaculat

Només es recull la fracció rica de l'ejaculat que es filtra i es dilueix 1:1 amb un diluent salí, com ara el *Belstville Thawing Solution* (BTS; Pursel i Johnson, 1975; Taula 1) temperat a 30-33 ºC per evitar el xoc tèrmic. Després, el semen es diposita en una cambra refrigerada a 16-17 ºC, on es deixa durant 3 hores per tal d'aconseguir un descens de la temperatura gradual i homogeni. Mentrestant, s'avalua la qualitat seminal determinant la concentració, el percentatge d'espermatozoides mòtils i la qualitat de moviment, així com el percentatge d'espermatozoides vius i les alteracions morfològiques i acrosòmiques. Els ejaculats destinats a la criopreservació han de tenir una qualitat elevada que superi uns valors mínims (Taula 2). Passades les 3 hores, el semen es centrifuga (600 *g* durant 10 minuts), s'elimina el sobrenedant per aspiració i el sediment es resuspèn en el diluent de congelació.

Taula 1. Composició del diluent *Belstville Thawing Solution* (BTS).

Component	**Concentració**
Glucosa	205 mM
Citrat sòdic	20,4 mM
Clorur potàssic	10 mM
Bicarbonat sòdic	15 mM
Àcid etilendiaminotetracètic (EDTA)	3,6 mM
Kanamicina	70 mg/L
pH	7,2 – 7,4
Osmolaritat	295 – 330 mOsm/L

2.2. Preparació dels diluents de congelació i dilució espermàtica

Els espermatozoides porcins són sensibles al refredament en el rang de temperatures entre 15 i 5 ºC. El dany per refredament es pot reduir per la inclusió de crioprotectors en el diluent de congelació (Woelders *et al*, 2005). Els diluents de criopreservació contenen un protector pel refredament i un protector per la congelació. El rovell d'ou és un dels protectors pel refredament més emprat en els diluents de congelació, i normalment representa un 20 % del volum total del diluent. S'ha demostrat que l'addició de rovell d'ou modifica els components proteics dels espermatozoides, millorant així la protecció durant l'emmagatzematge (Gilmore *et al*, 1998). A més, també protegeix la motilitat espermàtica, que es veu afectada tant per la refrigeració com per la congelació i la descongelació (Kikuchi *et al*, 1998). Com a protector per la congelació es sol utilitzar el glicerol. L'efecte crioprotector del glicerol

Taula 2. Valors mínims que haurien de tenir els ejaculats porcins destinats a la criopreservació.

Paràmetres de l'ejaculat	Valors mínims
Volum de la fracció rica (mL)	≥ 100
Concentració espermàtica ($\times 10^6$ spz/mL)	≥ 250
Motilitat espermàtica (%)	≥ 80
Viabilitat espermàtica (%)	≥ 80
Alteracions acrosòmiques (%)	≤ 10
Formes anormals (%)	≤ 15

és més evident a concentracions elevades, però s'ha de tenir en compte la seva toxicitat. La sensibilitat de l'espermatozoide a aquests efectes tòxics varia amb l'espècie, i s'ha observat que l'espermatozoide porcí és molt sensible a patir danys acrosomals a concentracions de glicerol relativament baixes (Curry, 2000). No existeixen diluents comercials formulats per a la criopreservació d'espermatozoides porcins. Per tant, aquests es preparen en el mateix laboratori i normalment el mateix dia en que s'han d'utilitzar. Els diluents més emprats contenen dos components bàsics, lactosa i rovell d'ou (LEY) i la seva composició es mostra a la Taula 3. A partir d'aquest diluent LEY es prepara un segon diluent (LEYGO) que incorpora el glicerol, com a crioprotector, i un detergent sintètic que afavoreix l'acció crioprotectora del rovell d'ou (Taula 3). La dilució del sediment espermàtic amb el diluent de congelació es fa en dues etapes. Una primera etapa immediatament després de la centrifugació amb el diluent LEY. Un cop diluïda la mostra, es torna a fer un recompte de la concentració espermàtica i s'ajusta la concentració a 15-30$\times 10^8$ espermatozoides/mL amb el diluent LEY. Tot seguit, el

Taula 3. Composició dels diluents de congelació lactosa – rovell d'ou (LEY) i LEY – glicerol – Equex Stem (LEYGO).

Composició	LEY	LEYGO
Rovell d'ou (%)	20	-
β-lactosa (mM)	248	-
Kanamicina (g/L)	0,1	-
LEY (%)	-	89,5 - 92,5
Glicerol (%)	-	6 - 9
Equex Stem® (%)	-	1,5
pH	6 - 6,3	6 - 6,3
Osmolaritat (mOsm/Kg)	330 - 390	1.650 - 1.750

semen diluït es porta a una cambra refrigerada a 5 ºC per induir un descens de la temperatura suau i progressiu de 16-17 ºC fins a 5 ºC en un temps de 2 hores. Aquest descens de temperatura lent i gradual permet disminuir el xoc fred que pateixen els espermatozoides porcins en aquestes temperatures (Woelders *et al*, 2005). Un cop a 5 ºC, es realitza la segona etapa de dilució, rediluint el semen amb el diluent LEYGO fins a una concentració final de 10-20×10^8 espermatozoides/mL. Finalment, el semen és envasat en els recipients escollits.

2.3. Envasat de les dosis seminals

Com ja s'ha mencionat anteriorment, els mètodes d'envasat més habituals són les palletes de 0,5 mL i els FlatPack® normals o mini. Aquests sistemes ofereixen

diferents possibilitats de volum i nombre d'espermatozoides per dosi, i es tria un o altre sistema depenent del mètode d'inseminació que es vulgui emprar. Els FlatPack® normals són adequats per a la inseminació tradicional ($5\text{-}6\times10^6$ espermatozoides/dosi d'inseminació), mentre que els mini-FlatPack® i les palletes de 0,5 mL són apropiades per la inseminació intrauterina profunda ($1\text{-}2\times10^9$ espermatozoides/dosi d'inseminació). L'envasat de les palletes es fa a 5 ºC, existint-ne diversos sistemes d'envasat automàtics i semi-automàtics.

2.4. Congelació

La congelació de les dosis seminals es fa de forma manual o mitjançant biocongeladors automatitzats (Figura 1). L'ús de biocongeladors té dos grans avantatges. En primer lloc, permet congelar un gran nombre de palletes alhora i en segon lloc, permet baixar la temperatura de forma controlada. La taxa de congelació és un factor clau a tenir en compte per tal de minimitzar el dany cel·lular causat a l'espermatozoide durant la congelació-descongelació. Si la velocitat de congelació és massa ràpida, l'aigua serà incapaç de sortir de l'espermatozoide i es congelarà formant cristalls. Si el refredament és massa lent, l'aigua pot sortir de l'espermatozoide prevenint la formació de cristalls intracel·lulars, però deixarà els soluts a dins la cèl·lula a una concentració massa elevada (Watson, 2000; Thurston *et al*, 2003). A més, tant a velocitats de refredament massa ràpides com massa lentes, la cèl·lula pateix danys per canvis osmòtics bruscs que provoquen un flux d'aigua molt elevat a través de la membrana plasmàtica. Aquests fluxos d'aigua elevats fan que la membrana plasmàtica de l'espermatozoide es desestabilitzi (Woelders *et al*, 2005). Per tant, una taxa de

refredament òptima ha de ser suficientment lenta per prevenir la formació de gel intracel·lular, però alhora prou ràpida per evitar una concentració de soluts massa elevada (Thurston *et al*, 2003). En el porc, la congelació és molt ràpida degut a que l'espermatozoide porcí és molt sensible al glicerol i per tant s'utilitzen concentracions baixes d'aquest que requereixen que la congelació sigui ràpida (Mazur, 1977). La corba de congelació emprada habitualment per congelar les dosis seminals porcines envasades en palletes de 0,5 mL sol seguir un perfil com el que es mostra a la Figura 1.

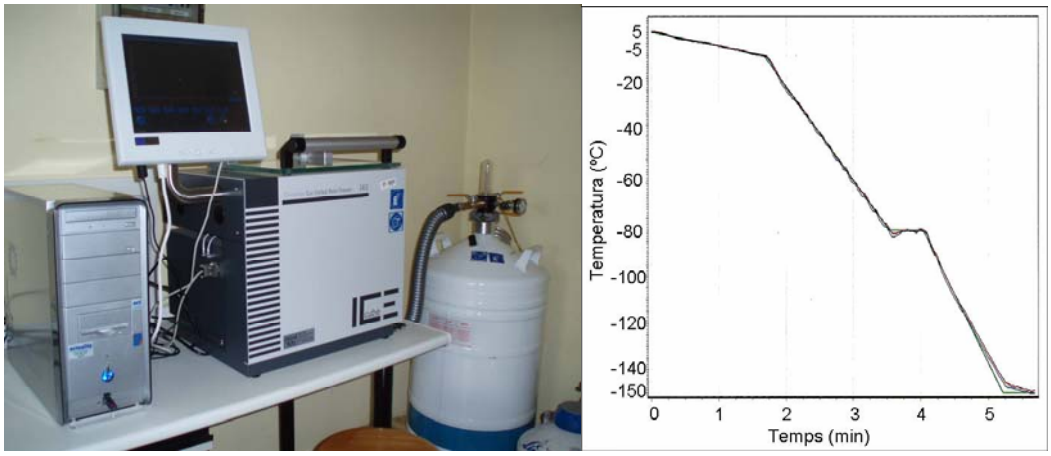

Figura 1. Biocongelador programable i corba de congelació emprats per a la congelació de mostres seminals porcines.

2.5. Descongelació

Cal tenir en compte que el procés de descongelació és tant o més perjudicial per a la supervivència espermàtica que la congelació en si (Thurston *et al*, 2003). Per tant, la velocitat de descongelació és un factor d'importància vital per tal d'explicar la supervivència espermàtica durant el procés. Fiser *et al* (1993) va demostrar que una taxa de descongelació relativament ràpida és beneficiosa per a l'espermatozoide porcí. Així,

per palletes de 0,5 mL es sol utilitzar una velocitat de 1.100 ºC/min (Hernández *et al*, 2006). La descongelació es fa introduint les palletes en un bany termostatitzat a 37 ºC durant 20 segons. Tot seguit, es treuen les palletes, s'assequen bé i es diposita el seu contingut en un tub prèviament temperat a 37 ºC. Finalment, les dosis descongelades es dilueixen en BTS fins a completar la dosi d'inseminació per al seu ús en els programes d'inseminació artificial (Hernández *et al*, 2006).

3. Analítica seminal porcina

És molt útil conèixer la fertilitat d'un ejaculat abans del seu ús en la inseminació artificial. Existeixen un gran nombre de proves que permeten saber la qualitat d'un ejaculat, encara que no prediuen amb exactitud la fecunditat d'aquest. Les tècniques més utilitzades en l'anàlisi seminal rutinari inclouen l'avaluació de la concentració, la motilitat, la viabilitat, la morfologia, la valoració acrosòmica i proves de funcionalitat espermàtica com són el test de resistència osmòtica (ORT; Schilling *et al*, 1986) o el test de resistència hiperosmòtica (HRT; Caiza de la Cueva *et al*, 1997b; González-Urdiales *et al*, 2006). Els intervals normals dels valors d'alguns paràmetres de qualitat d'un ejaculat porcí es mostren a la Taula 4. Altres tècniques més complexes també són emprades per conèixer més a fons com funciona l'espermatozoide. Entre elles destaquen l'avaluació de la integritat de l'ADN espermàtic, l'estudi de l'estructura de subpoblacions mòtils i l'estudi de la senyalització intracel·lular.

Taula 4. Característiques de l'ejaculat de verro.

Característiques de l'ejaculat	Valors normals
Volum fracció rica (mL)	40-100
Concentració fracció rica ($\times 10^9$ spz)	400-1.300
Motilitat total (%)	70-90
Viabilitat espermàtica (%)	80-95
Anomalies morfològiques (%)	15-20
Acrosomes alterats (%)	1-5
ORT (%)	65-80
HRT (Unitats arbitràries)	0,9-0,6

3.1. Concentració espermàtica

L'avaluació de la concentració espermàtica és una de les probes d'anàlisi seminal més important, ja que permet calcular el nombre de dosis seminals que es poden obtenir a partir d'un ejaculat. Es pot mesurar amb varis mètodes, com ara l'espectrofotometria, la citometria de flux, els sistemes computeritzats (CASA) i la utilització d'una cambra de recompte cel·lular com les de Bürke, Neubauer o Thoma (González-Urdiales *et al*, 2006). Tant l'espectrofotometria com la citometria de flux són mètodes indirectes. L'espectrofotometria mesura la llum monocromàtica absorbida per les partícules en suspensió, i es compara amb una corba estàndard patró prèviament validada. Permet mesurar la concentració de forma objectiva, ràpida i bastant exacta però té l'inconvenient que l'aglutinació espermàtica i la precipitació d'algunes proteïnes del plasma seminal proporcionen certa heterogeneïtat òptica als ejaculats. La citometria

de flux determina el nombre de partícules per unitat de volum, encara que no pot assegurar que totes les partícules siguin realment espermatozoides (Woelders, 1990). Aquest mètode és molt exacte i ràpid, però requereix d'una infraestructura molt cara. Els mètodes computeritzats d'anàlisi seminal (CASA) també permeten fer un recompte de la concentració, però al igual que amb la citometria de flux, no es pot assegurar que totes les partícules siguin espermatozoides, així, aquests sistemes tendeixen a fer una sobrevaloració de la concentració espermàtica. D'altra banda, la cambra de recompte cel·lular consisteix en realitzar un comptatge en el microscopi òptic a un augment de 400× de la mostra seminal prèviament diluïda en una solució fixadora. Aquest mètode és molt econòmic i fàcil de realitzar, però té alguns inconvenients com són el temps que es requereix per realitzar-lo i la possible variació entre duplicats.

3.2. Motilitat espermàtica

La motilitat espermàtica és el paràmetre que s'utilitza més freqüentment per a valorar la qualitat d'un ejaculat, tot i que presenta una baixa correlació amb la fertilitat en el porcí (Quintero-Moreno *et al*, 2004). Es pot valorar de diferents maneres, i tot i que cada vegada s'utilitzen més els sistemes d'anàlisi computeritzat (CASA; Figura 2), actualment encara s'utilitza la valoració visual amb microscòpia òptica pràcticament com a únic mètode en molts centres d'inseminació artificial porcina (González-Urdiales *et al*, 2006). No obstant, s'ha de tenir en compte que la valoració visual és un mètode molt subjectiu i dóna lloc a resultats molt dispars depenent de l'observador. Els sistemes CASA rebaixen en gran mesura la subjectivitat i estableixen de manera efectiva paràmetres precisos del moviment individual dels espermatozoides. Els sistemes CASA

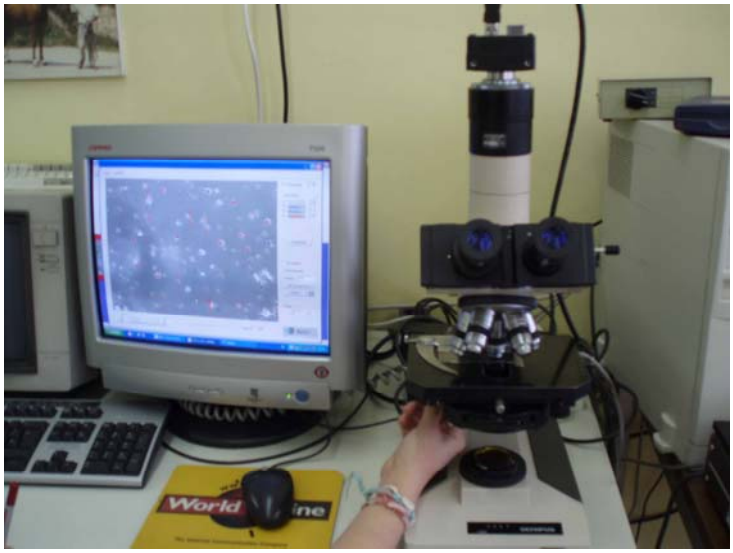

Figura 2. Sistema computeritzat d'anàlisi de la motilitat espermàtica (CASA). Consta d'un microscopi de contrast de fase connectat a una platina temperada, una càmera de vídeo d'alta resolució connectada a una pantalla de televisió i un software d'anàlisi d'imatges per ordinador.

es basen en la captura successiva d'imatges d'espermatozoides en moviment a través d'un microscopi. Aquestes imatges es digitalitzen, identificant en primer lloc les cèl·lules espermàtiques que conté la primera imatge. Després es procedeix a seguir aquestes cèl·lules en imatges successives i a establir les trajectòries definitives. Les trajectòries es processen matemàticament, per a obtenir així uns resultats numèrics precisos per a cada espermatozoide que donen informació de la velocitat del moviment en base a varis descriptors, la trajectòria que realitza el cap de l'espermatozoide i la freqüència dels canvis de direcció del cap (Serres, 1984). Els sistemes CASA consten d'un microscopi amb contrast de fase connectat a una platina temperada per mantenir les mostres a 37 ºC, una càmera de vídeo d'alta resolució connectada a una pantalla de televisió i un software d'anàlisi d'imatges per ordinador. A més d'augmentar la fiabilitat dels resultats obtinguts, l'anàlisi de la motilitat espermàtica mitjançant els sistemes

CASA ha permès estudiar aspectes de motilitat molt més subtils, com ara l'estructura de subpoblacions mòtils presents en els ejaculats de qualsevol mamífer, d'interès per a la millora de l'anàlisi de qualitat seminal (Abaigar *et al*, 1999; Rigau *et al*, 2001; Quintero-Moreno *et al*, 2003, 2004 i 2007; Martínez-Pastor *et al*, 2005; Rivera *et al*, 2006; Flores *et al*, 2008; Muiño *et al*, 2008; Ramió *et al*, 2008).

3.3. Viabilitat i morfologia espermàtiques

La integritat de la membrana plasmàtica ha estat un dels paràmetres més estudiats degut al seu paper clau en la funció espermàtica. Aquells espermatozoides que presenten la membrana plasmàtica estructuralment íntegra són classificats com a viables. El mètode més utilitzat per avaluar la viabilitat d'un ejaculat és la tinció dels espermatozoides. Una tinció vital és aquella que ens permet distingir els espermatozoides viables d'aquells que presenten alguna alteració en la membrana plasmàtica (no viables). La tinció més utilitzada és la Eosina-Nigrosina (Bamba, 1988) perquè és molt econòmica, ràpida i fàcil d'utilitzar. Aquesta tècnica tenyeix de color rosat aquells espermatozoides que presenten una membrana alterada degut a que el pigment penetra dins la cèl·lula, mentre que els espermatozoides vius s'observen de color blanc sobre un fons porpra. Una altra tinció vital és el Blau Tripà-Giemsa (Rodríguez-Gil *et al*, 1994), si bé requereix més temps que la tinció d'Eosina-Nigrosina. Actualment, s'estan utilitzant també diverses tincions fluorescents que presenten una major precisió, malgrat que necessiten d'un equipament més sofisticat (Harrison i Vickers, 1990). Les més utilitzades són la bisbenzimida (Hoechst 33258) i el iodur de propidi (IP) que ambdós s'uneixen a l'ADN dels espermatozoides morts. També

existeixen tincions dobles o triples que permeten valorar al mateix temps la viabilitat i altres estructures de l'espermatozoide com l'acrosoma i els mitocondris (Bussalleu *et al*, 2005)

La tinció de les cèl·lules espermàtiques permet també estudiar la morfologia d'aquestes i classificar-les en funció a les anomalies. Les anomalies es classifiquen segons el seu origen i alhora segons la zona de l'espermatozoide on es troba. Es consideren com a espermatozoides normals aquells en els que es distingeixen tres regions principals amb unes dimensions determinades (Taula 5): El cap (oval i pla, en el que s'observa amb microscòpia òptica una regió acrosòmica, una postacrosòmica i el segment equatorial), la peça intermèdia o mitocondrial i la cua (González-Urdiales *et al*, 2006). L'anomalia que es troba més freqüentment en els ejaculats porcins és la presència de gota citoplasmàtica, ja sigui proximal o distal. Aquesta anomalia apareix en un 5-15 % d'espermatozoides en un ejaculat normal. Els altres tipus d'anomalies morfològiques al cap, a la cua o a la zona intermèdia apareixen entre un 1 % i un 5 % en un ejaculat porcí normal (González-Urdiales *et al*, 2006).

3.4. Valoració acrosòmica

L'acrosoma té un paper molt important en la fecundació de l'oòcit i, per tant, és convenient realitzar una valoració acurada d'aquest. La valoració de la integritat acrosomal s'ha de fer amb microscòpia de contrast de fases o mitjançant tincions que poden ser fluorescents o no (González-Urdiales *et al*, 2006). Amb el microscopi de contrast de fases l'observació de l'acrosoma dels espermatozoides porcins no és tant fàcil com la d'altres espècies que tenen un acrosoma més gran. No obstant, és l'eina

Taula 5. Dimensions normals de l'espermatozoide de verro.

Regió espermàtica	Longitud	Amplada / Diàmetre
Espermatozoide complert	47 ± 1,5 μm	
Cap	7,4 ± 0,5 μm	3,7 ± 0,4 μm
Peça intermèdia	10,7 ± 0,2 μm	0,8 μm
Cua	29,1 ± 1,4 μm	
Cua (segment principal)	26,6 μm	0,4 μm
Cua (segment terminal)	2,5 μm	0,2 μm

més emprada en la valoració rutinària de molts centres d'inseminació, ja que és molt senzilla i ràpida de fer. De tincions no fluorescents hi ha un gran ventall, com ara la tinció de Giemsa, combinacions de dues o més tincions (triple tinció de Chacon), tincions comercials (Spermac Stain^{TM}) i fins i tot la d'Eosina-Nigrosina, que tot i ser una tinció vital, també es pot utilitzar per a valorar l'acrosoma (González-Urdiales *et al*, 2006). Pel que fa a les tincions fluorescents, les més utilitzades són les lectines conjugades amb fluorocroms. Les lectines són capaces d'unir-se, de forma reversible, a glicoproteïnes de l'acrosoma. La lectina de *Arachis hypogea* (PNA) s'uneix a la membrana acrosomal externa i la lectina de *Pisum sativum* (PSA) és específica de glicoconjugats de la matriu acrosòmica (Vázquez *et al*, 1996; Maxwell *et al*, 1996).

3.5. Proves de funcionalitat espermàtica

Les proves de funcionalitat espermàtica més utilitzades són les basades en l'anàlisi de les propietats osmòtiques. Aquestes proves consisteixen en sotmetre a

l'espermatozoide a un medi de pressió osmòtica diferent a la fisiològica. Això causa una entrada o sortida d'aigua de la cèl·lula per tal d'equilibrar la pressió osmòtica interna amb la del medi extern. Una resposta cel·lular adequada requereix que la membrana plasmàtica sigui íntegra i plenament funcional. Quan la membrana plasmàtica està alterada no és capaç d'adaptar-se als canvis d'osmolaritat i l'espermatozoide respon de manera anòmala. Les proves osmòtiques es basen en els estudis fets per Drevius i Eriksson (1966), que van demostrar la capacitat dels espermatozoides de toro i conill per captar aigua en un medi hipoosmòtic. Aquests autors van observar que quan l'espermatozoide s'inflava degut a l'entrada d'aigua, la cua s'enrotllava, i quan es tornava al medi isoosmòtic, la cua es tornava a desenrotllar. Aquests resultats van ser confirmats per altres autors en espermatozoides humans, obtenint-se una elevada correlació entre la capacitat de l'espermatozoide humà per adaptar-se a un medi hipoosmòtic amb la capacitat de penetració d'oòcits de hàmster lliures de zona pel·lúcida (Foote i Bredderman, 1969; Jeyendran *et al*, 1984). Actualment, les proves osmòtiques es poden classificar en tres tipus: test d'endosmosi (HOST), test de resistència osmòtica (ORT) i test de resistència hiperosmòtica (HRT).

3.5.1. Test d'endosmosi (HOST)

El test d'endosmosi (*Hypoosmotic Swelling test*, HOST) consisteix en sotmetre als espermatozoides a un medi hipoosmòtic. Això provoca l'entrada d'aigua a la cèl·lula per intentar equilibrar la pressió osmòtica interna amb la del medi extracel·lular. L'entrada dirigida d'aigua fa que l'espermatozoide s'infli i que la cua s'enrotlli. Aquesta prova s'ha utilitzat amb bons resultats de correlació amb la capacitat fecundant

in vivo i *in vitro* en espermatozoides humans (Jeyendran *et al*, 1992), bovins (Correa i Zavos, 1994), canins (Kumi-Diaka, 1993; Rodríguez-Gil *et al*, 1994), equins (Caiza de la Cueva *et al*, 1997a) i porcins (Vázquez *et al*, 1997), entre d'altres espècies. No obstant, en porcs s'ha observat que l'espermatozoide pot reaccionar de diferent manera a l'enrotllament de la cua quan es sotmet a un medi hipoosmòtic, provocant així alteracions en la interpretació dels resultats (Rodríguez-Gil i Rigau, 1996). Per aquesta raó, s'han desenvolupat altres tests per avaluar la resistència osmòtica en espermatozoides porcins.

3.5.2. Test de resistència osmòtica (ORT)

El test de resistència osmòtica (ORT) va ser descrit per primera vegada per Schilling *et al* (1986) com una prova de qualitat seminal, demostrant que tenia relació amb la capacitat fecundant *in vivo*. L'ORT es basa en la capacitat que tenen les membranes espermàtiques, en especial les acrosomals, de resistir un xoc hipoosmòtic. Qualsevol observació de cantells acrosomals irregulars, discontinus o absents implica la presència d'alguna alteració a l'estructura. Tenint en compte això, l'ORT determina la proporció d'acrosomes alterats en espermatozoides incubats en un medi isoosmòtic respecte a altres incubats en un medi equivalent hipoosmòtic. Un cop avaluat el percentatge d'acrosomes intactes, el valor del test es calcula aplicant la fórmula següent:

$$\text{ORT (\%)} = 100 - \frac{(\text{AIM} + \text{AHM})}{2}$$

on, AIM és el percentatge d'alteracions acrosomals en el medi isoosmòtic, i AHM és el percentatge d'alteracions acrosomals en el medi hipoosmòtic. Com més elevat sigui el

valor de ORT, millor serà la qualitat seminal.

3.5.3. Test de resistència hiperosmòtica (HRT)

El test de resistència hiperosmòtica (HRT) s'ha desenvolupat en el verro exclusivament (Caiza de la Cueva *et al*, 1997b). La capacitat de resistència a canvis sobtats d'osmolaritat dels espermatozoides porcins està relacionada amb la qualitat seminal porcina, tant quan es compara amb altres proves *in vitro* com quan s'utilitza com indicador de qualitat seminal *in vivo* (Quintero-Moreno *et al*, 2004). Aquest test consisteix a sotmetre als espermatozoides a un medi hiperosmòtic i posteriorment tornar-los al medi isosmòtic. Es calcula mesurant la relació entre els percentatges de viabilitat i d'acrosomes alterats dels espermatozoides sotmesos al canvi sobtat del medi hiperosmòtic a l'isosmòtic i la viabilitat i els acrosomes alterats inicials en el medi hiperosmòtic. El percentatge de resposta a l'HRT es calcula mitjançant les fórmules següents:

$$VHIPER = VD/VU$$

on, VHIPER és el resultant de viabilitats del test HRT, VD és el percentatge d'espermatozoides viables observats al medi isoosmòtic, i VU és el percentatge d'espermatozoides viables observats al medi hiperosmòtic.

$$ACROHIPER = AD/AU$$

on, ACROHIPER és el resultant d'acrosomes alterats del test HRT, AD és el percentatge d'espermatozoides amb acrosomes alterats observats al medi isoosmòtic, i AU és el percentatge d'espermatozoides amb acrosomes alterats observats al medi hiperosmòtic.

3.6. Tècniques per mesurar la fragmentació de l'ADN

L'avaluació de la integritat de l'ADN espermàtic és de gran interès, doncs aquest paràmetre pot estar darrera de l'aparició de mostres descongelades amb molt poca capacitat fecundant, malgrat tenir-ne uns resultats normals a les proves estàndard de l'anàlisi de qualitat seminal. De fet, recentment ha estat demostrat que la intensitat de fragmentació d'ADN en porcs està correlacionada amb la fecundació o el fracàs en la fecundació (Rybar *et al*, 2004). Varies tècniques són efectives detectant la fragmentació de l'ADN, com la *terminal deoxynucleotidyl transferase-mediated nick end-labeling assay* (TUNEL), *in situ nick translation*, *neutral comet assay*, i *sperm chromatin structure assay* (SCSA). No obstant, algunes d'aquestes tècniques requereixen instrumentació cara o necessiten la utilització d'enzims d'activitat i accessibilitat als talls d'ADN irregular (De Ambrogi *et al*, 2006). Com a conseqüència, alguns d'aquests procediments no són encara convenients per al seu ús en el diagnòstic de rutina (Fernández *et al*, 2005). Per tant, el desenvolupament d'una tècnica simple per determinar l'índex de fragmentació d'ADN (DFI) en qualsevol laboratori bàsic és de gran interès. Recentment, s'ha desenvolupat un nou procediment per a la determinació de la fragmentació de l'ADN en les cèl·lules espermàtiques humanes, l'anomenat *sperm chromatin dispersion test* (SCD; Fernández *et al*, 2005). L'SCD és una tècnica simple,

altament reproduïble i econòmica, els resultats de la qual estan fortament correlacionats amb aquells obtinguts amb altres tècniques, com l'SCSA (Fernández *et al*, 2005). Aquesta tècnica ha estat també adaptada a l'espermatozoide porcí i ha estat utilitzada per avaluar la fragmentació de l'ADN en el semen fresc, encara que la interpretació dels resultats no és tant fàcil com per als espermatozoides humans. Actualment, està disponible comercialment un kit per l'anàlisi de la fragmentació de l'ADN en el semen porcí (Sperm-Sus-Halomax®). L'Sperm-Sus-Halomax® és un procediment simple i ràpid per determinar la fragmentació de l'ADN de manera específica en les cèl·lules espermàtiques porcines. La discriminació dels espermatozoides amb ADN fragmentat és extremadament clara, amb elevada reproductibilitat. Això pot permetre la valoració rutinària de la fragmentació de l'ADN de les mostres espermàtiques porcines, així com una investigació bàsica i clínica en aquest àmbit en qualsevol laboratori d'anàlisis de semen (Enciso *et al*, 2006).

3.7. Estudi de l'estructura de subpoblacions espermàtiques mòtils

L'existència d'espermatozoides amb característiques funcionals i de motilitat diferents dins un mateix ejaculat és un fenomen ben conegut (Holt, 1996; Abaigar *et al*, 1999; Rigau *et al*, 2001). Aquestes subpoblacions espermàtiques mòtils s'han estudiat en nombroses espècies com el porc (Abaigar *et al*, 1999; Quintero-Moreno *et al*, 2004; Rivera *et al*, 2006), el gos (Rigau *et al*, 2001), el cavall (Quintero-Moreno *et al*, 2003), el conill (Quintero-Moreno *et al*, 2007), el toro (Muiño *et al*, 2008), el burro (Miró *et al*, 2005), el hàmster (Holt, 1996), la gasela (Abaigar *et al*, 1999) i el cérvol (Martínez-Pastor *et al*, 2005). També s'ha demostrat que el percentatge d'espermatozoides

inclosos en cadascuna de les subpoblacions canvia quan els ejaculats es sotmeten a processos com ara la congelació-descongelació (Flores *et al*, 2008) o la capacitació *in vitro* (Ramió *et al*, 2008). La presència de les subpoblacions espermàtiques amb característiques específiques de motilitat es poden analitzar mitjançant l'anàlisi computeritzat de motilitat (CASA). El CASA realitza medicions ràpides i objectives dels paràmetres de motilitat individual i aquests resultats obtinguts en el CASA es processen posteriorment mitjançant anàlisis estadístics multivariats, que permeten fer l'estudi de les característiques de les subpoblacions espermàtiques amb un alt grau de precisió (Holt, 1996; Abaigar *et al*, 1999; Quintero-Moreno *et al*, 2003, 2004 i 2007). L'anàlisi de les subpoblacions espermàtiques és de gran importància, ja que si es considera l'ejaculat com a una unitat homogènia, es perd una gran quantitat d'informació valuosa i rellevant a l'hora de predir la capacitat fecundant d'un ejaculat.

3.8. Tècniques per l'estudi de la senyalització intracel·lular en espermatozoides

L'estudi de la senyalització cel·lular s'ha convertit en un dels aspectes més importants de la biologia cel·lular moderna. Comprendre les vies de senyalització cel·lular és de vital importància per a un ampli rang d'especialitats científiques. Les proteïnes són els principals components d'aquesta complexa senyalització cel·lular, i l'estudi d'aquestes proteïnes de senyalització millorarà substancialment el coneixement de processos com la motilitat espermàtica, la supervivència, la capacitació, la reacció acrosòmica i la fecundació. Les tècniques que s'utilitzen per a estudiar les proteïnes dins de l'espermatozoide són sobretot tècniques immunològiques com serien el Western blot

o la immunocitoquímica (Tapia *et al*, 2006).

3.8.1. Tècnica de Western blot

La tècnica de Western blot consisteix a transferir les mostres biològiques d'un gel a una membrana i a la seva detecció. Per tant, per a realitzar un Western blot prèviament s'haurà de fer una electroforesis. L'electroforesi és una tècnica que permet separar i analitzar barreges de proteïnes que es troben en mostres biològiques. Es basa en la capacitat de migració de les proteïnes carregades quan es veuen sotmeses a un camp elèctric. La direcció de migració dependrà de la seva càrrega neta total. El protocol consta dels següents passos:

1. Extracció i solubilització de les proteïnes cel·lulars. Per tal d'extreure les proteïnes s'ha de trencar l'estructura cel·lular. En l'espermatozoide això s'aconsegueix mitjançant la sonicació (amb ultrasons) i amb l'ús d'un tampó de lisi que permet solubilitzar les proteïnes un cop extretes. El tampó de lisi conté un detergent, que pot ser iònic (dodecil sulfat sòdic, SDS) o no iònic (Tritó X-100), i inhibidors de proteases (leupeptina, benzamidina, fluorur de fenilmetil sulfonil [PMSF]).
2. Electroforesis de proteïnes en gel d'acrilamida. Les proteïnes es separen en funció a la seva càrrega i la seva massa. Les proteïnes més grans tenen una mobilitat més baixa en el gel, mentre que les proteïnes més petites tenen una mobilitat més gran. L'electroforesi es sol fer en un gel discontinu que conté un gel de càrrega amb una baixa concentració d'acrilamida que concentra les proteïnes, i un gel separador amb la concentració d'acrilamida adient per a la

separació de les proteïnes que volem.

3. Transferència de les proteïnes a una membrana. Les membranes poden ser de nitrocel·lulosa o de polivinilè-difluorur (PVDF). Per a transferir les proteïnes es necessita una electrotransferència ja que la difusió simple no és suficient. La migració de les proteïnes es fa del gel, orientat cap a l'ànode, a la membrana orientada cap al càtode.
4. Western blot o Immunoblotting. Aquesta tècnica es va desenvolupar l'any 1979 per Towbin *et al* (1979) i es basa en la especificitat de la interacció antigen-anticòs. En aquest cas la proteïna d'estudi és l'antigen. L'anticòs reconeix amb més o menys especificitat la proteïna d'interès dins una barreja proteica. La tècnica consta de dues fases:
   4.1. Bloqueig de la membrana. El primer pas del Western blot és el bloqueig de les unions inespecífiques de l'anticòs. Els tampons de bloqueig més utilitzats contenen albúmina sèrica bovina (BSA) o llet liofilitzada descremada.
   4.2. Immunodetecció. Després del bloqueig, la membrana s'incuba amb l'anticòs que reconeix específicament la proteïna que busquem (anticòs primari). En el mètode directe, l'anticòs primari està marcat amb un enzim o amb una sonda fluorescent que ens permet la detecció directa, mentre que en el mètode indirecte, necessitarem un anticòs secundari que reconeixerà i s'unirà a l'anticòs primari, i serà aquest anticòs secundari el que estarà marcat amb l'enzim o amb la sonda fluorescent. El mètode indirecte sol ser el més utilitzat. Finalment, la detecció de la proteïna es fa normalment per un substrat quimiluminescent que emet llum degut a una reacció química que es produeix en presència de l'enzim adequat (que està unit a l'anticòs). La llum

emesa es pot quantificar a l'exposar-la a una pel·lícula fotosensible.

3.8.2. Tècnica de Immunocitoquímica

La tècnica immunocitoquímica permet localitzar la proteïna d'estudi dins la cèl·lula i, a l'igual que el Western blot, es basa en la capacitat dels anticossos d'unir-se específicament a l'antigen corresponent. Per tal d'aplicar aquesta tècnica prèviament s'hauran de fixar els espermatozoides perquè mantinguin les seves característiques inicials. Un cop fixades les cèl·lules, es treballarà de diferent manera si la proteïna d'estudi es troba a la membrana plasmàtica o a dins de l'espermatozoide. En el cas que sigui una proteïna situada al citosol, necessitarem un pas previ de permeabilització per tal de fer la proteïna accessible a l'anticòs. La permeabilització es realitza amb un detergent com el Tritó X-100 que desestructura la membrana plasmàtica, permetent la penetració de l'anticòs a l'interior cel·lular. A partir d'aquest punt, es treballarà igual per ambdós tipus de proteïnes. Igual que en el Western blot, es fa un bloqueig de les unions inespecífiques de l'anticòs amb BSA previ a la incubació amb l'anticòs. Si l'anticòs està unit a un fluorocrom que permet la seva detecció directe parlarem del mètode directe, mentre que si s'utilitza un segon anticòs (anticòs secundari) marcat amb fluorocroms que s'uneix a l'anticòs primari (que està unit a la proteïna d'estudi) parlarem del mètode indirecte. Tal i com passava en el Western blot, el mètode indirecte sol ser el més utilitzat. Aquesta tècnica és molt valuosa ja que ens dóna informació de la situació de la proteïna dins de la cèl·lula.

4. Efecte perjudicial de la congelació

Com ja s'ha fet palès anteriorment, els espermatozoides porcins són molt sensibles al procés de congelació-descongelació. Diferents fases del procés de criopreservació són potencialment perjudicials per a l'espermatozoide. Dins d'aquestes fases, les més perjudicials serien els rentats per centrifugació, les dilucions, els canvis de temperatura i els canvis osmòtics amb la corresponent exposició als crioprotectors i formació de cristalls de gel intra- i extracel·lulars (Watson, 2000).

4.1. Centrifugació i dilució

La centrifugació és un pas necessari en el protocol de criopreservació, si bé s'ha demostrat que provoca danys a la cèl·lula espermàtica. Així, s'ha observat que la centrifugació abans de la congelació té un efecte advers en la motilitat de l'espermatozoide congelat-descongelat (Salamon, 1973). També s'ha demostrat que la centrifugació provoca la producció d'espècies reactives de l'oxigen (ROS) que indueixen danys irreversibles a l'espermatozoide (Aitken i Clarkson, 1988). Actualment no existeix una explicació completa i acceptada de com la centrifugació indueix el dany espermàtic. No obstant, s'ha hipotetitzat que es deu a un efecte mecànic directe a les membranes espermàtiques (Álvarez *et al*, 1993), així com a un efecte advers indirecte causat per una formació excessiva de ROS (Aitken i Clarkson, 1988; Mortimer, 1991). A més, la centrifugació, així com la dilució, eliminen el plasma seminal que conté elements antioxidants que eviten l'efecte negatiu del ROS (Brouwers *et al*, 2005). Per tant, la centrifugació i la dilució fan augmentar de manera indirecta el dany espermàtic

causat per la formació de ROS.

4.2. Canvis tèrmics i osmòtics

El protocol de congelació-descongelació sotmet a les mostres espermàtiques a un estrès tèrmic i un a xoc osmòtic com a mínim dos cops, un durant el refredament i la congelació, i l'altre durant la descongelació (Holt *et al*, 2005). Els espermatozoides porcins són molt sensibles a tots dos canvis. El xoc per fred produeix alteracions de funcionalitat de les membranes plasmàtiques i la viabilitat cel·lular es veu compromesa. L'elevada sensibilitat al xoc tèrmic de l'espermatozoide porcí es deu a l'especial composició de la membrana plasmàtica (Torre *et al*, 2002). Durant el procés de congelació-descongelació, les cèl·lules pateixen canvis de volum, ja que l'aigua i els soluts entren i surten de la cèl·lula. Aquesta resposta osmòtica pot ser potencialment letal per als espermatozoides si causa que aquests s'inflin o es desinflin per sobre dels seus límits de tolerància osmòtica (Gao *et al*, 1995; Gilmore *et al*, 1998; Holt *et al*, 2005). A més, durant l'addició dels agents crioprotectors, les cèl·lules es desinflen transitòriament, ja que l'aigua surt de la cèl·lula a un ambient hiperosmòtic. Tot seguit, les cèl·lules s'inflen quan l'aigua i el crioprotector entren. Quan s'elimina el crioprotector passa a la inversa, les cèl·lules inicialment s'inflen quan l'aigua entra, i després es desinflen quan l'aigua i el crioprotector deixen la cèl·lula. Com a resultat, aquests dos passos de la criopreservació poden ser perjudicials per la cèl·lula (Gao *et al*, 1995). Amb tot, els crioprotectors són essencials ja que protegeixen l'espermatozoide durant el refredament i la congelació (Curry, 2000).

4.3. Producció de ROS

Totes les cèl·lules vives sota condicions aeròbiques produeixen ROS que s'originen principalment per l'activitat metabòlica normal de la cèl·lula, i els espermatozoides no en són una excepció (Brouwers *et al*, 2005). Els dos llocs principals de producció de ROS són els mitocondris i la membrana plasmàtica de l'espermatozoide, essent-ne els mitocondris el principal centre generador (Agarwal *et al*, 2005a). La manipulació *in vitro* del semen ejaculat afecta a la formació de ROS degut a dos factors: l'eliminació del plasma seminal, que conté una gran font de substàncies protectores contra els ROS, i l'emmagatzematge de les dosis seminals en condicions aeròbiques (Gaczarzewicz *et al*, 2003; Shimatsu *et al*, 2002). S'assumeix que els espermatozoides manipulats són menys capaços de fer front a l'estrès oxidatiu. Això explica que la baixa generació de ROS fisiològica en un ambient normal activa la hiperactivació, la capacitació i la reacció acrosòmica de l'espermatozoide en el tracte genital femení, mentre que la generació de ROS elevada en espermatozoides manipulats *in vitro* causa deteriorament espermàtic (Brouwers *et al*, 2005). Els espermatozoides són sensibles a l'estrès oxidatiu perquè no tenen prou elements antioxidants de defensa (Donnelly *et al*, 1999; Saleh i Agarwal, 2002). A més, els àcids grassos poliinsaturats de la membrana plasmàtica espermàtica són altament vulnerables a l'atac per ROS. Així, els ROS, en presència d'àcids grassos poliinsaturats, provoquen una cadena de reaccions químiques que s'anomenen peroxidació lipídica (Kobayashi *et al*, 2001; Agarwal *et al*, 2005a), donant lloc a canvis en la fluïdesa de la membrana (De Lamirande i Gagnon, 1992b; Armstrong *et al*, 1999). Els ROS també poden danyar l'ADN, causant-ne delecions, mutacions, i altres efectes genètics letals (Aitken *et al*,

1998; Moustafa *et al*, 2004). En definitiva, s'ha demostrat que els ROS també causen la disminució del moviment espermàtic (Armstrong *et al*, 1999) i el bloqueig en la fusió espermatozoide-oòcit (Mammoto *et al*, 1996). L'estimació dels nivells de ROS és, per tant, important per valorar els efectes adversos en la fecundació no identificats per la mesura dels paràmetres seminals rutinaris (Agarwal *et al*, 2005b).

Els espermatozoides porcins són especialment sensibles al dany peroxidatiu degut al seu elevat contingut d'àcids grassos insaturats en els fosfolípids de la membrana plasmàtica i la relativament baixa capacitat antioxidant del plasma seminal porcí (Breininger *et al*, 2005). S'ha demostrat en espermatozoides humans (Álvarez i Storey, 1992), de toro (O'Flaherty *et al*, 1997) i de ratolí (Mazur *et al*, 2000) que la criopreservació provoca estrès oxidatiu. A més, s'ha vist que la congelació i descongelació dels espermatozoides bovins incrementa la generació de ROS (Chatterjee i Gagnon, 2001), la qual cosa produeix dany a l'ADN (Lopes *et al*, 1998), alteracions al citoesquelet (Hinshaw *et al*, 1986), inhibicions de la fusió espermatozoide-oòcit (Aitken *et al*, 1989) i canvis en l'axonema espermàtic associats amb pèrdua de motilitat (De Lamirande i Gagnon, 1992a). De tots els ROS que es formen, el peròxid d'hidrogen sembla ser el principal responsable d'aquests canvis (De Lamirande i Gagnon, 1992a). L'addició d'antioxidants o enzims antioxidants al medi de congelació-descongelació ha estat utilitzat per reduir els efectes adversos de la criopreservació en el semen porcí i s'ha observat que milloren la capacitat fecundant espermàtica (Peña *et al*, 2003; Roca *et al*, 2005), si bé encara no és del tot clar com la congelació-descongelació està associada, en el semen porcí, amb la formació de ROS i les subsegüents alteracions associades.

5. Danys que provoca la congelació a les diferents estructures espermàtiques

En definitiva, sigui quina sigui la causa, els efectes provocats per el procés de congelació-descongelació inclouen dany a la membrana plasmàtica, l'ADN espermàtic i altres orgànuls cel·lulars com a resultat de l'estrès osmòtic, el xoc tèrmic i la formació de gel intracel·lular (Guthrie i Welch, 2005).

5.1. La membrana plasmàtica

La membrana plasmàtica de l'espermatozoide és una de les estructures claus afectades durant la criopreservació, sent la seva avaluació de gran importància quan es valoren els protocols de congelació. El procés de congelació-descongelació provoca una gran distorsió a les membranes espermàtiques, ja que la bicapa lipídica esdevé inestable per la baixada de temperatura (Silva i Gadella, 2006). Les nombroses funcions de la membrana estan relacionades amb el manteniment del metabolisme cel·lular general, servint-se així per a regular la motilitat espermàtica, la capacitació, la reacció acrosòmica, les interaccions entre l'espermatozoide i l'epiteli del tracte genital femení, i les interaccions espermatozoide-oòcit (Rodríguez-Martínez, 2003; Peña *et al*, 2005). Buhr *et al* (2001) van observar que la membrana plasmàtica del cap de l'espermatozoide s'afectava per la congelació-descongelació de manera que es produïen canvis moleculars que interferien en la fecundació. No obstant, la zona més susceptible de patir danys durant la descongelació és la que envolta els mitocondris de la peça intermèdia (Holt i North, 1994; Mohammad *et al*, 1997; Medrano *et al*, 2002). En aquest sentit,

nivells elevats de ROS, originats en principi als mitocondris, estan associats amb dany a les membranes espermàtiques per la peroxidació lipídica (Shekarriz *et al*, 1995). Aquesta peroxidació altera la funció espermàtica portant a una pèrdua de la motilitat i la viabilitat, així com a una reducció de la capacitat fecundant de l'espermatozoide (Carvajal *et al*, 2004).

5.1.1. Diferències entre el porc i altres espècies

La refrigeració i la congelació són esdeveniments traumàtics per a l'espermatozoide i l'extensió d'aquests efectes varia amb l'espècie. Les diferències en la composició de lípids de la membrana plasmàtica de l'espermatozoide semblen ser el factor clau en la diferència en la congelabilitat entre els espermatozoides d'espècies diferents (Graham i Foote, 1987; Parks i Lynch, 1992). Així, la quantitat relativa de colesterol i àcids grassos poliinsaturats són determinants per a l'estabilitat de la membrana (Holt *et al*, 2005). En aquest sentit, les membranes de les cèl·lules espermàtiques porcines són extremadament riques en fosfolípids poliinsaturats (Flesch *et al*, 2001) que són fàcilment atacats pels ROS. El colesterol és també un component lipídic important de la membrana plasmàtica espermàtica. Nivells alts de colesterol estabilitzen les cèl·lules espermàtiques, donant lloc a una millor criopreservació en aquelles espècies que presenten nivells elevats de colesterol a les seves membranes espermàtiques. Les cèl·lules espermàtiques humanes, canines i bovines contenen nivells elevats de colesterol, essent-ne aquestes les espècies que tenen millors propietats crioresistents (Brouwers *et al*, 2005).

5.2. Integritat de l'ADN espermàtic

La fragmentació de l'ADN espermàtic es pot donar per un empaquetament deficient de la cromatina durant l'espermiogènesi (Sailer *et al*, 1995) o per una producció excessiva de ROS en l'ejaculat (Aitken *et al*, 1998; Lopes *et al*, 1998). Per tant, la congelació-descongelació pot causar fragmentació de l'ADN associada a alteracions en la producció i/o desaparició de ROS. La integritat de l'ADN espermàtic és de crucial importància per al desenvolupament de l'embrió. El dany a l'ADN està clarament correlacionat amb els problemes de desenvolupament de l'embrió i danys severs a l'ADN causen infertilitat masculina (Seli *et al*, 2004). No obstant, és important de destacar que experiments de fecundació *in vitro* amb espermatozoides irradiats amb rajos gamma van mostrar espermatozoides que presentaven danys severs a l'ADN però que persistien funcionalment intactes al nivell de la membrana, els orgànuls i els paràmetres de motilitat (Silva i Gadella, 2006). De fet, els espermatozoides amb danys a l'ADN mostraven característiques normals d'unió a la zona pel·lúcida i fins i tot les taxes de fecundació i divisió dels oòcits fecundats es mantenien normals. No obstant, quasi tots els embrions de 4 a 8 cèl·lules iniciaven apoptosi (Bordignon i Smith, 1999; Ahmadi i Ng, 1999; Fatehi *et al*, 2006). Així, el fracàs reproductiu causat per les aberracions a l'ADN no apareix al nivell de la fecundació, sinó al començament de l'expressió de l'ADN embriònic (Silva i Gadella, 2006), i per tant no és reconeixible en un anàlisi estàndard de qualitat seminal.

La reparació del dany en l'ADN no és possible en l'espermatozoide madur (Dadoune, 2003). Per tant, la defensa de la cèl·lula espermàtica contra el dany a l'ADN depèn de dos factors: L'estructura de la cromatina espermàtica, i els antioxidants

presents al plasma seminal que protegeixen l'espermatozoide contra el dany oxidatiu causat pel ROS (Boe-Hansen *et al*, 2005). El dany que finalment es dóna en l'ADN de l'espermatozoide, tot i els mecanismes de defensa, pot ser reparat només parcialment pel zigot després de la fecundació (Ahmadi i Ng, 1999).

5.2.1. Estructura de l'ADN espermàtic

La cromatina dels espermatozoides madurs està extremadament condensada mitjançant la unió íntima amb protamines en una estructura helicoïdal (Balhorn, 1982). Les protamines són unes proteïnes bàsiques, petites i molt riques en arginina que s'uneixen fortament a l'ADN i l'estabilitzen. La condensació del nucli espermàtic té lloc durant l'espermiogènesi, on les histones són eliminades dels nucleosomes per proteïnes nuclears de transició (Brewer *et al*, 2002; Meistrich *et al*, 2003). A continuació, l'ADN despullat és revestit de protamines i reempaquetat en l'últim estadi de l'espermiogènesi (Fuentes-Mascorro *et al*, 2000). La maduració nuclear final es dóna durant el trànsit epididimari, on es formen ponts disulfur entre les protamines per assegurar l'estabilització de l'ADN (Fraser i Strzeżek, 2005). La deposició de les protamines a la cromatina espermàtica i la condensació de la cromatina sembla estar controlada per processos de fosforilació-defosforilació. Les protamines es fosforilen molt, poc després de la seva síntesi i abans de la unió a l'ADN, mentre que es defosforilen àmpliament durant la maduració espermàtica (Lewis *et al*, 2003).

Les cites anteriors, doncs, mostren que les protamines són les proteïnes clau involucrades en la condensació final de l'ADN de l'espermatozoide (Brewer *et al*, 2002 i 2003; Meistrich *et al*, 2003). Una condensació adequada estabilitza l'ADN i el fa

menys vulnerable al dany oxidatiu (Silva i Gadella, 2006). A més, un nivell d'empaquetament de la cromatina correcte és essencial per expressar totalment la capacitat fecundant de l'espermatozoide, i defectes en l'estructura cromatínica espermàtica es poden associar amb un contingut de nucleoproteïnes anormal i/o trencaments de la cadena d'ADN (Sergerie *et al*, 2005). En aquest sentit, s'ha observat que la deficiència en protamina està relacionada amb nivells més elevats de dany a l'ADN (Bianchi *et al*, 1993; Manicardi *et al*, 1995).

En la majoria d'espècies de mamífers, el nucli espermàtic conté un únic tipus de protamina, la protamina-1. Un segon tipus de protamina, la protamina-2, és present en el nucli d'alguns mamífers, com ara els humans, els ratolins i els cavalls (Pirhonen *et al*, 1994), havent-ne poques diferències estructurals entre ambdues proteïnes i entre la mateixa proteïna de diferents espècies.

5.3. Els mitocondris espermàtics

Els mitocondris espermàtics estan localitzats a la peça intermèdia, enrotllats sobre la part principal del flagel. Els mitocondris produeixen ATP per fosforilació oxidativa, i de manera clàssica s'ha considerat que l'ATP mitocondrial era la principal font d'energia per al manteniment de la motilitat (Silva i Gadella, 2006). Malgrat això, darrerament la importància dels mitocondris en el manteniment de la motilitat espermàtica s'està reconsiderant per vàries raons:

- Els espermatozoides ejaculats frescos produeixen molt ATP per glicòlisi (>90 %) inclús en solucions que estan en contacte amb l'aire (16 % oxigen; Marin *et al*, 2003).

- El lliscament de la tubulina que depèn d'ATP i és responsable de la motilitat espermàtica, té lloc a la part distal del flagel, a la cua. Així, el consum d'ATP és bastant lluny de la producció aeròbica d'ATP.
- La beina fibrosa de la cua conté enzims involucrats en la glicòlisi (producció anaeròbica d'ATP) i ratolins *knock out* que fallen en l'expressió d'un d'aquests enzims són immòtils (Miki *et al*, 2004).
- Espermatozoides sotmesos a substàncies químiques que separen la fosforilació oxidativa de la cadena d'electrons a la membrana mitocondrial interna es mantenen mòtils i vius (Mukai i Okuno, 2004).

Sigui quin sigui el paper dels mitocondris sobre la motilitat espermàtica, el cert és que aquestes estructures tenen d'altres papers, igualment importants, en el manteniment de la funcionalitat espermàtica. Així, els mitocondris proporcionen a la zona intermèdia i al cap l'ATP necessari per els processos de manteniment de les membranes, tal i com la sustentació del gradient de Na^+/K^+ a la membrana plasmàtica (Silva i Gadella, 2006). D'aquesta forma, el manteniment de la funcionalitat mitocondrial es bàsic per a la vida de l'espermatozoide.

D'altra banda, els mitocondris sembla que són de les estructures més sensibles de l'espermatozoide a la congelació i la descongelació (Cummins *et al*, 1994). Per avaluar la integritat funcional dels mitocondris existeixen fluorocroms, com ara el Mitotracker X-Rosamina CM-H_2XROS que esdevé fluorescent després de l'oxidació, un procés que només té lloc sota la respiració oxidativa. Com que aquest procés només es dóna en mitocondris funcionals, aquesta proba és apropiada per discriminar espermatozoides amb mitocondris deteriorats d'espermatozoides amb mitocondris funcionals (Gadella i Harrison, 2002; De Vries *et al*, 2003). Una manera alternativa per

detectar una producció d'ATP adequada és observar les característiques de motilitat espermàtica amb un anàlisi computeritzat de motilitat espermàtica (CASA). Totes aquestes proves són, doncs, importants per a l'avaluació de la integritat espermàtica després de la congelació-descongelació, tenint en compte, a més, que la resta de proves de funcionalitat espermàtica donen molt poca informació sobre aquest aspecte fonamental en la vida de l'espermatozoide.

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OBJECTIUS DE L'ESTUDI / AIMS OF THE STUDY

Objectius de l'estudi / Aims of the study

The overall aim of this study was to assess the effect of the cryopreservation process on several molecular aspects of boar spermatozoa function. More specifically, the studied aspects were the following:

I. Evaluation of the DNA fragmentation and related changes on the overall protamine-DNA structure during a standard freezing-thawing protocol of boar spermatozoa.

II. Analysis of putative differences in both the motile-sperm subpopulations structure and the overall mitochondrial activity on boar semen samples with different resistance to freezability.

III. Evaluation of the putative relationship between mitochondrial alterations and changes in the expression and location of two important mitochondria regulatory proteins, mitofusin-2 and midpiece actin network, during the cryopreservation process of boar spermatozoa.

CAPÍTOL I / CHAPTER I

Freeze-thawing induces alterations in the protamine-1−DNA overall structure in boar sperm

Abstract

The main aim of this work was to test the effects that freeze-thawing could have on the overall nuclear structure of boar sperm. This was done by analyzing both the DNA fragmentation and the protamine-1–DNA interaction of the boar-sperm nucleus. Our results indicate that freezing-thawing did not induce a significant degree of DNA fragmentation, as manifested through both the Sperm-Sus-Halomax® stain and a random primed analysis prior to partial DNA digestion with enzymes BamHI-HinDIII. On the other hand, freeze-thawing induced significant changes in the protamine-1–DNA interaction, as revealed through both Western blot analysis and immunocytochemistry for protamine-1. These alterations caused, in turn, significant changes in the overall nuclear structure of boar sperm after thawing. Protamine-1–DNA alterations started to be apparent during the cooling phase of the freeze-thawing protocol. These results imply that one of the alterations that may be responsible for the loss of fertilizing ability of boar sperm after freeze-thawing may be an alteration in the correct formation of the overall nuclear structure, which, in turn, would induce alterations in the correct formation of the first nuclear structure after oocyte penetration.

Introduction

It is well established that freeze-thawing induces a clear decrease in the fertilizing ability of boar sperm. This decrease has been related to a wide range of both functional and structural alterations associated with the freeze-thawing process, such as changes in the cell membrane structure, increase of oxidative reactions, mitochondrial alterations and others (Holt & North, 1994; Mohammad *et al*, 1997; Shekarriz *et al*, 1995; Medrano *et al*, 2002; Carvajal *et al*, 2004; Brouwers *et al*, 2005; Silva & Gadella, 2006). Moreover, in the last few years, several authors have studied sperm DNA alterations as a source of decreased fertility of frozen-thawed boar sperm. Studies have been conducted being based on the previous work of several authors, which has shown a close relationship between DNA fragmentation and sperm fertilizing ability in several species (see Silva & Gadella, 2006 as a review). Following this, freezing-induced DNA fragmentation has been described in species such as horse (Baumber *et al*, 2003). However, results obtained in boar varied from authors that indicated that freeze-thawing induced a clear impairment of DNA integrity (Fraser & Strezeżek, 2005) to others that indicated that DNA integrity was not significantly affected by freezing (Evenson *et al*, 1994; Hernández *et al*, 2006). A similar lack of effect has been shown in ram (Martínez-Pastor *et al*, 2004), opening doubts about a freezing-induced effect on sperm DNA integrity. This is an important question since, as indicated above, the relationship between DNA integrity and sperm fertilizing ability has been well established (Silva & Gadella, 2006), and this could be a major point in explaining cases of lack of fertilizing ability of thawed samples despite reasonably good results in other parameters of semen quality, like viability and motility. In fact, it has been described that sperm with a high

ratio of DNA fragmentation can maintain its motility characteristics and is even able to undergo oocyte penetration. However, embryos obtained after *in vitro* fertilization with DNA-damaged sperm died through an apoptotic process during the 4-to-8-cell stage, possible due to aberrant embryo DNA expression (Silva & Gadella, 2006). Thus the establishment of the mechanisms by which freeze-thawing can affect DNA integrity in species like the boar will be of the greatest importance to improve the fertilizing ability of these samples.

The mammalian sperm nucleus has a very peculiar structure, which differentiates it from all other eucaryotic cells. This differentiation is not only due to the fact that spermatozoa are haploid cells, but also to a very specific nucleoproteinic structure (Wykes & Krawetz, 2003; O'Brien & Zini, 2005; Biegeleisen, 2006). Thus, the most important nuclear proteins of mammalian sperm are from the protamine family, instead of histones commonly found in somatic eucaryotic cells. Protamines are very small, acidic proteins that form very complex structures with sperm DNA, thus inducing a highly compacted DNA (Wykes & Krawetz, 2003; O'Brien & Zini, 2005; Biegeleisen, 2006). There are two different protamines associated with mammalian sperm DNA. They are named protamine-1, which is present in all of the studied species, and protamine-2, only present in species such as human, mouse and horse (Biegeleisen, 2006). The maintenance of an appropriate protamine–DNA structure in the sperm nucleus is very important in order to maintain sperm fertilizing ability. In this sense, it has been described in humans that alterations in protamine–DNA and protamine-1–protamine-2 ratios are associated with a decrease of fertility (Aoki *et al*, 2005a; Aoki *et al*, 2005b). This is probably due to the fact that decondensation of the sperm nucleus after oocyte penetration involves a strict, sequential process, which is

initiated by the loss of nuclear protamines before sperm nuclear decondensation (Shimada *et al*, 2000; Nakazawa *et al*, 2002). Notwithstanding, there is a noticeable lack of information regarding the effect of freeze-thawing on the specific sperm protamine–DNA structure. This is especially important in species such as boar, where a putative, freezing-induced destabilization of the protamine–DNA structure could cause a significant loss of sperm fertilizing ability without a great modification of the results of the routinely performed semen analysis. Taking this into account, the main aim of this work is to evaluate how a standard freeze-thawing protocol can affect the overall protamine–DNA structure of boar sperm. For this purpose, DNA integrity was analyzed through two separate techniques. First, via a specific stain for DNA fragmentation and, secondly, by a random priming analysis of sperm DNA after digestion with the combination of restriction enzymes BamHI and HinDIII. Finally, the protamine-1–DNA interaction was analyzed by Western blot and immunocytochemistry of samples utilizing a specific anti-protamine-1 antibody.

Materials and methods

Animals and samples collection

Twelve healthy boars of 2-3 years of age from a commercial farm and one ejaculate per boar were used in this study. The boars were from three separate lines (four Landrace, three Large White and five Pietrain). All boars had proven fertility after artificial insemination (AI) using extended, liquid semen. The sperm-rich fraction of each ejaculate utilized in this study was manually collected twice weekly using the

gloved-hand method and analyzed to ensure the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was suspended (1:2; v/v) in a commercial extender (MR-A). The extended semen samples were cooled and maintained at 17 ºC for shipment to the laboratory of the Autonomous University of Barcelona within 24 h post-collection, for further processing and analyses.

Semen cryopreservation

Immediately after receiving the shipped semen samples, an aliquot was taken to perform the appropriate semen assessments, as well as those regarding protamine-1–DNA studies (fresh semen sample). Only those samples displaying a minimum of 70 % progressive motile and 80 % of morphologically normal spermatozoa were further processed by adapting a proven protocol (Eriksson & Rodríguez-Martínez, 2000). The extended semen was centrifuged in a programmable refrigerated centrifuge set at 17 ºC, at 600 *g* for 10 min. After centrifugation, the supernatant was discarded. The remaining pellets were re-extended with a lactose-egg yolk (LEY) extender (80 % [v/v] 310 mM β-lactose, 20 % 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 Thoma or Neubauer haemocytometer. At this point, and after thorough mixing, the semen was further cooled to 5 ºC for 2 h in the centrifuge. Then, an aliquot of the refrigerated semen was taken to carry out the appropriate semen quality parameters, as well as those regarding protamine-1–DNA studies (refrigerated or 5 ºC semen sample) and then the semen was slowly mixed with a third extender consisting of 89.5 mL LEY extender, 9 mL glycerol and 1.5 mL of Equex STM, 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 ºC, which was verified by counting in a Thoma or Neubauer haemocytometer. Spermatozoa were packaged at 5 ºC in a cool cabinet in 0.5-mL polyvinyl chloride (PVC) plastic straws, 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 set at 5 ºC. The cooling/freezing rate used was: 6 ºC/min from 5 ºC to -5 ºC, 40 ºC/min from -5 ºC to -80 ºC, 30 sec for crystallization, and thereafter 60 ºC/min from -80 ºC to -150 ºC. The samples were then plunged into liquid nitrogen (N_2; -196 ºC) for storage. Frozen samples were stored in liquid N_2 for at least 21 days. After this, samples were thawed by the plunging of samples in a water bath at 37 ºC for 20 sec. Immediately afterwards, straws were carefully dried and opened, and samples were immediately analyzed to determine the appropriate semen quality parameters, as well as those regarding protamine-1–DNA studies (frozen-thawed semen sample).

Analysis of semen quality parameters

Percentages of viability and altered acrosomes were determined by using the staining bis-benzamidine/propidium iodide-Mitotracker®Green FM-Alexa Fluor® 488-conjugated lectin trypsin-inhibitor from soybean (SBTI) as described in Bussalleu *et al* (2005). In this technique, an aliquot of sperm suspension was firstly incubated with a solution of 15 μM bis-benzamidine (proportion 1:1000, v/v) for 10 min at 37 ºC. Afterwards, a 2-mm propidium iodide solution was added (proportion 6:1000, v/v) and the sperm were subjected to further incubation for 10 min at 37 ºC. After this

incubation, the sperm suspension was centrifuged at 1500 *g* for 10 min and the supernatant discarded. The obtained sperm pellet was resuspended in 1 mL of a solution of 100 nM Mitotracker® Green FM and FM-Alexa Fluor® 488-conjugated SBTI in IVC medium without BSA. The sperm suspension was incubated in this solution for 20 min at 37 ºC and then was immediately centrifuged at 1500 *g* for 12 min. The resultant supernatant was discarded, the sperm pellet was resuspended in 100 µL of IVC medium without BSA at 37 ºC. The sperm suspension was spread onto slides and fluorescence was immediately determined in a Zeiss Axioskop-40 fluorescence microscope with the appropriate filters. Viability and altered acrosome percentages were determined after counting 200-300 spermatozoa per slide at 1000×. Unaltered acrosomes were considered to be those which showed a faint-to-moderate and uniform STBI lectin stain. Furthermore, viable sperm showed a blue stain of the sperm head, whereas non-viable cells showed an intense red stain of the head. Using this technique, the percentages of both viability and altered acrosomes were determined after counting 200-300 spermatozoa per slide at 1000×.

The osmotic resistance test (ORT test) was carried out as described in Rodríguez-Gil & Rigau (1995), whereas the hyperosmotic resistance test (HRT Test) was carried out as in Quintero-Moreno *et al* (2004). Total motility was evaluated through analysis by using a commercial computer-assisted analysis of sperm motility (CASA system). Samples were previously warmed at 37 ºC for 5 min in a water bath and 5-µL aliquots of these samples were then placed on a warmed (37 ºC) slide and covered with a 25-mm^2 coverslip. Total motility was defined as the percentage of spermatozoa which showed a mean velocity (VAP, defined as the mean velocity of the sperm head along a straight line from its first to its last position) above 10 µm/s.

Analysis of sperm DNA damage

The analysis of sperm DNA damage was carried out through two separate techniques. First, via a specific stain for DNA fragmentation and, secondly, by a random priming analysis of sperm DNA after digestion with the combination of restriction enzymes BamHI and HinDIII.

Analysis of DNA fragmentation through the Sperm-Sus-Halomax® stain

The utilized stain for DNA fragmentation was the commercial kit Sperm-Sus-Halomax©. This kit is specifically designed for boar sperm. It is based on the different response that intact and fragmented DNA show after a de-proteinization treatment. It has been described that the results obtained with this technique strongly correlated with those obtained with other previously utilized, but much more difficult to apply tests, like the neutral comet assay (Fraser *et al*, 2007). This suggests that the Sperm-Sus-Halomax© is a good technique to determine DNA fragmentation in boar sperm. For applying this stain, samples were diluted in phosphate-buffered saline (PBS) to a final sperm concentration of (5-10) × 10^6 sperm/mL. At the same time, vials containing agarose from the commercial kit were heated to 90-100 ºC for 5 min and then placed in a water bath at 37 ºC for 5 min in order to liquify the commercial agarose solution and maintain it at an optimal temperature for sperm. When commercial agarose solutions were liquified and stabilized at 37 ºC, 25-µL aliquots of the diluted sperm samples were placed into the agarose commercial vials and mixed thoroughly. Afterwards, a 25-µL

aliquot of the sperm mixed with the commercial agarose solution was placed onto a siliconized 76 mm × 26 mm slide and was then immediately covered with a 22 mm × 22 mm coverslip, avoiding the formation of air bubbles. Samples were placed at 4 ºC for 5-10 min to solidify the agarose-sperm mixture. Afterwards, the coverslips were carefully removed and slides containing samples were covered by the commercial proteolysis solution included in the kit at 20 ºC for 5 min. Next, samples were rinsed thoroughly with distilled water and further dehydrated through sequential treatments of 70 % (v/v), 90 % (v/v) and 100 % (v/v) ethanol. Samples were left to air-dry and were then stained for 10 min at room temperature with a commercial Wright solution contained in the kit. Finally, samples were thoroughly rinsed with distilled water and were then mounted by utilizing a commercial mounting solution (DPX®). Following the application of the protocol of the commercial kit, boar sperm can be classified according to its response to this protocol in the following four types (see Enciso *et al*, 2006):

Type I sperm: these cells showed a compact head after treatment. Intact DNA.

Type II sperm: slightly swollen sperm head, without DNA fragmentation.

Type III sperm: moderately swollen sperm head, without the presence of a DNA fragmentation halo.

Type IV sperm: cells which show a clear DNA halo around their heads. These spermatozoa are the only ones which present DNA fragmentation.

Percentages of sperm included in each sperm type defined in the kit were determined after counting 200-300 spermatozoa per slide at 1000×.

Fractioning of sperm genomic DNA and random priming analysis

The random priming analysis of boar sperm DNA was based on a previous, specific fractioning of sperm DNA by incubation with two specific restriction enzymes. This digestion induces the formation of a specific digestion pattern after DNA electrophoresis in agarose gel. If sperm DNA is in a fragmented state, the specific digestion pattern obtained after the enzyme digestion will vary, since some of the specific DNA sequences that are sensitive to the enzyme action will be affected by the DNA fragmentation. The further random priming analysis will increase the sensitivity of the recognition of differences in the obtained digestion parameters by amplification of the signal related to the specific digestion pattern after agarose gel electrophoresis. Because of this, random priming analysis of boar sperm DNA was preceded by a sperm genomic DNA extraction followed by genomic DNA electrophoresis in agarose gel. This electrophoretic step was performed in extracted DNA with or without previous combined digestion of restriction enzymes BamHI and HinDIII. The utilized protocol for sperm genomic DNA extraction was a standard proteinase K-phenol-chloroform extraction (Powell & Gagnnon, 2002). The DNA concentration of samples was determined through spectrophotometric analyses at a wavelength of 260 nm. When stated, genomic DNA was digested with a 1:1 mixture of restriction enzymes BamHI-HinDIII. Enzyme digestion was performed on 5 μg DNA for each sample, and incubation with the enzyme mixture was maintained either for 2 h at 37 ºC, which induced a partial digestion of genomic DNA, or for 18 h at 37 ºC, which induced a complete digestion of sperm genomic DNA. One microgram of each isolated, native and digested DNA was fractionated by electrophoresis in 1 % agarose gels. Several

experiments were finished at this point, through a direct revealing of the genomic DNA fractioning after electrophoresis by incubation with 0.1 μg/mL ethidium bromide in a 0.45-M Tris-borate buffer (pH 8.3) containing 10 mM ethylene diamino tetra-acetic acid (EDTA) and further ultraviolet photographic exposition. The other experiments were further transferred to nitrocellulose membranes to be subjected to random priming analysis.

Digoxigenin (DIG)-conjugated primers for the random priming analysis were obtained after using the "DIG High Prime DNA Labeling and Detection Starter Kit II®". One microgram of sperm genomic DNA from fresh samples previously digested for 18 h at 37 ºC with the above-mentioned combination of restriction enzymes BamHI-HinDIII was primed after utilizing the above-mentioned kit following the kit's instructions. The DIG-conjugated random primers obtained were then utilized to carry out hybridizations on the DNA samples previously transferred to nitrocellulose membranes. The hybridizations were performed at 65 ºC for 10 min. Filters were then washed at 65 ºC to a final stringency of 0.1× SSC and 0.1 % (w/v) sodium dodecyl sulfate (SDS). The 0.1× SSC was composed of a buffer solution (pH 7.0) of 15 mM NaCl and 1.5 mM sodium citrate. Finally, the presence of DIG-primed DNA was assessed with the aid of the appropriate materials included in the "DIG High Prime DNA Labeling and Detection Starter Kit II®".

Immunological techniques regarding protamine-1 detection

Both Western blot analyses and immunocytochemical detection of boar sperm protamine-1 were performed by using the same commercial anti-goat protamine-1

antibody. To perform Western blot analyses, boar spermatozoa were homogenized by sonication in ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 1 % (w/v) SDS, 15 mM EDTA, 150 mM KF, 0.6 M sacarose, 14 mM β-mercaptoethanol, 10 μg/mL leupeptin, 1 mM benzamidine and 1 mM phenylmethyl sulfonyl fluoride (PMSF). The homogenates were then centrifuged at 10,000 *g* for 5 min at 4 ºC and the resultant pellets were discarded. Western blot was only then performed on soluble sperm fractions of homogenates. This analysis was based on SDS gel electrophoresis (Laemmli, 1970), followed by transfer to nitrocellulose (Burnette, 1981). To carry out the SDS gel electrophoresis, 20 μg of total protein per sample were loaded in each lane. Total protein contents of samples was determined by the Bradford method (Bradford, 1976), after applying a commercial kit (BioRad). The transferred samples were tested with the anti-protamine-1 antibody at a dilution (v/v) of 1:1000. Immunoreactivity was tested using peroxidase-conjugated donkey anti-goat secondary antibody and the reaction was developed with an ECL-Plus detection system. Moreover, the specificity of the observed immunoreactivity was tested after subjecting several experiments to Western blot in the presence of the anti-protamine-1 antibody previously pre-adsorbed with a commercial, specific peptide to a final concentration of 20 μg/mL. Lastly, since the first results seemed to indicate that the specific reaction obtained in samples could be due to the presence of protamine-1–DNA low-molecular-weight complexes, Western blot analyses of supernatants from sperm homogenates were carried out, which involved incubation with 40 μg/mL DNAase-1 for 2 h at 25 ºC prior to being applied to the SDS gel electrophoresis that started the Western blot process.

Immunocytochemistry was initially performed with spermatozoa seeded onto gelatin-coated slides (76 mm × 26 mm). However, initial tests showed that the anti-

protamine-1 antibody was unable to penetrate into the sperm head, even when spermatozoa were previously permeabilized by using Triton X-100 solutions. This leads to the need to carry out immunocytochemistry on sperm sections. For this purpose, sperm samples were washed three times with PBS and fixed with 500 μL of a 2 % (w/v) paraformaldehyde solution in PBS for 15 min at 25 ºC. Fixed samples were centrifuged at 600 *g* for 3 min, and the supernatants were discarded. The cellular pellet was resuspended in 500 μL of PBS and again centrifuged at 600 *g* for 3 min. Supernatants were again discarded, and the obtained pellets were embedded in 40 μL of the OCT® inclusion medium. Samples were immediately frozen with liquid N_2 and stored until their processing at -80 ºC. When stated, the included samples were sectioned in slices of 1 μm of thickness by using a cryostat. The obtained sections were then seeded onto gelatin-coated slides (76 mm × 26 mm). Immediately, the slides were covered with a PBS solution containing 0.1 (v/v) commercial Hoechst 33258 solution. This stain allowed for the determination of an exact co-localization between the protamine-1 signal obtained with the specific antibody and the sperm nuclear DNA. Incubation with Hoechst 33258 was maintained for 15 min at 38.5 ºC, preventing any light source from reaching the slides. From this moment on, all of the further steps were carried out preventing a direct incidence of any light source on the samples. After this, the excess liquid on the slides was eliminated by decantation, and slides were thoroughly washed three times with PBS. Protamine-1 immunocytochemistry was started by incubation with 1 mg/mL $NaBH_4$ for 15 min to prevent autofluorescence. This step was followed by a permeabilization with 0.2 % (v/v) Triton X-100 in PBS for 30 min and a blocking step with 3 % (w/v) bovine serum albumin (BSA) for 30 min. The sperm sections were then incubated with the anti-protamine-1 antibody (dilution 1/100; v/v) for 1-2 h at

15 ºC, washed with PBS, and treated with an Alexa 594-conjugated donkey anti-goat secondary antibody. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope adapted to an inverted Leitz DMIRBE microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo Lens. The light source was an argon/krypton laser (75 mW). Finally, arbitrary colors shown here (green and red) were chosen in order to obtain the best possible contrast between both stains. Confocal images were stored as TIFF-format images. These images were simultaneously observed and stored under visible light in a phasecontrast system. The combination of visible light and laser images allowed for the exact location of the positive reactions in sperm-head sections, thus permitting for a better analysis of the obtained data.

Finally, the specificity of the observed immunoreactivity was tested after subjecting several experiments to immunocytochemistry in the presence of the antiprotamine-1 antibody previously pre-adsorbed with a commercial, specific peptide to a final concentration of 20 μg/mL.

Statistical analyses

Data were analyzed by using the SAS statistical package for Windows (SAS, 1996). The determination of putative differences among the studied phases of the freeze-thawing protocol was performed by applying the GLM procedure included in the SAS package. For an optimal application of the statistical procedures, data were normalized through an arcsin[$\sqrt{x}/100$] transformation, x being the transformed data. Differences among data were considered as being significant from $P < 0.05$.

Results

Cooling- and thawing-induced changes in the mean semen quality parameters of boar spermatozoa

The cooling phase of the freeze-thawing protocol induced several significant changes in the majority of the tested semen quality parameters of boar spermatozoa. As shown in Table 6, cooling induced a significant ($P<0.05$) decrease in the percentages of viability, total motility and ORT, which were concomitant to a significant ($P<0.05$) increase in the percentage of altered acrosomes. Furthermore, freeze-thawing induced greater changes in the tested semen quality parameters of boar spermatozoa. In this way, the percentages of viability, total motility and ORT suffered a further, significant ($P<0.05$) decrease when compared with samples after cooling, which were also concomitant to a further increase in the percentage of altered acrosomes, which increased from 24.1 ± 1.0 % in cooled samples to 49.2 ± 2.1 % in thawed sperm (Table 6).

Effects of freeze-thawing on DNA fragmentation in boar spermatozoa

Staining of boar spermatozoa through the Sperm-Sus-Halomax©, specific DNA fragmentation kit did not show any significant increase on the freeze-thawing induced DNA fragmentation rate. Thus, as shown in Table 7, the majority of sperm in fresh samples were included in Type I class spermatozoa (intact sperm head), following the

Table 6. Effect of freezing-thawing on the mean semen quality parameters of boar spermatozoa

	Fresh sperm	5 ºC	Frozen-thawed sperm
Viability (%)	85.1 ± 1.2[a]	76.0 ± 1.2[b]	41.9 ± 1.2[c]
Altered acrosomes (%)	13.7 ± 0.4[a]	24.1 ± 1.0[b]	49.2 ± 2.1[c]
ORT (%)	60.6 ± 2.9[a]	34.8 ± 2.9[b]	14.8 ± 2.9[c]
HRT (arbitrary units)	1.08 ± 0.03[a]	0.99 ± 0.03[a]	1.01 ± 0.03[a]
Total motility (%)	75.9 ± 0.2[a]	53.3 ± 0.3[b]	46.9 ± 0.9[c]

Semen quality parameters have been defined in Materials and methods section. Results are means ± S.E.M. of 12 separate experiments. Fresh sperm. 5 ºC: sperm after the cooling phase of the freezing protocol. Frozen-thawed sperm. Different letters in a row indicate significant ($P<0.05$) differences among groups.

Sperm-Sus-Halomax© scale (92.9 ± 0.7 % of total sperm) and sperm with distinct DNA fragmentation were practically absent (0.1 ± 0.7 % of total sperm included in Type IV class spermatozoa of the Sperm-Sus-Halomax© scale). The cooling phase of the freeze-thawing process did not affect sperm DNA fragmentation, as estimated by the Sperm-Sus-Halomax© technique. Furthermore, additional freeze-thawing only induced a slight decrease in the percentage of spermatozoa included in Type I class spermatozoa (intact sperm head), following the Sperm-Sus-Halomax© scale, which was concomitant to an equivalent increase in the percentage of sperm included in the Type II class (slightly swollen heads, without DNA fragmentation) of the Sperm-Sus-Halomax© scale (Table 7).

The analysis of boar-sperm genomic DNA by both agarose electrophoresis and random priming analysis after BamHI-HinDIII digestion yielded results that were compatible with the results obtained through the Sperm-Sus-Halomax© technique. In this sense, agarose electrophoresis of native genomic DNA showed the presence of a single, wide band of molecular weight ranging from 21.2 to 4.3 kb. This band was

Table 7. Effects of freezing-thawing on boar-sperm DNA fragmentation determined through the Sperm-Sus-Halomax® staining kit

	Fresh sperm	5 ºC	Frozen-thawed sperm
Type I sperm (%)	90.0 ± 0.7[a]	90.7 ± 0.7[a]	78.3 ± 0.7[b]
Type II sperm (%)	6.7 ± 1.6[a]	7.2 ± 1.6[a]	19.2 ± 1.6[a]
Type III sperm (%)	1.3 ± 0.3[a]	1.1 ± 0.1[a]	1.4 ± 1.8[a]
Type IV sperm (%)	1.1 ± 0.6[a]	1.0 ± 0.6[a]	1.1 ± 0.6[a]

Type I sperm: sperm head with normal morphology, no DNA fragmentation. Type II sperm: sperm heads slightly swollen, no DNA fragmentation. Type III sperm: sperm heads moderately swollen, no DNA fragmentation. Type IV sperm: positive sperm-head halo, fragmentation of sperm DNA. Results are means ± S.E.M. of nine separate experiments. 5 ºC: sperm after the cooling phase of the freezing protocol. Different letters in a row indicate significant ($P<0.05$) differences among groups.

similar in fresh, cooled and frozen-thawed samples, and was transformed after the digestion of samples for 3 h with BamHI-HinDIII in a uniform smearing, which was also similar in fresh, cooled and thawed samples (Fig. 3A). This smearing did not change in samples digested for 12 h, thus yielding complete digestion of the genomic DNA (data not shown). Concomitantly, the random priming DNA analysis on Bam HI-HinDIII digested DNA of fresh boar-sperm samples for 3 h showed a similar smearing, and the greatest intensity in the obtained markings was observed in a weight range from about 21.2 kb to about 3.5 kb (Fig. 3B). DNA extracts from both cooled and frozen-thawed sperm showed a similar pattern of random priming to that of fresh samples, indicating that both processes did not strongly modify DNA structure or behavior in front of the Random Priming analysis (Fig. 3B).

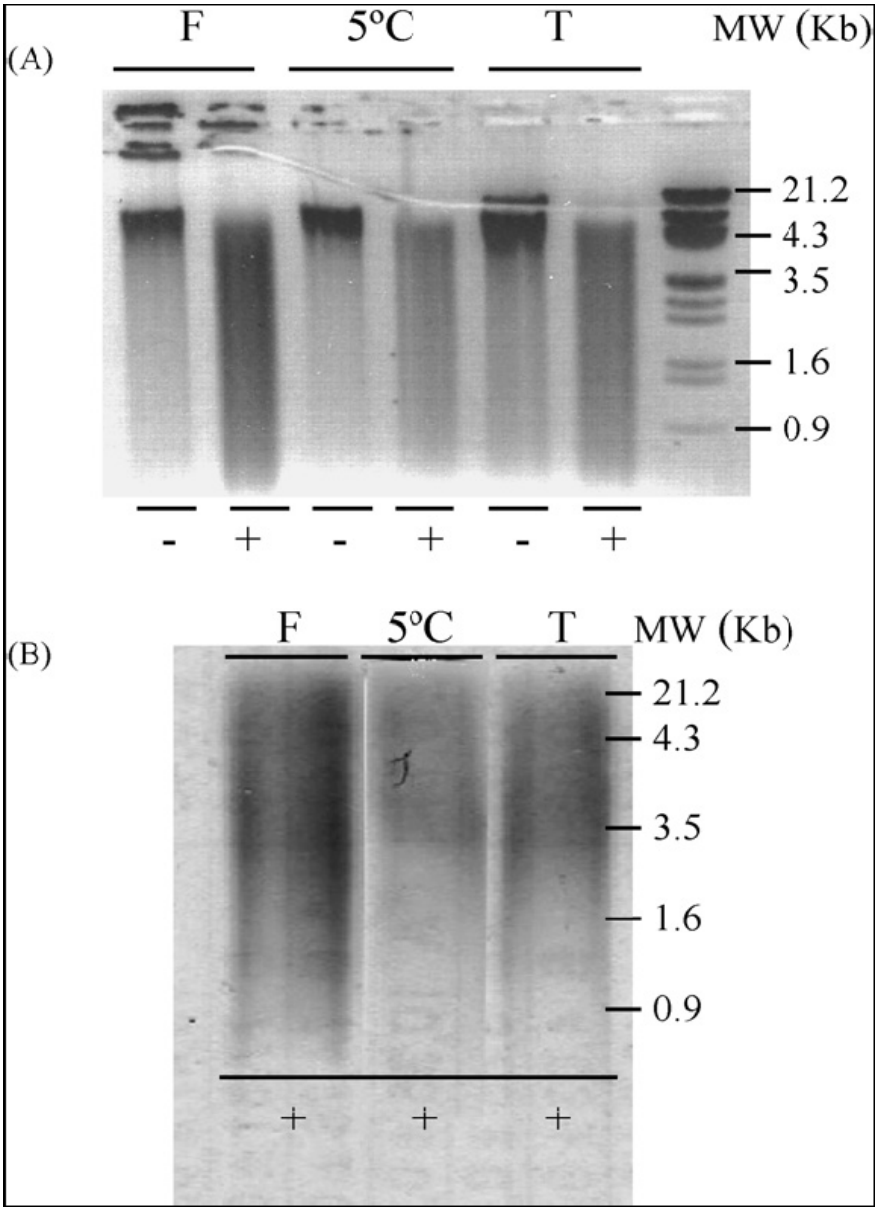

Fig. 3. Agarose electrophoresis and Southern blot associated with Random Priming of boar sperm subjected to freezing-thawing. Both the agarose electrophoresis and the Southern blot associated with Random Priming analysis were performed as described in Materials and methods section. (A) Agarose electrophoresis. (B) Southern blot associated to Random Priming analysis. (F) Fresh sperm. 5 ºC: sperm after the cooling phase of the freezing protocol. (T) Frozen-thawed sperm. (-) Samples that were not digested with BamHI-HinDIII restriction enzymes. (+) Samples digested with BamHI-HinDIII restriction enzymes as described in Materials and methods section. (MW) Molecular weight markers. The figure shows representative results obtained from eight separate replicates from eight different ejaculates.

Effects of freeze-thawing on protamine-1/DNA interaction in boar sperm

The Western blot against protamine-1 in boar sperm from fresh samples showed the presence of several specific bands with a molecular weight ranging from about 15 kDa to about 200 kDa (Fig. 4A). These bands were made up of protamine-1–DNA complexes, since the pre-treatment of samples with DNAase-1 induced a clear increase in the intensity of the band corresponding to the free protamine-1 monomer, of about 15 kDa, concomitantly with the decrease, and even disappearance of the other bands (Fig. 4A). Cooling and further freeze-thawing induced clear changes in the specific band pattern. Thus, as shown in Fig. 4A, cooling induced the near total disappearance of protamine-1 bands of less than 200 kDa, whereas thawed samples showed the presence of three majoritary bands ranging from about 100 to 200 kDa. As in fresh samples, the protamine-1 bands detected in both cooled and thawed samples were composed of protamine-1–DNA complexes, since the pre-treatment of samples with DNAase-1 induced the appearance of the 15-kDa band corresponding to free protamine-1 (Fig. 4A). Notwithstanding, it is noteworthy that the band profile obtained in samples pre-treated with DNAase-1 was also different in both cooled and thawed samples when compared to fresh sperm. Thus, whereas the DNAase-1 treatment induced the appearance of a single, 15-kDa band in fresh samples, the same treatment induced the appearance of a 100-kDa band in cooled sperm and the presence of several bands from 30 to 100 kDa in thawed cells, besides the 15-kDa, free protamine-1 band (Fig. 4A).

Immunocytochemistry against protamine-1 in 1-μm sections of boar sperm from fresh samples showed the presence of a specific immunoreactivity at the apical region of the sperm head, together with several very small, positive points located at the

posterior region of the spermhead (Fig. 5A). Cooling increased the specific reactivity against the protamine-1 antibody at the posterior region of the sperm head, and frozen-thawed sperm showed the greatest positive reaction, with the presence of intense immunoreactive zones at the posterior region of the head, as well as clear immunoreactive points located over all of the anterior region of the head (Fig. 5B and C).

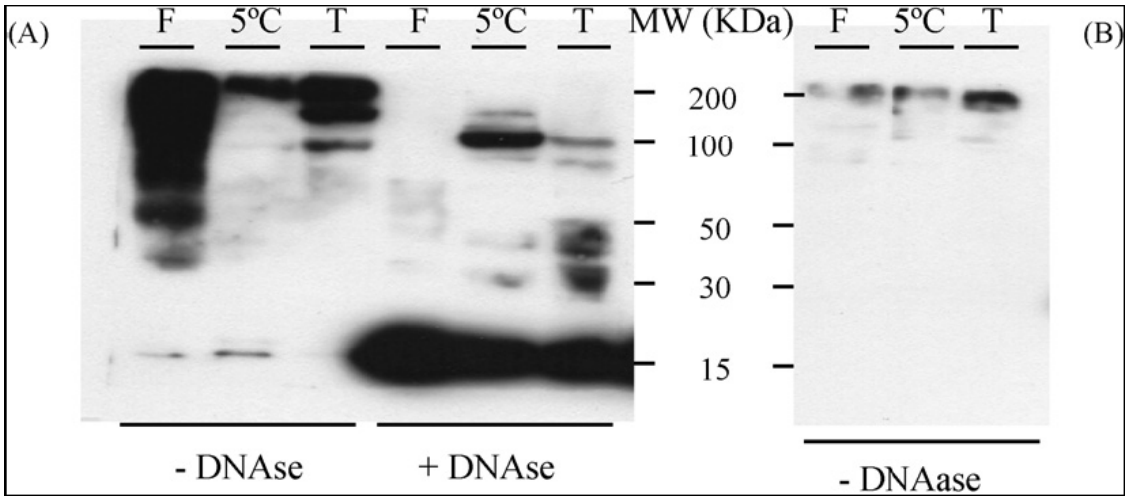

Fig. 4. Western blot analysis of DNA-associated protamine-1. Supernatants of sperm homogenates were processed as described in Materials and methods section. (A) Standard Western blot analysis. (B) Western blot performed with a previous pre-absorption of the protamine-1 antibody with the corresponding, specific synthetic peptide. -DNAase: samples which were applied to Western blot without a previous digestion with DNAase-1. +DNAase: samples applied after a previous digestion with DNAase-1 as described in Materials and methods section. (F) Fresh sperm. 5 ºC: sperm after the cooling phase of the freezing protocol. (T) Frozen-thawed sperm. The figure shows a representative Western blot analysis obtained from eight separate replicates from eight different ejaculates.

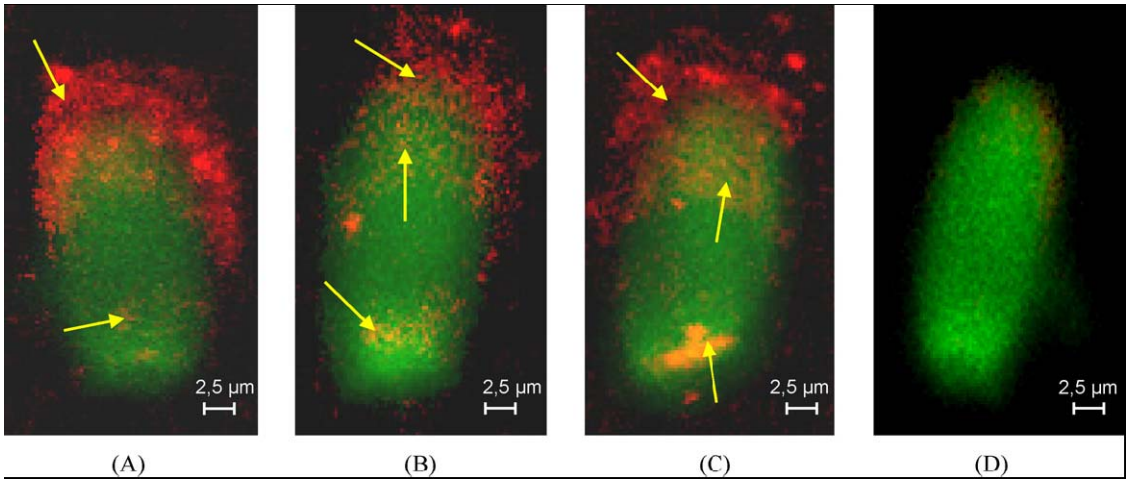

Fig. 5. Immunocytochemistry of protamine-1 in boar spermatozoa. The co-localization protamine-1–DNA was performed as described in Materials and methods section. (A) A representative sperm head from fresh samples. (B) Sperm after the cooling phase of the freeze-thawing protocol. (C) Sperm head from a thawed sample. (D) Image of a sperm head from a thawed sample in which immunocytochemistry was performed with a previous pre-absorption of the protamine-1 antibody with the corresponding, specific synthetic peptide. Arrows indicate the presence of specific signaling for protamine-1 (orange-to-red spots). DNA has been highlighted by marking it as green in the utilized false-colors system. The increase in the orange-to-red marking in both the anterior and the posterior poles of the sperm head after the cooling phase and also after further freeze-thawing is noteworthy. Bars indicate the actual size of the image. The figure shows representative images obtained from eight separate replicates from eight different ejaculates.

Discussion

Our results clearly show that a standard freezing-thawing protocol induces a significant alteration of the protamine-1–DNA boar sperm-head structure without a significant increase in DNA fragmentation. This can be inferred when analyzing both the results regarding DNA integrity and protamine immunodetection. Thus, both the

Sperm-Sus-Halomax© staining and the random priming technique indicate the lack of a significant effect of freeze-thawing on DNA integrity. These results are similar to those described by Evenson *et al* (1994) and Hernández *et al* (2006), where no significant alteration of DNA integrity in frozen-thawed boar spermatozoa was found.

The results concerning Western blot against protamine-1 need a careful explanation, since a correct interpretation is not evident at first glance. Firstly, it must be pointed out that the protamine-1 signal obtained both through Western blot analysis and immunocytochemistry was specific, as the results obtained with the pre-absorbed antibody indicate. On the other hand, the Western blot analysis was only performed on supernatants from sperm homogenates obtained after sonication. This indicates that the observed signal against the anti-protamine-1 antibody was obtained in fragments of low molecular weight that were bound to the rest of the nuclear structure in a manner such that the mechanical traction originated by sonication was able to detach them. That these low-molecular-weight fragments were constituted by protamine-1–DNA aggregates was demonstrated when Western blot was performed in samples previously treated with DNAase-1. The treatment with this enzyme separated protamine-1, which appeared then basically as its monomeric, low-molecular-weight form of about 15 kDa in Western blot. Summing up all of these data, our results indicate that freeze-thawing induced a significant change in the proportion and types of low-weight protamine-1–DNA aggregates that can be separated from the rest of the nuclear structure by sonication, since the number and distribution of specific bands that showed the Western blot clearly changed between fresh and frozen-thawed samples. This warrants at least two comments. The first is that freeze-thawing induced enough changes on the overall protamine-1–DNA structure of the boar-sperm nucleus to be

intense enough to modify Western blot results. The second is that our results indicate that the boar-sperm nuclear structure is not a homogeneous one, since sonication is able to detach several concrete protamine-1–DNA, low-molecular-weight aggregates from a very tight nuclear structure. The heterogeneous organization of the sperm nucleus has already been described. In this way, it has already been described as containing DNA domains where the associated proteins are not protamines but rather histones (Wykes & Krawetz, 2003; O'Brien & Zini, 2005). The percentage of sperm DNA that is structured around histones is about 15 %, and these domains are less compact, located on the telomeric sequences (O'Brien & Zini, 2005). The combination of zones with different compactness characteristics would lead to the formation of zones more or less sensitive to mechanical disruption, thus originating the formation of the observed low-molecular-weight protamine-1–DNA aggregates after sonication. Freeze-thawing would induce changes in the distribution of these sensitive nuclear zones, thus inducing the observed changes in the Western blot specific bands. However, these changes are not due to the presence of DNA fragmentation prior to sonication, since DNA fragmentation analysis on whole cells (the Sperm-Sus-Halomax© stain) was negative. In this regard, it is noteworthy that, in our conditions, freeze-thawing induced an increase of spermatozoa included in the Type II class of the Sperm-Sus-Halomax© that was accompanied by a concomitant decrease of the percentage of Type I sperm. This indicates, following the classification published in Enciso *et al* (2006), that freeze-thawing induced a slight increase of sperm nucleus volume that could indicate a loss of the compactness of the protamine-1–DNA structure. This result could be related to those published in Hernández *et al* (2006), which indicate that boar-semen ejaculates with poor survival after freeze-thawing showed less homogeneous sperm chromatin than those with good

freezing characteristics. Taking into account all of this information, it can be then inferred that freeze-thawing induces local, specific changes in concrete nuclear zones that modify its compactness. This effect would lead to the detachment of different zones of the protamine-1–DNA nuclear structure that were sensitive to the mechanical forces induced by sonication.

The results obtained through immunocytochemistry agree with those of Western blot, although they also need a careful explanation. Firstly, the overall compactness of the boar-sperm nuclear structure is highlighted by the fact that immunocytochemistry had to be performed on sperm slices, since the antibody was unable to penetrate inside the nuclear structure in whole cells. Moreover, the anti-protamine-1 antibody was also unable to completely penetrate into the nuclear structure in cell slices, and only the zones that had a less compact protamine-1–DNA organization allowed the protein-antibody interaction. This explains the results obtained in fresh samples, where the antibody-associated mark only appeared in some concrete zones of the sperm, such as the peri-acrosomal area. This positive reaction could indicate the presence of the protamine–DNA complexes loosely bound that further appeared after sonication in the Western blot analysis. The increase of antibody-associated marking in frozen-thawed samples could then be due to a loss of nuclear compactness in these zones, which allowed for this observed increasing reactivity. Thus, these results also indicate the existence of an alteration in the protamine-1–DNA structure induced by the freeze-thawing process.

We can only speculate about the mechanism/s by which freeze-thawing could cause the observed alteration in the protamine-1–DNA structure of boar sperm. Notwithstanding, we believe that one of the main mechanisms would be the mechanical

stress induced by the freeze-thawing-related osmotic stress. In this sense, it has been reported that similar mechanical processes, such as those caused through sex-sorting, can cause significant alterations of the nuclear structure in boar sperm (De Ambrogi *et al*, 2006a). Osmotic changes, especially if they are very intense, like those induced during the very fast temperature changes associated with the freeze-thawing process, can induce a similar mechanical stress, mainly due to the fact that the fast entry (or releasing) of cell water will provoke fast changes in the form and strength of all internal sperm structures, including the nucleus. Thus, this could lead, at least partially, to the observed alterations, especially when considering that the protamine-1–DNA interaction resulted in the formation of a very peculiar structure in which the DNA chains are forming a torodial and linear, not helicoidal, skeleton. In this structure, protamines are located inside both DNA chains, thus forming cellular, repetitive structural cells randomly distributed throughout the entire nucleus (Biegeleisen, 2006). This structure is more rigid than that observed in the nucleus of eucaryotic, somatic cells. This rigidness would imply that the sperm nuclear structure would be sensitive to the mechanical tractions induced by the freeze-thawing process, specially affecting the protamine–DNA bounds. However, it must be stressed that, although osmotic changes can be determinant in explaining the observed effects, this mechanism would not be responsible for all of the effects, since boar sperm nuclear alterations started to manifest themselves during the cooling phase of the freeze-thawing process, in which osmotic stress was not very high. Thus, other mechanism/s would be implied in the boar-sperm nuclear alterations. In this sense, it has been suggested that oxidative damage, such as that induced in human patients with varicocele, can also cause nuclear alterations to different degrees (Smith *et al*, 2005). Moreover, it has been also described that storage of boar sperm in

refrigerated conditions (17-18 ºC) can also cause nuclear alterations, although there are contradictory publications regarding this point (Boe-Hansen *et al*, 2005; De Ambrogi *et al*, 2006b). Taking into account that boar spermatozoa are cells which are very sensitive to oxidative damage, in part due to their characteristic composition of cellular membranes (Flesch *et al*, 2001; Breininger *et al*, 2005; Holt *et al*, 2005), it would be possible that an oxidative process, especially during the cooling phase of the freezing protocol, could act as one of the mechanisms underlying the observed alteration of the boar-sperm nuclear structure.

In conclusion, our results show that freeze-thawing induced a specific nuclear alteration of boar sperm. This alteration is related to changes in the formation of the protamine-1–DNA complex rather than to direct DNA fragmentation, and the final consequence is the formation of a less rigid and compact structure of the sperm-head nucleus. This subtle alteration could be important when sperm has to induce syngamia after oocyte penetration, thus inducing a loss of the fertilizing ability of the affected cells. Thus, this phenomenon has to be controlled in order to optimize the efficiency of boar-sperm freeze-thawing.

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CAPÍTOL II / CHAPTER II

The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa

Abstract

The main aim of this work was to analyze the possible relationship between the structures of motile-sperm subpopulations and boar (*Sus scrofa domesticus*) sperm resistance to freezability. For this purpose, 45 boar ejaculates were subjected to a standard freezing-thawing protocol, and afterwards they were classified into three groups, in accordance with their resistance to freezing-thawing. Our analysis yielded four separate motile-sperm subpopulations in all of the studied ejaculates, both in fresh samples and after freezing-thawing. Furthermore, whereas curvilinear velocity (VCL), mean velocity (VAP) and dance (DNC) of sperm from Subpopulation 1 underwent significant increases after freezing-thawing in samples with a good response to freezing-thawing, the same parameters of Subpopulation 1 either did not undergo significant variations (VCL and DNC) or even showed a decrease (VAP) (from 20.4 ± 0.4 μm/sec in fresh samples to 15.2 ± 2.2 μm/sec after freezing-thawing) in samples with the poorest response. Similarly, the behavior of other motility parameters in each subpopulation was also very different in the worst samples when comparing them with

those with a good or average response to cryopreservation. Additionally, the DNC of all four subpopulations was in all cases lower in samples with the poorest characteristics of freezability. This was not the only difference, and significant changes in parameters such as the VCL of Subpopulations 2 and 4, linearity coefficient (LIN) of Subpopulations 1, 2 and 3, and wobble coefficient (WOB) of Subpopulations 2 and 3 were also observed in samples with different response to freezing-thawing. Meanwhile, the determination of mitochondrial activity and mitochondrial-linked reactive oxygen species formation indicated that the samples with the poorest freeazibility characteristics were also those with the lowest mitochondrial activity. We conclude that boar ejaculate resistance to cryopreservation seems to be related to the specific, initial motile-sperm subpopulation structure. In turn, this structure would be closely related to the specific, overall mitochondrial activity, which would be a very important indicator of sperm function. Furthermore, and as a practical conclusion, an in-depth analysis of motile sperm subpopulation structure together with functional tests could improve the design of predictive strategies for the freezability of boar sperm.

Introduction

Currently, one of the most conflicting points regarding freezing of boar semen is the very high variability that is observed in the resistance to freezability among boars and even among ejaculates from single male pigs (Larsson & Einarsson, 1976; Thurston *et al*, 2001; Medrano *et al*, 2002; Roca *et al*, 2006). This phenomenon, which has also been observed in other species, such as cattle (Parkinson & Whitfield, 1987), horse (Janett *et al*, 2003), sheep (D'Alessandro & Martemucci, 2003) or dog (Yu *et al*, 2002)

is of the utmost practical importance, as it makes a better approximation of the freezing technology applied to the porcine industry difficult. Furthermore, these difficulties are even worsened by the fact that the variations in semen freezability cannot be related to the results obtained in the standard semen-quality analysis of the semen sample before freezing, thus impeding a predictive analysis of freezing resistance for a single boar semen sample (Gil *et al*, 2005; Roca *et al*, 2006; Hernández *et al*, 2006 and 2007). It is obvious, then, that the appearance of tools that are able to be applied in a predictive form will greatly improve both the application and the results of frozen semen for artificial insemination (AI).

In recent years, there has been an increasing of information reporting that ejaculates for a very great and increasing number of mammalian species are composed of well-defined subpopulations, which have been characterized by a precise, quantitative evaluation of both motility and morphometry parameters through different computer-assisted motility (CASA; Holt, 1996; Abaigar *et al*, 1999; Rigau *et al*, 2001; Quintero-Moreno *et al*, 2003 and 2007; Martínez-Pastor *et al*, 2005; Miró *et al*, 2005; Muiño *et al*, 2008) and morphometry (ASMA) systems (Thurston *et al*, 2001; Hirai *et al*, 2001; Peña *et al*, 2005; Rubio-Guillén *et al*, 2007). Centering our attention on boar semen, the existence of three or four separate subpopulations, with specific characteristics of either motility (Abaigar *et al*, 1999; Quintero-Moreno *et al*, 2004; Rivera *et al*, 2005 and 2006; Ramió *et al*, 2008; Flores *et al*, 2008) or morphometry (Peña *et al*, 2005), has been well established. Moreover, regarding motility, it has also been reported that the motility changes associated with processes such as *in vitro* capacitation and further acrosome reaction (Ramió *et al*, 2008) and freezing-thawing (Flores *et al*, 2008) are associated with changes in the specific percentage of motile

sperm included in each subpopulation and, to a lesser extent, with changes in the specific motility characteristics of each subpopulation.

One of the mechanisms involved in the control of sperm motility is the modulation of sperm mitochondrial activity. Several authors have established that mitochondrial activity is absolutely necessary to maintain overall sperm motility (Nevo *et al*, 1970; Ford & Harrison, 1985; Halangk *et al*, 1985; Folgero *et al*, 1993; Ruíz-Pesini *et al*, 1998). However, this affirmation seems not to be universal as, in species such as mice, sperm motility from freshly obtained samples is maintained after the complete inhibition of their mitochondrial activity (Mukai & Okuno, 2004), and in freshly obtained boar sperm, the amount of energy that is derived from mitochondrial sources accounts for only about 5 % of the total energy consumed by the cells (Marín *et al*, 2003). Notwithstanding, it is doubtless that mitochondrial activity plays a key role in modulating different aspects of boar-sperm function. One of the most studied to date is the mitochondrial control and production of reactive oxygen species (ROS). Intrinsic mitochondrial activity caused the formation of important amounts of ROS as an unwanted by-product, and cells have several mechanisms to counteract the deleterious effects that ROS have on cell function (see Proctor, 1989 for a review). In mammalian sperm, ROS are especially important, as spermatozoa are not very efficient in counteracting mechanisms against ROS effects (Donnelly *et al*, 1999; Saleh & Agarwal, 2002; Brouwers *et al*, 2005). This implies that spermatozoa are specially sensitive to undergoing ROS-induced alterations such as excessive lipid peroxidation (De Lamirande & Gagnon, 1992b; Aitken *et al*, 1998; Armstrong *et al*, 1999; Kobayashi *et al*, 2001; Moustafa *et al*, 2004; Agarwal *et al*, 2005). Concerning freezing-thawing, it has been well established that one of the most important mechanisms that explains

freezing-associated sperm alterations and death is an excessive accumulation of ROS (Hinshaw *et al*, 1986; Aitken *et al*, 1989; Alvarez & Storey, 1992; De Lamirande & Gagnon 1992a; O'Flaherty *et al*, 1997; Lopes *et al*, 1998; Mazur *et al*, 2000; Chatterjee & Gagnon, 2001). This is especially important in boar, in which its very specific cellular-membrane structure, with a very high percentage of unsaturated fatty acids, is especially prone to undergoing ROS-mediated membrane lesions (Breininger *et al*, 2005). Moreover, the accumulation of ROS during freezing-thawing seems to be clearly associated with alterations of mitochondrial function, and, in fact, it has been reported that mitochondria are among the cellular structures most sensitive to freezing-thawing (Cummins *et al*, 1994). All of these data clearly indicate that one of the most important points that explain sperm resistance to freezing-thawing would be the maintenance of correct mitochondrial activity, which allows for the modulation of intracellular ROS levels within tolerable limits.

The main aim of this work was to evaluate a putative relationship between the resistance to freezing-thawing of boar-semen samples and the structures of specific motile-sperm subpopulations. This was done by analyzing and comparing these subpopulation structures in samples that showed poor, average, and good resistance to cryopreservation by using a CASA system. Additionally, the mitochondrial activity of ejaculates with poor, average, and good freezability was also compared. For this purpose, mitochondrial activity was evaluated through two techniques. First, changes in the stain intensity of a specific marker of mitochondrial activity, the MitoTracker Red CM-H_2Xros, were evaluated. Second, mitochondrial formation of ROS (mROS) was simultaneously evaluated by quantifying changes in the stain intensity of a specific marker, proxylfluorescamine.

Materials and methods

Animals and samples collection

Forty-five healthy boars (*Sus scrofa domesticus*) of about 2 to 3 years of age from a commercial farm and one ejaculate per boar were used in this study. The boars were from three separate lines (Landrace, Large White, and Pietrain). All boars had proven fertility after AI using extended, liquid semen. The sperm-rich fraction of each ejaculate used in this study was manually collected twice weekly using the gloved-hand method and analyzed to ensure the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was suspended (1:2; v/v) in a commercial extender (MR-A). The extended semen samples were cooled and maintained at 17 ºC for shipment to the laboratory of the Autonomous University of Barcelona within 24 h after collection for further processing and analyses.

Semen cryopreservation

Immediately after receiving the shipped semen samples, an aliquot was taken to perform the appropriate semen quality parameters, as well as those regarding sperm-motility studies (fresh semen sample). Only those samples displaying a minimum of 70 % progressive motile and 80 % of morphologically normal spermatozoa were further processed by adapting a proven protocol (Sancho *et al*, 2007). The extended semen was centrifuged in a programmable refrigerated centrifuge, set at 17 ºC, at 600 *g* for 10 min. After centrifugation, the supernatant was discarded. The remaining pellets were

re-extended with a lactose-egg yolk (LEY) extender (80 % [v/v] 310 mM β-lactose, 20 % egg yolk) at a ratio that led to a final concentration of 1.5 x 10^9 spermatozoa/mL. The sperm concentration was manually assessed in a Thoma or Neubauer hemocytometer. At this point, and after thorough mixing, the semen was further cooled to 5 ºC for 2 h in the centrifuge. Afterwards, 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 at a ratio of two parts of semen to one part of extender, yielding a final concentration of 3 % (v/v) glycerol and a concentration of 1 x 10^9 spermatozoa/mL at 5 ºC, which was verified by counting in a Thoma or Neubauer hemocytometer. Spermatozoa were packaged at 5 ºC in a cool cabinet in 0.5-mL polyvinyl chloride (PVC) plastic straws, which were sealed with PVC powder and placed on racks for freezing. The racks were transferred to the chamber of a programmable freezer set at 5 ºC. The cooling/freezing rate used was 6 ºC/min from 5 ºC to -5 ºC, 40 ºC/min from -5 ºC to -80 ºC, 30 sec for crystallization, and thereafter 60 ºC/min from -80 ºC to -150 ºC. The samples were then plunged into liquid N_2 (-196 ºC) for storage. Frozen samples were stored in liquid N_2 for at least 21 days. After this, samples were thawed by plunging the samples into a water bath at 37 ºC for 20 sec. Immediately afterwards, straws were carefully wiped and opened, and samples were immediately analyzed to determine the appropriate semen-quality parameters, as well as those regarding sperm-motility studies (frozen-thawed semen sample).

Analysis of semen-quality parameters

Percentages of viability, altered acrosomes and morphologic abnormalities were determined by using the eosin-nigrosin stain (Bamba, 1988). This technique shows viable spermatozoa as being those with a uniform, whitish tonality in the entire cell as a result of the lack of staining of these cells, which were translucent over the stained background. The presence of a partial or a totally pinkish stain was indicative of nonviable sperm cells. Moreover, acrosome integrity was evaluated by observing the presence of a regular and intact acrosomal ridge after the eosin-nigrosin staining. Any part of the acrosomal ridge that did not have a regular and intact aspect was considered as representing an altered acrosome. The percentages of both viability and altered acrosomes were obtained after analyzing a minimum of 200 spermatozoa/sample through optical microscopy (magnification: 1000×). The osmotic resistance test (ORT) was carried out as described in Rodríguez-Gil & Rigau (1995), whereas the hyperosmotic resistance test (HRT) was carried out as in Caiza de la Cueva *et al* (1997).

The computer-assisted analysis of sperm motility (CASA) was carried out by using a commercial system. In this system, samples were previously diluted with Beltsville Thawing Solution (BTS; Pursel & Johnson, 1975) to a final concentration of 2 x 10^7 spermatozoa/mL and warmed at 37 ºC for 5 min in a water bath, and 5-µL aliquots of these samples were then placed onto a warmed (37 ºC) slide and covered with a 22-mm^2 coverslip. Our CASA system was based on the analysis of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 200× on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image-capturing of one photograph

every 40 msec. Two to three separate fields were taken for each sample, which yielded a minimum of 200 spermatozoa per sample. Finally, total motility was defined as the percentage of spermatozoa which showed a mean velocity (VAP) above 10 μm/sec.

Determination of mitochondrial activity and mitochondrial-associated ROS formation

Both the overall mitochondrial activity and mROS levels were simultaneously evaluated following the technique of MitoTracker Red/proxylfluorescamine double staining (Wenzel *et al*, 2005), with the following modifications:

Samples from fresh and frozen-thawed semen were incubated with a solution of proxylfluorescamine in phosphate-buffered saline (PBS; pH 7.4) at a final concentration of 50 μM for 10 min at 37 ºC. Immediately afterwards, samples were centrifuged at 600 *g* for 10 min at 20 ºC, and the resultant pellet was resuspended in 1 mL of a solution of 500 nM MitoTracker Red CM-H_2Xros in PBS. Sperm cells were incubated in this medium for another 10 min at 37 ºC. Then, cell suspensions were again centrifuged at 600 *g* for 10 min at 20 ºC and the cellular pellet was resuspended in 50 μL PBS. This final suspension was spread onto 76 mm x 26 mm slides, which were left to dry. Once dry, the slides were covered with a paraformaldehyde solution (2 %, v/v) in PBS for 10 min at 20 ºC. Fixed samples were thoroughly rinsed with distilled water and were then allowed to air-dry at room temperature. Until this moment, all of the steps of this procedure were carried out in a dark chamber, to avoid any unspecific photostimulation of fluorochromes. Samples were finally stored at 4 ºC in the dark until their evaluation, which was performed over a maximal period of 3 days after treatment. Fluorescence

was analyzed through the Leica TCS 4D confocal laser microscope, which was adapted to a Leitz DMIRBE inverted microscope with a Nomarsky interferential contrast objective and a Leitz-Plan-Apo 63× lens. The energy of the argon/krypton laser source was of 74 mW, and serial images were taken with a depth of 0.5 to 1 μm/image. In this process, the Nomarsky interferential contrast was used as a precise control of the location of the analyzed cells. Fluorescence detection was performed by utilizing an excitation wavelength of 543 nm and an emission length of 440 to 480 nm (mROS) and 590 to 650 nm (MitoTracker Red). Finally, special care was taken to maintain the exact same conditions of background and intensity in all cases, as this was absolutely essential for a correct analysis of the signal intensity in each sperm.

The analysis of the signal intensity for both MitoTracker Red and mROS was performed on the digitalized images through the Adobe Photoshop® image program. For this purpose, midpieces for at least 200 sperm per point were selected using the appropriate tool of the program and the intensity of brightness in these selected midpieces was quantified individually by utilizing the command "Histogram". This quantification was only performed after a careful homogenization of brightness and contrast among all of the analysed images in all cases. After this, mean values of brightness were adjusted to a mean, arbitrary value of 100 ± S.E.M. for control, fresh sample points, and the other points were compared taking as reference this value of control samples. Differences among treatments were analyzed after applying the GLM and LSMEANS procedures included in the SAS statistical package.

Statistical analysis of motility descriptors

Data were processed by using the SAS statistical package (SAS, 2000). Normality of data distributions was assessed by the Shapiro-Wilks test, which is included in the UNIVARIATE procedure. After the assessment of normality, a VARCLUS clustering procedure was applied to reduce the number of sperm motility descriptors, as CASA analysis yielded an elevated number of separate motion parameters (as high as 12) that were often closely related among themselves. This allows for the determination of the optimal number of motion parameters that can be used to define sperm motion characteristics. The motility parameters that were chosen to keep the maximal statistical information with the minimal number of parameters are described in Table 8. Afterwards, a clustering procedure, FASTCLUS, was used to categorize the whole ejaculates by their postthawed sperm-quality parameters. Three groups were finally obtained by the clustering procedure:

Group I: Samples included in this group showed a decrease in their percentages of viability below 42 %. This group thus included ejaculates considered as being resistant to freezing-thawing, and are called “good freezers”.

Group II: Samples included in this group showed a decrease in their percentages of viability between 42 % and 55 %. This group thus included ejaculates considered as being moderately resistant to freezing-thawing, and are called “moderate freezers”.

Group III: Samples included in this group showed a decrease in their percentages of viability equal to or above 55 %. This group thus included ejaculates

considered as being not resistant to freezing-thawing, and are called "bad freezers".

After these observations, the next step was to use a statistical clustering procedure, FASTCLUS. This procedure performs a disjointed cluster analysis based on Euclidean distances computed from one or more quantitative parameters. In this case, these variables are the different sperm-motility parameters measured by the CASA system. Spermatozoa were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster, whereas spermatozoa that differed in motility characteristics were assigned to different clusters. A GLM procedure was applied to evaluate significant differences ($P<0.05$), and the LSMEANS procedure was applied to test these differences. Finally, a chi-square procedure was applied to determine the subpopulational distribution percentage in every single experiment. Once the percentage distribution per experiment was determined, new GLM and LSMEANS procedures

Table 8. Descriptions of the motility parameters used in this study.

Name	Units	Description
Curvilinear velocity (VCL)	μm/sec	The instantaneously recorded sequential progression along the entire trajectory of the spermatozoon.
Mean velocity (VAP)	μm/sec	The mean trajectory of the spermatozoon per unit of time.
Linear coefficient (LIN)	%	(VSL/VCL) × 100.
Straightness coefficient (STR)	%	(VSL/VAP) × 100.
Wobble coefficient (WOB)	%	(VAP/VCL) × 100.
Dance (DNC)	μm^2/sec	The product of the multiplication between VCL and the mean lateral head displacement around the sperm mean trajectory.
Absolute angular mean displacement (absMAD)	º	The absolute value of the advancing angle of the sperm trajectory.
Total motility	%	The percentage of spermatozoa with a VAP above 10 μm/sec.

were applied to determine and test, respectively, the differences among the different treatments.

Results

Effects of freezing-thawing on the mean parameters of semen-quality parameters of ejaculates as a whole and classified in accordance with their freezability

Freezing-thawing induced a significant decrease in the percentages of viability, ORT, and total motility, which were concomitant with a significant increase in the percentage of altered acrosomes (from 10.8 ± 1.9 % in fresh samples to 78.1 ± 1.9 % in frozen-thawed samples, see Table 9). These alterations were also associated with significant changes in motility parameters. Thus, as shown in Table 9, freezing-thawing induced an increase of curvilinear velocity (VCL; from 46.7 ± 0.3 μm/sec in fresh samples to 82.6 ± 0.8 μm/sec after freezing-thawing), mean velocity (VAP; from 29.0 ± 0.3 μm/sec in fresh samples to 47.5 ± 0.6 μm/sec after freezing-thawing), straightness coefficient (STR; from 63.2 ± 0.2 % in fresh samples to 69.0 ± 0.5 % after freezing-thawing) and dance (DNC; from 114.5 ± 1.9 μm^2/sec in fresh samples to 335.8 ± 4.8 μm^2/sec after freezing-thawing).

We must highlight several points regarding analyzed samples being categorized according to their freezability. First, the impairment of freezability, quantified as the percentage decrease in viability, was accompanied by a concomitant impairment in the

Table 9. Mean values of the semen-quality analysis of fresh and frozen-thawed boar semen.

Parameter	Fresh	Frozen-thawed
Viability (%)	83.9 ± 1.8^a	48.6 ± 1.8^b
Altered acrosomes (%)	10.8 ± 1.9^a	78.1 ± 1.9^b
ORT (%)	67.6 ± 4.9^a	9.4 ± 4.9^b
HRT (arbitrary units)	0.75 ± 0.06^a	0.75 ± 0.06^a
Total motility (%)	64.4 ± 0.1^a	18.5 ± 0.3^b
VCL (μm/sec)	46.7 ± 0.3^a	82.6 ± 0.8^b
VAP (μm/sec)	29.0 ± 0.3^a	47.5 ± 0.6^b
LIN (%)	40.5 ± 0.2^a	41.5 ± 0.5^a
STR (%)	63.2 ± 0.2^a	69.0 ± 0.5^b
WOB (%)	61.2 ± 0.2^a	57.3 ± 0.4^b
DNC (μm^2/sec)	114.5 ± 1.9^a	335.8 ± 4.8^b
absMAD (angular degrees)	114.3 ± 0.4^a	112.8 ± 0.9^a

Semen-quality parameters shown here have been described in the Materials and methods section. Motility parameters have been defined in Table 8. Results are expressed as means ± S.E.M. of 45 different experiments with a total number of analysed sperm of 12,942 (fresh semen) and 9,866 (frozen-thawed semen). Different superscripts between rows indicate significant differences ($P<0.05$).

percentage of altered acrosomes and total motility. Thus, the increase of altered acrosomes in semen of Group I after freezing-thawing was about 370 % (from 12.3 ± 2.3 % in fresh samples to 58.2 ± 1.2 % in frozen-thawed semen), whereas the same increase in semen from Group III was of about 700 % (from 11.3 ± 1.7 % in fresh samples to 90.6 ± 2.5 % in frozen-thawed semen; Table 10). Accordingly, whereas the percentage decrease in total motility was about 38 % in samples of Group I (from 67.9 ± 0.2 % in fresh samples to 42.2 ± 0.5 % in frozen-thawed semen), the same decrease was of about 83 % in semen of Group III (from 61.6 ± 0.3 % in fresh samples

Table 10. Mean values of the semen-quality analysis of the three freezability groups of fresh and frozen-thawed boar semen.

Parameter	Group I		Group II		Group III	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
Viability (%)	83.3 ± 3.3^{a}	56.4 ± 2.6^{b}	88.0 ± 1.2^{a}	48.6 ± 3.0^{c}	80.8 ± 4.0^{a}	25.9 ± 4.5^{d}
Altered acrosomes (%)	12.3 ± 2.3^{a}	58.2 ± 1.2^{b}	7.7 ± 1.2^{a}	65.4 ± 3.2^{b}	11.3 ± 1.7^{a}	90.6 ± 2.5^{c}
ORT (%)	75.5 ± 4.9^{a}	25.1 ± 4.9^{b}	74.8 ± 5.6^{a}	16.9 ± 5.6^{b}	58.9 ± 5.0^{c}	1.9 ± 0.5^{d}
HRT (arbitrary units)	0.8 ± 0.1^{a}	0.8 ± 0.1^{a}	0.8 ± 0.1^{a}	0.9 ± 0.1^{a}	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}
Total motility (%)	67.9 ± 0.2^{a}	42.2 ± 0.5^{b}	66.3 ± 0.2^{a}	31.8 ± 0.6^{c}	61.6 ± 0.3^{a}	10.6 ± 1.4^{d}
VCL (μm/sec)	48.3 ± 0.5^{a}	89.7 ± 1.0^{b}	52.5 ± 0.5^{a}	84.2 ± 1.2^{b}	45.4 ± 0.7^{a}	65.3 ± 2.7^{c}
VAP (μm/sec)	26.6 ± 0.4^{a}	44.1 ± 0.9^{b}	27.8 ± 0.4^{a}	59.2 ± 1.1^{c}	31.4 ± 0.6^{a}	37.8 ± 2.3^{d}
LIN (%)	33.1 ± 0.3^{a}	33.3 ± 0.7^{a}	$36.3 \pm 0.3^{a,b}$	42.2 ± 0.9^{b}	48.0 ± 0.5^{c}	$37.8 \pm 1.9^{a,b}$
STR (%)	59.3 ± 0.3^{a}	64.9 ± 0.7^{b}	63.6 ± 0.3^{b}	73.7 ± 0.8^{c}	68.3 ± 0.4^{c}	$67.5 \pm 1.8^{b,c}$
WOB (%)	64.4 ± 0.3^{a}	59.8 ± 0.6^{b}	70.2 ± 0.3^{c}	$68.0 \pm 0.7^{a,c}$	66.8 ± 0.4^{a}	53.3 ± 1.6^{d}
DNC (μm^2/sec)	127.6 ± 2.9^{a}	397.5 ± 6.5^{b}	146.8 ± 2.9^{a}	331.2 ± 7.8^{c}	110.8 ± 4.1^{a}	220.4 ± 17.0^{d}
absMAD (angular degrees)	127.9 ± 0.6^{a}	124.8 ± 1.3^{a}	97.4 ± 0.6^{b}	95.6 ± 1.5^{b}	103.3 ± 0.8^{b}	$117.6 \pm 3.3^{a,b}$

The parameters shown here have been determined and described as explained in the Materials and methods section and Table 8. Results are expressed as means ± S.E.M. of 45 (15 Group I, 15 Group II, and 15 Group III) different experiments with a total number of analysed sperm of 8,613 (Group I), 8,641 (Group II), and 5,554 (Group III). Different superscript in a row indicates significant differences ($P<0.05$).

to 10.6 ± 1.4 % in frozen-thawed semen; see Table 10). Additionally, the response to the ORT was also different, and fresh semen from Group I already had a significant, better response than that of equivalent semen samples from Group III (Table 10). Regarding motility parameters, there were some slight but significant differences in several motion parameters when good, average, and bad freezability doses were compared. Thus, Groups II and III showed values of linearity coefficient (LIN) (only Group III), STR, and wobble coefficient (WOB) (only Group II) significantly higher than those from fresh semen samples of Group I (Table 10). On the contrary, values of absolute angular mean displacement (absMAD) were significantly lower in both Groups II and III than those in Group I (Table 10). Furthermore, the observed freezing-thawing increases in VCL, VAP, and DNC of samples from Group I were much less intense in Group III, whereas the observed decrease in WOB was also much more intense in Group III than that in Group I (Table 10). Moreover, LIN, which did not significantly change after freezing-thawing in samples from Group I (from 33.1 ± 0.3 % in fresh samples to 33.3 ± 0.7 % in frozen-thawed semen), underwent a significant decrease in semen from Group III (from 48.0 ± 0.5 % in fresh samples to 37.8 ± 1.9 % in frozen-thawed semen; see Table 10), and both STR and absMAD underwent an inversion in their tendency when compared with those of semen from Groups I and Group III (Table 10).

Effects of freezing-thawing on the structures of motile-sperm subpopulations of boar ejaculates as a whole and classified according to their freezability

Our analysis showed the presence of four separate subpopulations, with specific motility characteristics in fresh-boar semen samples. These subpopulations were classified in an ascending order following their values of VCL and VAP. The mean motion characteristics of these subpopulations and their frequency in fresh semen were similar to those previously published by our laboratory (Rivera *et al*, 2005 and 2006; Ramió *et al*, 2008; Flores *et al*, 2008) and, in this way, we shall not go further in-depth in their description here (Fig. 6 and Table 11). Freezing-thawing induced few important changes in the majority of motility parameters evaluated in any of the obtained subpopulations. Despite this, VCL underwent a significant increase after freezing-thawing in all four subpopulations, which ranged from an increase of about 55 % in Subpopulation 1 (from 36.0 ± 0.2 μm/sec in fresh samples to 55.8 ± 0.5 μm/sec after freezing-thawing, see Table 11) to about 32 % of a percentage increase in Subpopulation 4 (from 145.7 ± 1.2 μm/sec in fresh samples to 192.0 ± 3.3 μm/sec after freezing-thawing; see Table 11). The DNC was also significantly modified by freezing-thawing, showing an increase of about 120 % in Subpopulation 1 (from 63.5 ± 0.8 μm^2/sec in fresh samples to 138.5 ± 2.4 μm^2/sec after freezing-thawing) and about 30 % in both Subpopulations 3 and 4 (Table 11). The VAP experienced a similar increase, although this was only significant in Subpopulations 1 and 2 (Table 11). In contrast, freezing-thawing induced great changes in the percentages of motile sperm included in each subpopulation. Thus, a significant decrease in motile sperm from

Subpopulation 1 after freezing-thawing (from 75.8 ± 2.0 % to 54.8 ± 1.4 %) was observed (Fig. 6A). This decrease was concomitant with a parallel, significant increase in spermatozoa included in Subpopulation 2, which went from 17.4 ± 1.0 % in fresh samples to 35.5 ± 1.9 % after freezing-thawing (Fig. 6A).

Table 11. Effects of freezing-thawing on motility parameters of the motile-sperm subpopulations determined in boar samples.

Parameter	Subpopulation 1		Subpopulation 2	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	36.0 ± 0.2[a]	55.8 ± 0.5[b]	72.3 ± 0.3[a]	102.7 ± 0.6[b]
VAP (μm/sec)	21.4 ± 0.2[a]	36.3 ± 0.6[b]	48.4 ± 0.4[a]	59.1 ± 0.7[b]
LIN (%)	39.3 ± 0.2[a]	43.7 ± 0.7[b]	44.9 ± 0.5[a]	41.1 ± 0.8[b]
STR (%)	62.4 ± 0.2[a]	68.4 ± 0.7[b]	66.3 ± 0.4[a]	69.9 ± 0.8[b]
WOB (%)	60.1 ± 0.2[a]	59.8 ± 0.6[a]	65.4 ± 0.4[a]	57.3 ± 0.7[b]
DNC (μm^2/sec)	63.5 ± 0.8[a]	138.5 ± 2.4[b]	215.8 ± 1.5[a]	443.7 ± 2.7[b]
absMAD (angular degrees)	117.0 ± 0.4[a]	107.4 ± 1.3[b]	105.8 ± 0.8[a]	113.8 ± 1.5[b]
	Subpopulation 3		Subpopulation 4	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	107.2 ± 0.5[a]	140.1 ± 1.0[b]	145.7 ± 1.2[a]	192.0 ± 3.3[b]
VAP (μm/sec)	70.6 ± 0.6[a]	69.0 ± 1.2[a]	92.0 ± 1.4[a]	92.6 ± 3.9[a]
LIN (%)	40.1 ± 0.8[a]	34.6 ± 1.4[a]	36.2 ± 1.6[a]	31.8 ± 4.6[a]
STR (%)	61.1 ± 0.7[a]	67.3 ± 1.4[b]	57.5 ± 1.6[a]	63.8 ± 4.5[a]
WOB (%)	64.3 ± 0.6[a]	50.2 ± 1.1[b]	62.5 ± 1.3[a]	47.8 ± 3.7[b]
DNC (μm^2/sec)	467.1 ± 2.5[a]	822.1 ± 4.6[b]	868.8 ± 5.3[a]	1441.6 ± 15.1[b]
absMAD (angular degrees)	103.6 ± 1.3[a]	125.1 ± 2.5[b]	107.2 ± 2.9[a]	122.7 ± 8.1[a]

Motility parameters shown have been determined and described as explained in the Materials and methods section and Table 8. Results are expressed as means ± S.E.M. of 45 different experiments, which implies the total number of 12,942 (fresh semen) and 9,866 (frozen-thawed semen) analysed sperm. Different superscripts between rows in the same subpopulation indicate significant ($P<0.05$) differences.

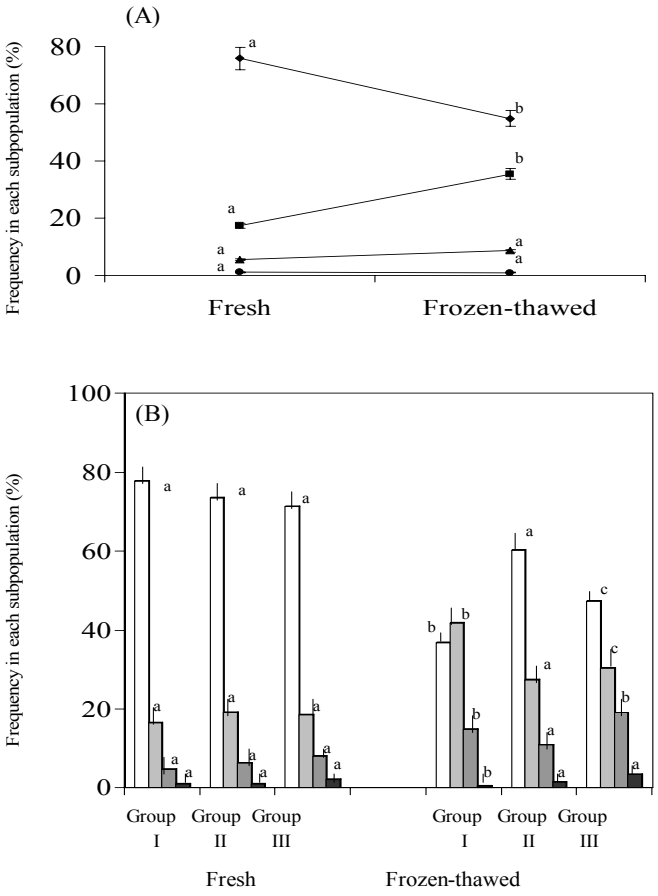

Figure 6. Changes in the proportion of each motile-sperm subpopulation in boar samples of fresh and frozen-thawed semen. (A) Mean frequency of each motile-sperm subpopulation taking all of the performed experiments together. The frequency of motile sperm in each subpopulation has been determined as described in the Materials and methods section. Results are means ± S.E.M. for 45 different experiments. Different superscripts indicate significant ($P<0.05$) differences between fresh and frozen-thawed samples in the same subpopulation. ◆: Subpopulation 1. ■: Subpopulation 2. ▲: Subpopulation 3. ●: Subpopulation 4. (B) Changes in the proportion of each motile-sperm subpopulation in boar samples of fresh and frozen-thawed semen taking into account the resistance to freezing-thawing of each ejaculate. The frequency of motile sperm in each subpopulation has been determined as described in the Materials and methods section. Results are means ± S.E.M. for 45 different experiments. Different superscripts in each subpopulation indicate significant ($P<0.05$) differences between fresh and frozen-thawed samples in the same subpopulation. White bars: Subpopulation 1. Light grey bars: Subpopulation 2. Dark grey bars: Subpopulation 3. Black bars: Subpopulation 4.

Table 12. Effects of freezing-thawing on motility parameters of the motile-sperm subpopulations determined in boars classified as good freezers (Group I).

Parameter	Subpopulation 1		Subpopulation 2	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	37.6 ± 0.2^{a}	50.0 ± 0.8^{b}	72.5 ± 0.5^{a}	94.0 ± 0.7^{b}
VAP (μm/sec)	20.2 ± 0.3^{a}	28.3 ± 1.0^{b}	40.4 ± 0.6^{a}	47.5 ± 0.9^{b}
LIN (%)	32.7 ± 0.4^{a}	34.9 ± 1.2^{a}	35.1 ± 0.8^{a}	34.7 ± 1.1^{a}
STR (%)	58.9 ± 0.4^{a}	61.8 ± 1.2^{a}	61.8 ± 0.8^{a}	67.6 ± 1.1^{b}
WOB (%)	54.0 ± 0.3^{a}	53.6 ± 1.0^{a}	55.2 ± 0.6^{a}	50.2 ± 0.9^{b}
DNC (μm^2/sec)	69.1 ± 1.1^{a}	127.1 ± 3.8^{b}	226.6 ± 2.4^{a}	396.8 ± 3.4^{b}
absMAD (angular degrees)	129.0 ± 0.6^{a}	118.5 ± 2.2^{b}	126.6 ± 1.4^{a}	125.6 ± 1.9^{a}
	Subpopulation 3		Subpopulation 4	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	110.6 ± 0.9^{a}	124.1 ± 1.2^{b}	154.7 ± 2.0^{a}	164.5 ± 1.8^{a}
VAP (μm/sec)	65.5 ± 1.1^{a}	57.2 ± 1.4^{b}	88.8 ± 2.4^{a}	64.7 ± 2.1^{b}
LIN (%)	33.9 ± 1.4^{a}	30.7 ± 1.7^{a}	30.2 ± 2.9^{a}	23.9 ± 2.6^{a}
STR (%)	57.9 ± 1.4^{a}	66.5 ± 1.7^{b}	54.6 ± 2.9^{a}	59.8 ± 2.6^{a}
WOB (%)	58.2 ± 1.1^{a}	45.9 ± 1.4^{b}	56.9 ± 2.4^{a}	39.4 ± 2.1^{b}
DNC (μm^2/sec)	$509.8 \pm 4,3^{a}$	663.4 ± 5.3^{b}	1007.4 ± 9.1^{a}	1005.5 ± 8.1^{a}
absMAD (angular degrees)	116.7 ± 2.5^{a}	131.7 ± 3.0^{b}	118.0 ± 5.1^{a}	132.8 ± 4.6^{a}

Motility parameters shown have been determined and described as explained in the Materials and methods section and Table 8. Results are expressed as means ± S.E.M. of 15 different experiments, which implies the total number of analysed sperm of 5,129 (fresh semen), and 3,484 (frozen-thawed semen). Different superscripts between rows in the same subpopulation indicate significant ($P<0.05$) differences.

The comparison of specific motility parameters and structures of motile-sperm subpopulations among good, average, and poor freezability samples also rendered several significant results. First of all, there were several significant differences when results from fresh samples among the three freezability groups were compared. Thus,

Table 13. Effects of freezing-thawing on motility parameters of the motile-sperm subpopulations determined in boars classified as average freezers (Group II).

Parameter	Subpopulation 1		Subpopulation 2	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	36.2 ± 0.3^{a}	59.3 ± 0.7^{b}	75.8 ± 0.5^{a}	109.7 ± 1.0^{b}
VAP (μm/sec)	26.0 ± 0.3^{a}	45.1 ± 0.9^{b}	55.9 ± 0.5^{a}	76.9 ± 1.3^{b}
LIN (%)	45.6 ± 0.4^{a}	55.3 ± 1.1^{b}	50.2 ± 0.7^{a}	50.9 ± 1.6^{a}
STR (%)	62.9 ± 0.4^{a}	75.4 ± 1.1^{b}	67.4 ± 0.6^{a}	73.2 ± 1.5^{a}
WOB (%)	69.5 ± 0.3^{a}	69.4 ± 0.9^{a}	72.8 ± 0.5^{a}	68.9 ± 1.3^{a}
DNC (μm^2/sec)	63.4 ± 1.2^{a}	138.1 ± 3.4^{b}	235.2 ± 2.1^{a}	472.8 ± 4.8^{b}
absMAD (angular degrees)	99.9 ± 0.7^{a}	92.1 ± 1.9^{a}	91.1 ± 1.2^{a}	96.0 ± 2.7^{a}
	Subpopulation 3		Subpopulation 4	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	109.9 ± 0.7^{a}	141.4 ± 1.9^{b}	143.9 ± 1.7^{a}	187.7 ± 4.4^{b}
VAP (μm/sec)	77.6 ± 0.9^{a}	83.8 ± 2.2^{a}	97.3 ± 2.1^{a}	107.3 ± 5.2^{a}
LIN (%)	42.9 ± 1.1^{a}	38.3 ± 2.7^{a}	38.6 ± 2.5^{a}	42.1 ± 6.5^{a}
STR (%)	60.4 ± 1.1^{a}	64.8 ± 2.7^{a}	56.7 ± 2.5^{a}	71.4 ± 6.3^{a}
WOB (%)	70.0 ± 0.9^{a}	58.5 ± 2.2^{b}	67.6 ± 2.1^{a}	57.7 ± 5.3^{a}
DNC (μm^2/sec)	486.1 ± 3.4^{a}	869.8 ± 8.5^{b}	835.1 ± 7.9^{a}	1590.0 ± 20.1^{b}
absMAD (angular degrees)	92.8 ± 1.9^{a}	114.0 ± 4.8^{b}	97.4 ± 4.5^{a}	105.7 ± 11.4^{a}

Motility parameters shown here have been determined and described as explained in the Materials and methods section and Table 8. Results are expressed as means ± S.E.M. of 15 different experiments, which implies the total number of analysed sperm of 5,226 (fresh semen) and 3,415 (frozen-thawed semen). Different superscripts between rows in the same subpopulation indicate significant ($P<0.05$) differences.

Group II showed a significant increase of LIN (45.6 ± 0.4 % vs. 32.7 ± 0.4 % in Group I) of its Subpopulation 1 when compared with Subpopulation 1 from Group I (Tables 12 and 13). Similarly, LIN and WOB of Subpopulation 3 were also significantly higher in fresh semen from Group II compared with that of Group I (Tables 12 and 13),

whereas DNC from Subpopulation 4 was significantly lower in fresh samples from Group II than that in fresh samples from Group I (835.1 ± 7.9 μm^2/sec vs. 1007.4 ± 9.1 $\mu^2 m$/sec in Group I; see Tables 12 and 13). These differences were even more pronounced when fresh semen samples from Groups I and III were compared (Tables 12 and 14). Moreover, the VCL from Subpopulations 2 and 4 and absMAD from Subpopulations 1 and 2 (Subpopulation 2: 87.4 ± 1.7 º in Group III vs. 126.6 ± 1.4 º in Group I) were also significantly lower in Group III with respect to those of fresh samples from Group I (Tables 12 and 14). On the contrary, LIN from Subpopulations 1, 2 and 3, and WOB from Subpopulations 2 and 3 were significantly higher in fresh samples of Group III compared with those of fresh samples from Group I (Tables 12 and 14).

Regarding resistance to freezing-thawing itself, sperm samples from Group III underwent fewer changes in motility parameters of each specific subpopulation than did those of both Group I and Group II. Thus, whereas VCL, VAP, and DNC of sperm from Subpopulation 1 experienced significant increases after freezing-thawing in Groups I and II, the same parameters of Subpopulation 1 either did not undergo significant variations after freezing-thawing (VCL and DNC) or showed a decrease (VAP) of about 25 % in Group III samples (Table 14). Similarly, the behavior of the other motility parameters in Subpopulation 1 were also very different in Group III samples, and though there were significant decreases of LIN, STR, and WOB in these samples after freezing-thawing, no changes or even significant increases in some of these parameters were observed in samples from Groups I and II (Tables 12-14). A similar pattern of changes can be observed in the other subpopulations, although these differences were especially evident in Subpopulation 4 (Tables 12-14).

Table 14. Effects of freezing-thawing on motility parameters of the motile-sperm subpopulations determined in boars classified as poor freezers (Group III).

Parameter	Subpopulation 1		Subpopulation 2	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	31.2 ± 0.4^{a}	34.1 ± 1.9^{a}	64.7 ± 0.7^{a}	78.5 ± 2.2^{b}
VAP (μm/sec)	20.4 ± 0.4^{a}	15.2 ± 2.2^{a}	48.8 ± 0.8^{a}	52.3 ± 2.6^{a}
LIN (%)	45.9 ± 0.5^{a}	27.1 ± 2.8^{b}	56.5 ± 1.0^{a}	49.5 ± 3.3^{a}
STR (%)	67.6 ± 0.5^{a}	59.3 ± 2.7^{a}	72.9 ± 1.0^{a}	74.2 ± 3.2^{a}
WOB (%)	64.3 ± 0.4^{a}	43.7 ± 2.3^{b}	74.5 ± 0.8^{a}	65.1 ± 2.7^{a}
DNC (μm^2/sec)	49.8 ± 1.6^{a}	66.5 ± 8.6^{a}	160.6 ± 3.1^{a}	237.6 ± 10.2^{b}
absMAD (angular degrees)	109.0 ± 0.9^{a}	122.9 ± 4.9^{a}	87.4 ± 1.7^{a}	112.2 ± 5.8^{b}
	Subpopulation 3		Subpopulation 4	
	Fresh sperm	Frozen-thawed	Fresh sperm	Frozen-thawed
VCL (μm/sec)	97.3 ± 1.0^{a}	104.4 ± 2.9^{a}	129.1 ± 2.0^{a}	122.8 ± 6.2^{a}
VAP (μm/sec)	69.1 ± 1.3^{a}	61.3 ± 3.4^{a}	82.7 ± 2.4^{a}	63.0 ± 7.4^{a}
LIN (%)	47.3 ± 1.6^{a}	43.8 ± 4.2^{a}	38.7 ± 3.0^{a}	36.9 ± 9.1^{a}
STR (%)	65.2 ± 1.5^{a}	74.9 ± 4.1^{a}	59.9 ± 3.0^{a}	70.7 ± 8.9^{a}
WOB (%)	70.1 ± 1.3^{a}	56.7 ± 3.5^{a}	63.3 ± 2.5^{a}	50.9 ± 7.5^{a}
DNC (μm^2/sec)	362.0 ± 4.8^{a}	445.1 ± 13.2^{b}	671.7 ± 9.4^{a}	730.5 ± 28.40^{a}
absMAD (angular degrees)	94.1 ± 2.7^{a}	114.2 ± 7.5^{a}	103.8 ± 5.3^{a}	118.3 ± 16.1^{a}

Motility parameters shown here have been determined and described as explained in the Materials and methods section and Table 8. Results are expressed as means ± S.E.M. of 15 different experiments, which implies the total number of analysed sperm of 2,587 (fresh semen) and 2,967 (frozen-thawed semen). Different superscripts between rows in the same subpopulation indicate significant ($P<0.05$) differences.

Regarding the percentage distribution of motile-sperm subpopulations, freezing-thawing induced a significant decrease in the percentage of spermatozoa included in Subpopulation 1, which went from 77.7 ± 2.5 % in fresh samples to 36.8 ± 1.8 % (Fig. 6B). This decrease was accompanied by concomitant, significant increases in the percentages of spermatozoa of Subpopulations 2 (from 16.5 ± 1.0 % in fresh samples to

41.8 ± 3.2 % after freezing-thawing), 3 (from 4.7 ± 0.7 % in fresh samples to 14.9 ± 1.2 % after thawing) and 4 (from 1.1 ± 0.1 % in fresh samples to 6.5 ± 0.8 % after thawing; see Fig. 6B). Samples from Group II did not show any significant change in the structures of their motile-sperm subpopulations after freezing-thawing, whereas those from Group III followed a similar pattern to that of Group I samples, although the observed changes were much less intense and even non-significant (Fig. 6B).

Changes in both MitoTracker Red and proxylfluorescamine staining intensity of midpiece after freezing-thawing

Freezing-thawing induced a significant decrease in the mean values of MitoTracker Red and proxylfluorescamine staining intensity of sperm midpieces. Thus, MitoTracker midpiece staining intensity decreased from 100.0 ± 2.4 (arbitrary units) to 71.4 ± 1.9 (arbitrary units) after freezing-thawing (Table 15 and Fig. 7). Similarly, proxylfluorescamine staining intensity decreased from 100.0 ± 1.9 (arbitrary units) to 60.3 ± 1.7 (arbitrary units) after freezing-thawing. Regarding the resistance to freezing-thawing, samples from Group I did not show any significant change in MitoTracker Red stain intensity after freezing-thawing, whereas proxylfluorescamine stain intensity only

Table 15. Mean values of midpiece staining intensity with both MitoTracker Red and proxylfluorescamine.

	Fresh	Frozen-thawed
MitoTracker Red stain (arbitrary units)	100.0 ± 2.4^{a}	71.4 ± 1.9^{b}
Proxylfluorescamine (arbitrary units)	100.0 ± 1.9^{a}	60.3 ± 1.7^{b}

Results are means ± S.E.M. of 11 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences.

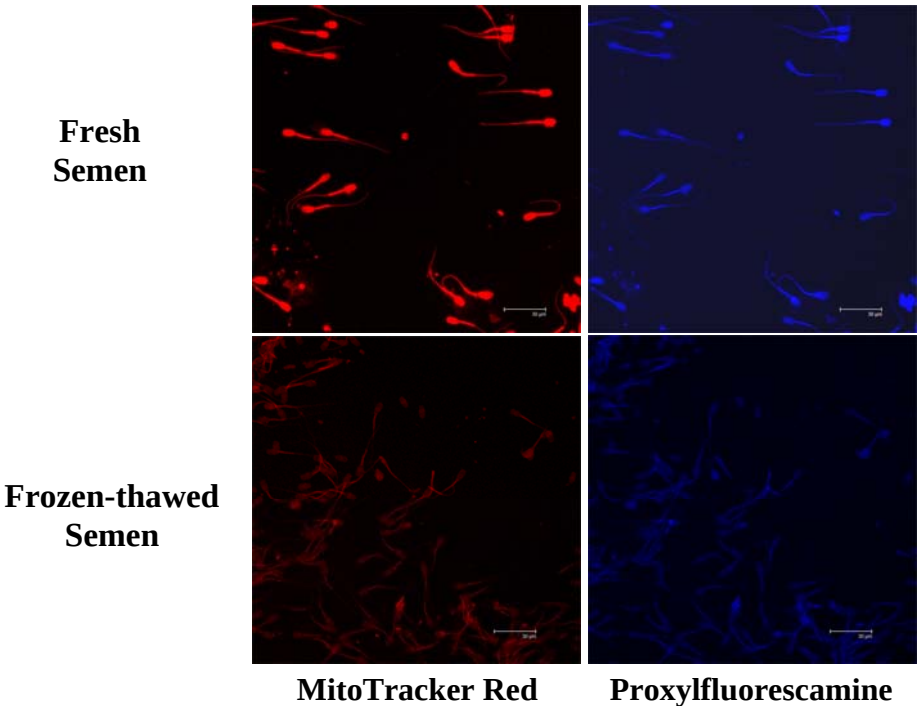

Figure 7. Changes in the stain intensity for both MitoTracker Red and proxylfluorescamine of boar spermatozoa subjected to freezing-thawing. Images show the observed stain for MitoTracker Red and proxylfluorescamine in spermatozoa from an ejaculate with poor resistance to freezing-thawing (Group III sample; see Material and methods section). Both MitoTracker Red and proxylfluorescamine stains, as well as the system used to quantify the intensity of the signal at the midpiece, have been described in the Materials and methods section. Images are representative for 11 different experiments. Bars indicate the actual magnification of the images.

decreased from 100.0 ± 1.8 (arbitrary units) to 83.3 ± 3.7 (arbitrary units). On the contrary, sperm from Group III showed an intense decrease of midpiece staining with both MitoTracker Red and proxylfluorescamine (Table 16). Sperm samples of Group II showed a decrease of midpiece stain intensity of both MitoTracker Red and proxylfluorescamine that was between those observed in Group I and Group III (Table 16 and Fig. 7). There were no significant differences in the signal intensity of both MitoTracker Red and proxylfluorescamine among fresh samples from Groups I, II and III (data not shown).

Table 16. Values of midpiece staining intensity with both MitoTracker Red and proxylfluorescamine in sperm samples classified in accordance with their resistance to freezing-thawing.

MitoTracker Red stain (arbitrary units)		
	Fresh	Frozen-Thawed
Group I	100.0 ± 3.1^{a}	98.2 ± 3.2^{a}
Group II	100.0 ± 3.0^{a}	84.0 ± 2.1^{b}
Group III	100.0 ± 2.5^{a}	48.4 ± 2.1^{c}
Proxylfluorescamine stain (arbitrary units)		
	Fresh	Frozen-Thawed
Group I	100.0 ± 1.8^{a}	83.3 ± 3.7^{b}
Group II	100.0 ± 3.7^{a}	65.1 ± 2.4^{c}
Group III	100.0 ± 2.5^{a}	40.7 ± 1.3^{d}

Results are means ± S.E.M. of 11 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences between fresh and frozen-thawed samples from the same experimental group, whereas different superscripts in a column indicate significant ($P<0.05$) differences between frozen-thawed samples of different experimental groups.

Discussion

The results presented in this article indicate that resistance to freezing-thawing in boar sperm induces specific changes in both the structures of motile-sperm subpopulations and the motion characteristics of each subpopulation. Furthermore, the existence of small but significant differences in several motility parameters among samples with different resistance to freezing-thawing, when these parameters are analyzed through the structures of subpopulations, strongly suggest that freezability can be related to a specific, initial motile sperm subpopulation structure, thus opening the possibility to use this type of analysis to greatly improve the standard boar-semen quality analysis.

Regarding specific changes in structures of subpopulations related to resistance to freezing-thawing, it is noteworthy that there were not great differences among semen

samples with separate resistance when comparing their changes in the specific percentage of motile sperm that were included in each subpopulation after freezing-thawing. This is remarkable as, in a previous study, our laboratory showed that the changes in overall motility that were linked to freezing-thawing in boar semen were mostly induced by changes in the percentage of motile spermatozoa included in each subpopulation rather than by specific changes in motility parameters of these subpopulations (Flores *et al*, 2008). This apparent contradiction could be explained by the fact that the results obtained separately in each freezability group were masked when motility data were analyzed without considering the resistance to freezing-thawing as a differentiating factor. In this way, when analyses were carried out on samples without considering their freezability, the differences related to this aspect were dismissed in the final results. Taking this into consideration, the interpretation of our results can be made into a more understandable form.

The results showed here indicate that samples with the worst resistance to freezing-thawing were also those that showed fewer changes in motility parameters in subpopulations, and there was not even any change in motility parameters of Subpopulation 4 after freezing-thawing. This suggests that the samples with a stronger resistance to freezing-thawing display resistance mechanisms that involve changes in the motility properties of spermatozoa, in accordance with the freezing-thawing-associated stress mechanisms. On the contrary, samples with poor resistance included sperm that were not sufficiently able to engage resistance mechanisms that involve these adaptive changes in sperm motility, depending on the ascription of a sperm to a specific subpopulation. In this sense, a relationship between resistance to freezing-thawing and the ability to adapt to osmotic stress has already been described (Caiza de la Cueva *et al*, 1997). This is logical, as osmotic stress is one of the most important factors that

explain cell death during freezing-thawing (Holt *et al*, 2005). Regarding this point, it has been described that the Na^+/K^+, ouabain-dependent ATPase activity is an important osmotic resistance mechanism of boar sperm (Caiza de la Cueva *et al*, 1997). Remarkably, the Na^+/K^+, ouabain-dependent ATPase activity is also instrumental in the maintenance of the proper functioning of flagellar mechanisms in eucaryotic cells (Lingrel & Kuntzweiler, 1994). In this manner, changes in the activity of this ATPase would be associated with concomitant changes in the flagellar movements of sperm subjected to osmotic stress, thus modifying their motility patterns. On the other hand, those sperm that lack an active and functional Na^+/K^+ ATPase do not show these ATPase-linked motility changes when subjected to osmotic stress.

Another interesting point of the results shown is the existence of slight, but significant differences in fresh samples in accordance with further resistance to freezing-thawing. These slight differences were, basically, decreases in the results of the ORT and the mean values of both STR and absMAD, as well as changes in the subpopulational structure, in samples with less resistance. The importance of subtle differences already observed in fresh samples is evident, both from a practical and a functional point of view. Thus, under a practical view, the existence of subtle but detectable differences in some parameters of fresh samples that can be detected can be used to greatly improve semen-quality analysis when studying freezability. In this way, our results also confirm others already published, such as the importance of the ORT as a functional test with some predictive value in both resistance to storage (Schilling & Vengust, 1985) and *in vivo* fertility (Quintero-Moreno *et al*, 2004). Moreover, there is not a clear relationship between the specific parameters of motility, either when considering the ejaculate as a whole or when analyzing motility as a subpopulational structure and the overall functional characteristics of boar sperm (Gadea *et al*, 1998;

Quintero-Moreno *et al*, 2004). Hence, the rigorous analysis of the subtle differences obtained in the structures of motile-sperm subpopulations, combined with the results of other sperm function parameters such as ORT, could introduce some predictive value in boar-semen analysis when ability to resist cryopreservation is concerned.

Regarding our results of mitochondrial activity, it is evident that the resistance to freezing-thawing of boar sperm is closely related to its ability to maintain a minimal level of mitochondrial function. This would be explained through a freezing-induced increase in ROS generation during cryopreservation, as suggested by different authors (Hinshaw *et al*, 1986; Aitken *et al*, 1989; Alvarez & Storey, 1992; De Lamirande & Gagnon, 1992a; O'Flaherty *et al*, 1997; Lopes *et al*, 1998; Mazur *et al*, 2000; Chatterjee & Gagnon, 2001; Silva & Gadella, 2006). However, we must remember that proxylfluorescamine stain analyzes the ROS formed in mitochondria as hydroxyl radicals, but not the total amount of ROS accumulated by the cell (Wenzel *et al*, 2005). In this way, our results indicate that freezing-thawing would decrease the *de novo* formation of mitochondrial ROS, as a result of the already described decrease in the overall mitochondrial function (Cummins *et al*, 1994). Thus, the most likely explanation of our results is that the overall increase of ROS linked to freezing-thawing would be associated with a decrease in the activity of mitochondrial-linked mechanisms involving the elimination and destruction of ROS more than in an increase in the mitochondrial mechanisms producing hydroxyl radicals.

In conclusion, our results indicate that the resistance to cryopreservation of boar sperm seems to be related to specific changes in both the structures of motile-sperm subpopulations and the motion characteristics of each subpopulation. Furthermore, the existence of small but significant differences in the overall structures of motile-sperm subpopulations strongly suggests that freezability can be related to a specific, initial

structure of a motile-sperm subpopulations. This structure would be closely related to the specific, overall functional levels of the whole ejaculate, of which the mitochondrial activity would be a very important indicator. Finally, a more in-depth analysis of the subtle differences in structures of subpopulations together with functional tests could aid in the design of predictive strategies for the freezability of boar sperm.

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CAPÍTOL III / CHAPTER III

Cryopreservation-induced alterations in boar spermatozoa mitochondrial function are related to changes in the expression and location of midpiece mitofusin-2 and actin network

Abstract

Here we analyzed changes in mitochondrial activity of boar semen during a standard cryopreservation protocol. For this purpose, mitochondrial activity was evaluated simultaneously with the rhythm of mitochondrial formation of reactive oxygen species (mROS) through a double MitoTracker Red/proxylfluorescamine stain. Moreover, we analyzed changes in the expression and location of two key regulatory elements of mitochondrial function, namely mitofusin-2 (Mfn-2) and actin, during the freezing-thawing protocol. Our results indicate that mitochondrial activity and mROS formation decreased during cryopreservation, with an initial decrease during the cooling phase of the protocol. This decrease was accompanied by an increase in the amount of solubilised Mfn-2, which was concomitant to a progressive extension of Mfn-2 location from de apical zone of the midpiece to the whole midpiece. Simultaneously, cryopreservation induced a decrease in solubilized actin, which was concurrent with significant changes in the midpiece actin location. The observed changes in the expression and location of both Mfn-2 and actin were already present after the cooling

phase of the cryopreservation protocol. Our results suggest that freezing-thawing impaired mitochondrial function. This impairment was concomitant with a decrease in the mitochondrial capacity to synthesize mROS. This impairment is attributed to changes of mitochondrial volume as a result of alterations in the expression and location of both Mfn-2 and actin network. Finally, the alterations of mitochondrial function induced by the cryopreservation protocol were already apparent at the cooling phase. This observation indicates that the cooling phase is a crucial stage in which mitochondrial alterations occur during cryopreservation.

Introduction

The cryopreservation of boar semen offers considerable advantages in the swine production system. However, its use is currently restricted as a result of lower fertility rates and higher economical costs than artificial insemination (AI) with refrigerated semen (Breininger *et al*, 2005; Cremades *et al*, 2005). In addition, porcine sperm are very sensitive to low temperatures and the freezing-thawing process causes significant reductions in farrowing rates and litter size compared with AI with refrigerated semen (Corcuera *et al*, 2007; Bailey *et al*, 2008). One of the most important reasons for this reduction is that cryopreservation causes considerable damage to the plasma membrane, the cytoskeleton, the motion system and sperm nuclei. Consequently, the damage to boar sperm caused by cryopreservation includes motility impairment, nuclear alterations, significant membrane alterations, decreased mitochondrial membrane potential and oxidative damage with reactive oxygen species (ROS) generation (Correa *et al*, 2007; García-Herreros *et al*, 2007). In this regard, in previous work we

demonstrated that freezing-thawing induced a clear decrease in mitochondrial activity of boar sperm, which was accompanied by a concomitant decrease in the rhythm of mitochondria-synthesised ROS (Flores *et al*, 2009). However, little is known about the mechanism/s underlying these alterations.

Growing evidence indicates that mitochondrial morphology is instrumental in the modulation of mitochondrial function. Thus, changes in mitochondrial shape have been related to apoptotic cell death, development, neurodegeneration, calcium signaling, cell division, and ROS production (de Brito & Scorrano, 2008). The mitochondrion is a highly dynamic organelle and its morphology is heterogeneous, ranging from small spheres to interconnected tubules. This morphologic heterogeneity is due to frequent and continual cycles of mitochondrial fusion and fission, opposing processes that act in equilibrium to maintain the architecture of these organelles (Chen *et al*, 2003; de Brito & Scorrano, 2008). Mitofusin-2 (Mfn-2) is an outer mitochondrial membrane protein that participates in mitochondrial fusion (de Brito & Scorrano, 2008). Previous studies revealed that Mfn-2 is crucial for the maintenance of the morphology and operation of the mitochondrial network and in mitochondrial metabolism (Bach *et al*, 2003), thus being one of the key modulators in the shape-induced changes of mitochondrial function.

However, other external proteins and structures have a profound influence on mitochondrial reshaping. One of the most relevant of these structures is the actin cytoskeleton that surrounds mitochondria, which constituted in somatic cells the mitochondrial network in which Mfn-2 is localised (Bach *et al*, 2003). Furthermore, midpiece mitochondria are associated with an equivalent actin cytoskeleton, which is in fact one of the most important elements of the midpiece (Clarke *et al*, 1982; Holt &

North, 1991; de las Heras *et al*, 1997). The actin cytoskeleton is organized in a dynamic intracellular network and plays a crucial role in regulating cell shape, migration and cellular interaction with extracellular matrices (Liu *et al*, 2005; Correa *et al*, 2007). The actin network undergoes reorganization in response to osmotic stress in all organisms and it has been proposed that plasma membrane defects are caused by a loss of structure of the actin cytoskeleton (Correa *et al*, 2007). In mammalian spermatozoa, actin contributes to sperm motility (Azamar *et al*, 2007), capacitation and the acrosome reaction (Breitbart *et al*, 2005; Liu *et al*, 2005). However, little is known about the relationship between the actin network and the modulation of sperm mitochondrial function.

Given the data described above, here we tested whether the alterations in boar sperm mitochondrial function caused by freezing-thawing are linked to concomitant changes in the expression and location of two key mitochondria regulatory proteins, Mfn-2 and midpiece actin. For this purpose, first we evaluated the mitochondrial activity of ejaculates at the following stages: before freezing, after the cooling phase of the freezing procedure, and after freezing-thawing. We assessed the mitochondrial activity of the samples using two techniques. The first involved the evaluation of changes in the stain intensity of a specific marker of mitochondrial activity, the MitoTracker Red CM-H_2Xros while the second evaluated mitochondrial formation of ROS (mROS) by quantifying changes in the stain intensity of a specific marker, proxylfluorescamine. We then applied Western blot and immunocytochemistry to analyzed Mfn-2 and actin in samples of fresh, cooled to 5ºC and frozen-thawed boar semen. Our results indicate that sperm express Mfn-2. This is the first report of this expression. Moreover, the freezing-thawing process caused significant alterations to

both Mfn-2 and midpiece actin. This observation indicates that these mitochondrial alterations are, at least partially, related to concomitant changes in the shape-modulated control mechanisms of mitochondria function.

Materials and Methods

Animals and samples collection

Fifty-five healthy boars ranging between 2 and 3 years of age from a commercial farm and one ejaculate per boar were used. The boars were from 3 separate lines (Landrace, Large White and Pietrain). AI using extended liquid semen from these boars demonstrated their fertility. The sperm-rich fraction of each ejaculate used in this study was collected manually twice weekly using the gloved-hand method and analyzed to ensure the quality and homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was suspended (1:2; v/v) in a commercial extender (MR-A). The extended semen samples were cooled and maintained at 17 ºC for shipment to the laboratory of the Autonomous University of Barcelona within 24 h-post-collection, where they were processed and analyzed.

Semen cryopreservation

Immediately after receiving the semen samples, we took an aliquot to determine the appropriate semen quality parameters, as well as those regarding mitochondrial activity studies (fresh semen sample). Only those samples displaying a minimum of

70 % progressive motility, and 80 % of morphologically normal spermatozoa were further processed by adapting a proven protocol (Sancho *et al*, 2007). The extended semen was centrifuged at 600 *g* for 10 minutes at 17 ºC in a programmable refrigerated centrifuge. After centrifugation, the supernatant was discarded. The remaining pellets were re-extended with a lactose-egg yolk (LEY) extender (80 % [v/v] 310 mM β-lactose, 20 % egg yolk), at a ratio that led to a final concentration of 1.5 x 10^9 spermatozoa/mL. The sperm concentration was assessed manually in a Thoma or Neubauer haemocytometer. At this point, and after thorough mixing, the semen was further cooled to 5 ºC for 2 h in the centrifuge. An aliquot of this semen was then taken to examine the appropriate semen quality parameters, as well as those regarding mitochondrial activity (refrigerated or 5 ºC semen sample). Semen was then slowly mixed with a third extender consisting of 89.5 mL LEY extender, 9 mL glycerol and 1.5 mL of Equex STM, which is equivalent to Orvus Es Paste (Graham *et al*, 1971) at a ratio of two parts semen to one part extender, yielding a final concentration of 3 % (v/v) glycerol and a concentration of 1 x 10^9 spermatozoa/mL at 5 ºC. This concentrations was verified by counting in a Thoma or Neubauer haemocytometer. Spermatozoa were packaged in 0.5-mL polyvinyl chloride (PVC) plastic straws, which were sealed with PVC powder, at 5 ºC in a cool cabinet. These straws were then placed on racks for freezing (Saravia *et al*, 2005). The racks were transferred to the chamber of a programmable freezer set at 5 ºC. The cooling/freezing rate used was as follows: 6 ºC/min from 5 ºC to -5 ºC, 40 ºC/min form -5 ºC to -80 ºC, 30 sec for crystallization, and thereafter 60 ºC/min from -80 ºC to -150 ºC. The samples were then plunged into liquid N_2 (-196 ºC) for storage for at least 21 days. After this time, samples were placed in a water bath at 37 ºC for 20 sec to cause thawing. Afterwards, straws were carefully

wiped and opened, and samples were immediately analyzed to measure the semen quality parameters, and parameters regarding mitochondrial activity (frozen-thawed semen sample).

Analysis of semen quality parameters

Percentages of viability, altered acrosomes and morphological abnormalities were determined using the Eosin-Nigrosin stain (Bamba, 1988). This technique shows viable spermatozoa as those with a uniform whitish colour in the entire cell while the presence of a partial or a totally pinkish stain is indicative of non-viable sperm cells. Moreover, acrosome integrity was evaluated by observing the presence of a regular and intact acrosomal ridge after the Eosin-Nigrosin stain. Acrosomal ridges that did not have a regular and intact appearance were considered altered acrosomes. The percentages of viability and altered acrosomes were established after analyzing a minimum of 200 spermatozoa/sample through optical microscopy (magnification: 1000×).

The computer-assisted analysis of sperm motility (CASA) was carried out by using a commercial system. In this system, samples were previously warmed at 37 ºC for 5 min in a water bath, and 5-µL aliquots were then placed on a warmed (37 ºC) slide and covered with a 22-mm^2 coverslip. Our CASA analysis examined 25 consecutive, digitized photographic images obtained from a single field at a magnification of 200× on a dark field. These photographs were taken in a time lapse of 1 sec, which implied a velocity of image-capturing of one photograph every 40 msec. Two to three separate

fields were taken for each sample. The sperm motility descriptors follow Quintero-Moreno *et al* (2003). The CASA analyses provide the following motility descriptors:

Curvilinear velocity (VCL): the mean path velocity of the sperm head along its trajectory (units: μm/sec).

Linear velocity (VSL): the mean path velocity of the sperm head along a straight line from its first to its last position (units: μm/sec).

Mean velocity (VAP): the mean velocity of the sperm head along its average trajectory (units: μm/sec).

Linearity coefficient (LIN): (VSL/VCL)x100 (units: %).

Straightness coefficient (STR): (VSL/VAP)x100 (units: %).

Wobble coefficient (WOB): (VAP/VCL)x100 (units: %).

Mean amplitude of lateral head displacement (ALH): the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: μm).

Frequency of head displacement (BCF): the frequency with which the sperm trajectory crosses the average path trajectory (units: Hz).

Dance (DANCE): VCL x ALH (units: μm^2/sec).

Mean dance (MeanDANCE): ALH/LIN (units: μm).

Absolute angular mean displacement (absMAD): the absolute value of the advancing angle of the sperm trajectory (units: angular degrees).

Algebraic angular mean displacement (algMAD): the algebraic value of the advancing angle of the sperm trajectory. Negative values indicate a clockwise displacement (units: angular degrees).

Finally, total motility was defined as the percentage of spermatozoa that showed a VAP above 10 μm/sec.

Determination of mitochondrial activity and mitochondrial-associated ROS formation

The overall mitochondrial activity and mROS levels were simultaneously evaluated by MitoTracker Red/proxylfluorescamine double staining (Wenzel *et al*, 2005), with the modifications as described in Flores *et al* (2009). For this purpose, samples were incubated with a solution of proxylfluorescamine in phosphate-buffered saline (PBS; pH 7.4) at a final concentration of 50 μM for 10 min at 37 ºC. Immediately afterwards, samples were centrifuged at 600 *g* for 10 min at 20 ºC and the resultant pellet was resuspended in 1 mL of a solution of 500 nM MitoTracker Red CM-H_2Xros in PBS. Sperm cells were incubated in this medium for another 10 min at 37 ºC. Cell suspensions were then again centrifuged at 600 *g* for 10 min at 20 ºC and the cellular pellet was resuspended in 50 μL of PBS. This final suspension was spread onto 76 mm x 26 mm slides, which were left to dry. Once dry, the slides were covered with a paraformaldehyde solution (2 %; v/v) in PBS for 10 min at 20 ºC. Fixed samples were thoroughly rinsed with distilled water and were then allowed to dry at room temperature. Until this point, all of the steps of this procedure were carried out in a dark chamber in order to prevent unspecific photostimulation of fluorochromes. Samples were finally stored at 4 ºC in the dark until their evaluation, which was performed within 3 days after treatment. Fluorescence was analyzed through a Leica TCS 4D confocal laser microscope, which was adapted to a Leitz DMIRBE inverted microscope

with a Nomarsky interferential contrast objective and a Leitz-Plan-Apo 63X lens. The energy of the argon/krypton laser source was of 74 mW, and serial images with a depth of 0.5-1 μm/image were taken. In this process, the Nomarsky interferential contrast was used as a precise control of the location of the cells analyzed. Fluorescence detection was performed using an excitation wavelength of 543 nm and an emission length of 440-480 nm (mROS) and 590-650 nm (MitoTracker Red). Finally, special care was taken to maintain the same conditions of background and intensity in all cases.

We used the Adobe Photoshop® image-analyzing program to examine the signal intensity for MitoTracker Red and mROS on the digitalized images. For this purpose, the midpieces of at least 200 sperm per point were selected using the appropriate tool of the program and the intensity of brightness in these selected midpieces was quantified individually using the command "Histogram". This quantification was performed only after careful homogenization of brightness and contrast among all of the images analyzed. The mean values of brightness were then adjusted to a mean, arbitrary value of 100 ± S.E.M. for control fresh sample points. The other points were compared taking this control value as reference. Differences between treatments were analyzed after applying the GLM and LSMEANS procedures included in the SAS statistical package.

Immunological techniques for Mfn-2 and actin detection

Western blot analyses and immunocytochemical detection of boar sperm Mfn-2 and actin were performed using the same commercial anti-goat Mfn-2 and anti-rabbit total actin antibodies. To perform Western blot analyses, boar spermatozoa were homogenized by sonication in ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 1 %

(w/v) SDS, 15 mM EDTA, 150 mM KF, 0.6 M sacarose, 14 mM β-mercaptoethanol, 10 μg/mL leupeptin, 1 mM benzamidine and 1 mM phenylmethyl sulfonyl flouride (PMSF). The homogenates were then centrifuged at 10,000 *g* for 15 min at 4 ºC and the resultant pellets were discarded. For the detection of actin, homogenates were mixed in a proportion 1:10 with acetone and kept on ice for 30 min. Finally the precipitates were centrifuged at 10,000 *g* for 15 min at 4 ºC and the supernatants were discarded. The resultant pellets were dissolved in distilled water. Western blot was then performed only on soluble sperm fractions of homogenates. This analysis was based on SDS gel electrophoresis (Laemli, 1970), followed by transfer to nitrocellulose (Burnette, 1981). To carry out the SDS gel electrophoresis, 10 μg of total protein per sample for Mfn-2 and 20 μg for actin were loaded in each lane. Total protein content of samples was determined by the Bradford method (Bradford, 1976), after applying a commercial kit (BioRad). The transferred samples were tested with the anti-Mfn-2 antibody at a dilution (v/v) of 1:500 and anti-actin antibody at a dilution (v/v) of 1:20,000. Immunoreactivity was tested using peroxidase-conjugated donkey anti-goat secondary antibody for Mfn-2 and goat anti-rabbit secondary antibody for actin and the reaction was developed with an ECL-Plus detection system. Moreover, the specificity of the immunoreactivity was tested after subjecting several experiments to Western blot in the presence of the anti-Mfn-2 antibody previously pre-adsorbed with a commercial, specific peptide to a final concentration of 4 μg/mL. In the Western blot for actin, we ran a positive control of actin from rabbit muscle.

Immunocytochemistry was performed with spermatozoa seeded onto gelatin-coated slides (76 mm × 26 mm). For this purpose, sperm samples were washed three times with PBS and fixed with 1 mL of a 4 % (w/v) paraformaldehyde solution in PBS

for 15 min at 25 ºC. Fixed samples were centrifuged at 600 *g* for 3 min, and the supernatants were discarded. The cellular pellet was resuspended in 500 µL of PBS. This step was followed by permeabilization with 0.2 % (v/v) Triton X-100 in PBS for 30 min and a blocking step with 1 % (w/v) bovine serum albumin (BSA) for 30 min. The slides were then incubated overnight at 4 ºC with the appropriated antibody, anti-mitofusin-2 (dilution 1/200; v/v) and anti-actin (dilution 1/100; v/v). After the incubation, the samples were washed with PBS and treated with Alexa 488-conjugated donkey anti-goat or Alexa 647-conjugated goat anti-rabbit secondary antibody. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope adapted to an inverted Leitz DMIRBE microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo Lens. The light source was an argon/krypton laser (75 mW). Confocal images were stored as TIFF-format images. These images were simultaneously observed and stored under visible light in a phase-contrast system. The combination of visible light and laser images allowed the exact location of the positive reactions in sperm-head sections, thus permitting a better analysis of the data obtained.

Statistical analysis of semen quality parameters

Data were processed using the SAS statistical package (SAS/STAC Software, 2000). The normality of data distributions was assessed by the Shapiro-Wilks Test, which is included in the UNIVARIATE procedure. Putative differences in the semen quality parameters among the phases of the freeze-thaw protocol were determined by applying the GLM procedure, and the LSMEANS procedure was used to compare the results obtained. Differences among data were considered as significant from $P<0.05$.

Results

Effects of cryopreservation on the mean semen quality parameters

The cooling phase of the cryopreservation protocol induced several significant changes in most of the semen quality parameters of boar spermatozoa tested. Cooling induced a significant ($P<0.05$) decrease in the percentage of viability (83.9 ± 1.8 % in fresh semen vs. 77.2 ± 1.8 % after the cooling phase to 5 ºC, means ± S.E.M.) and a concomitant significant ($P<0.05$) increase in the percentage of altered acrosomes (Table 17). For the motility descriptors, cooling to 5 ºC caused a significant ($P<0.05$) decrease in total motility (64.5 ± 0.1 % in fresh semen vs. 37.3 ± 0.1 % in cooled semen), STR and absMAD (see Table 17). A significant ($P<0.05$) increase in VCL (46.7 ± 0.3 μm/sec in fresh semen vs. 95.5 ± 0.4 μm/sec in cooled semen), VSL, VAP (29.0 ± 0.3 μm/sec in fresh semen vs. 71.8 ± 0.4 μm/sec in cooled semen), LIN, WOB, ALH, BCF, DANCE (114.5 ± 1.9 μm^2/sec in fresh semen vs. 363.8 ± 2.6 μm^2/sec in cooled semen) and Mean DANCE was also observed (see Table 17).

Cryopreservation induced a subsequent significant ($P<0.05$) decrease in the percentage of viability, and a concomitant significant ($P<0.05$) increase in the percentage of altered acrosomes (34.9 ± 1.9 % in cooled semen vs. 71.4 ± 2.3 % in frozen-thawed semen; see Table 17). Percentages of total motility and WOB also underwent a significant ($P<0.05$) decrease after thawing (Table 17). Furthermore, thawed sperm showed a significant ($P<0.05$) increase in STR, ALH and Mean DANCE (see Table 17). Thawed sperm also showed a significant ($P<0.05$) increase in VCL, VSL, VAP and DANCE when compared to fresh samples (Table 17).

Table 17. Mean values of the semen quality analysis of fresh, cooled to 5 ºC and frozen-thawed boar semen.

	Fresh	5 ºC	Frozen-thawed
Viability (%)	83.9 ± 1.8^{a}	77.2 ± 1.8^{b}	48.6 ± 1.8^{c}
Altered acrosomes (%)	10.8 ± 1.9^{a}	34.9 ± 1.9^{b}	71.4 ± 2.3^{c}
Total motility (%)	64.5 ± 0.1^{a}	37.3 ± 0.1^{b}	18.6 ± 0.3^{c}
VCL (μm/sec)	46.7 ± 0.3^{a}	95.5 ± 0.4^{b}	82.6 ± 0.8^{c}
VSL (μm/sec)	19.4 ± 0.2^{a}	44.0 ± 0.3^{b}	34.5 ± 0.5^{c}
VAP (μm/sec)	29.0 ± 0.3^{a}	71.8 ± 0.4^{b}	47.5 ± 0.7^{c}
LIN (%)	40.5 ± 0.2^{a}	45.0 ± 0.3^{b}	41.5 ± 0.5^{a}
STR (%)	63.2 ± 0.2^{a}	62.4 ± 0.3^{b}	69.0 ± 0.5^{c}
WOB (%)	61.2 ± 0.2^{a}	70.5 ± 0.2^{b}	57.3 ± 0.4^{c}
ALH (μm)	2.1 ± 0.01^{a}	3.3 ± 0.01^{b}	3.5 ± 0.03^{c}
BCF (Hz)	6.41 ± 0.03^{a}	6.89 ± 0.04^{b}	6.80 ± 0.08^{b}
DANCE (μm^2/sec)	114.5 ± 1.9^{a}	363.8 ± 2.6^{b}	335.8 ± 4.8^{c}
Mean DANCE (μm)	0.08 ± 0.001^{a}	0.11 ± 0.001^{b}	0.12 ± 0.002^{c}
absMAD (angular degrees)	114.3 ± 0.4^{a}	92.9 ± 0.5^{b}	112.8 ± 0.9^{a}
algMAD (angular degrees)	0.03 ± 0.08^{a}	-0.01 ± 0.11^{a}	-0.21 ± 0.21^{a}

The parameters showed here have been determined as explained in the Materials and methods section. Results are expressed as means ± S.E.M. of 55 different experiments with a total number of analysed spermatozoa of 12,942 (fresh semen), 6,705 (semen cooled to 5 ºC) and 1,866 (frozen-thawed semen). Different superscript between rows indicates significant ($P<0.05$) differences.

Changes in both MitoTracker and proxylfluorescamine staining intensity of the midpiece after cryopreservation

The cooling phase of the cryopreservation procedure induced a significant ($P<0.05$) decrease in the mean values of MitoTracker Red and proxylfluorescamine staining intensity of sperm midpieces. Thus, the intensity of MitoTracker in the

midpiece decreased from 100.0 ± 1.5 (arbitrary units) to 70.6 ± 1.0 (arbitrary units) after the cooling phase (Table 18). Similarly, the intensity of proxylfluorescamine staining decreased from 100.0 ± 0.7 (arbitrary units) to 77.6 ± 0.9 (arbitrary units) after the cooling phase. However, the completion of the freezing-thawing protocol did not induce any further significant change in the mean values of mitochondrial MitoTracker Red, although there was a further, significant decrease in mROS values, which reached 63.7 ± 1.0 (arbitrary units) after thawing (Table 18).

Table 18. Mean relative values of both mitochondrial activity and mitochondria-associated ROS of fresh, cooled to 5 ºC and frozen-thawed boar semen.

	Fresh	5 ºC	Frozen-thawed
Mitocondrial activity (arbitrary units)	100.0 ± 1.5^{a}	70.6 ± 1.0^{b}	72.7 ± 1.2^{b}
mROS (arbitrary units)	100.0 ± 0.7^{a}	77.6 ± 0.9^{b}	63.7 ± 1.0^{c}

The parameters showed here have been determined as explained in the Materials and methods section. Results are expressed as means ± S.E.M. of 7 different experiments. Different superscript between rows indicates significant ($P<0.05$) differences.

Effects of cryopreservation on the expression and location of Mfn-2

Western blot analysis of Mfn-2 in fresh boar semen showed a two band pattern of about 85 kDa and 70 kDa (Fig. 8A). This pattern was similar to those reported in other cell types (Rojo *et al*, 2002). The cooling phase to 5 ºC of the cryopreservation process induced an increase of the intensity of these two bands, and subsequent freezing-thawing induced an additional increase in intensity (Fig. 8A).

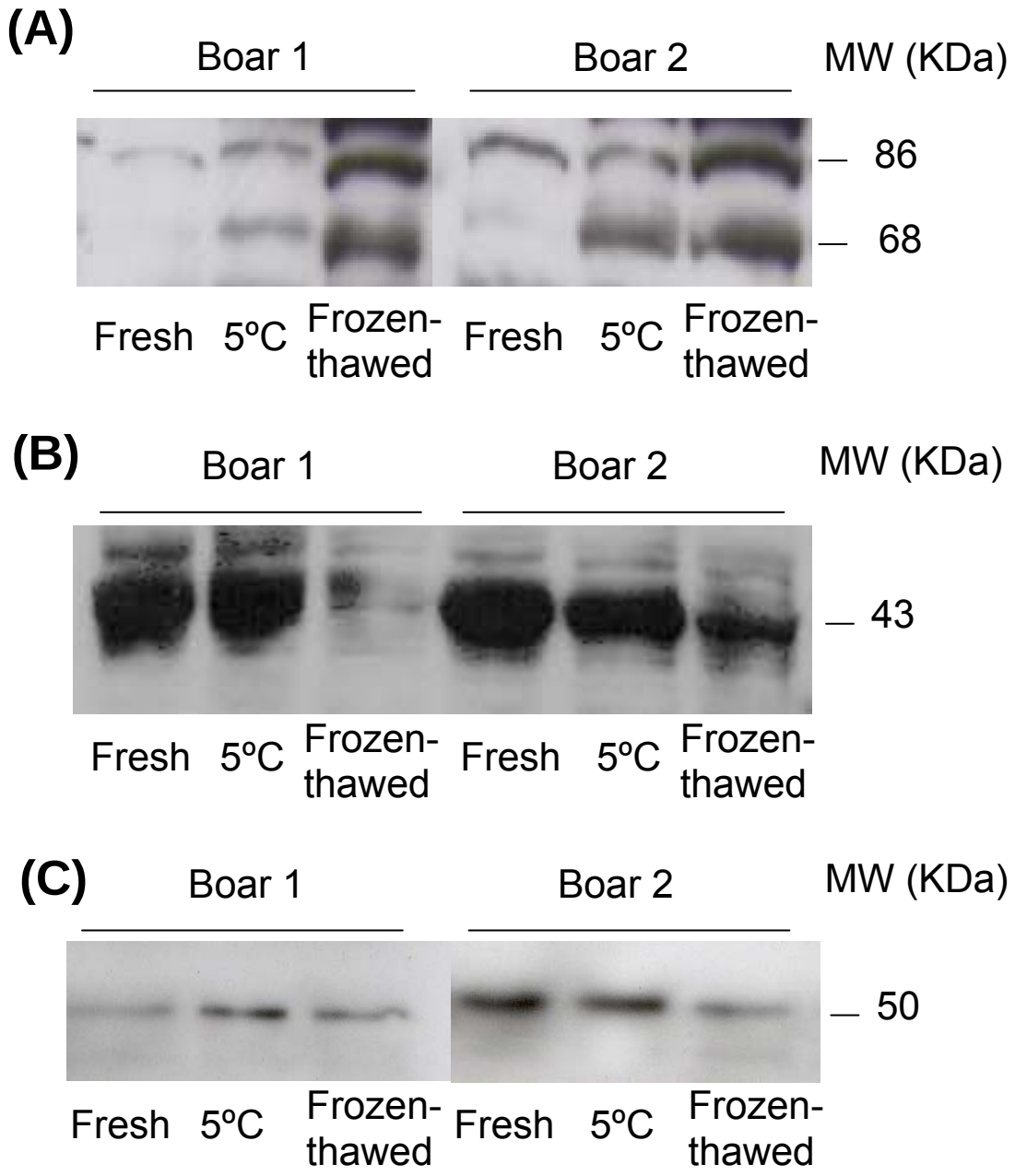

Fig. 8. Western blot analysis of mitofusin-2 (A) and actin (B). Supernatants of sperm homogenates were processed as described in Materials and methods section. The Figure shows the pattern of fresh, cooled to 5 ºC (5ºC), and frozen-thawed boar semen of two representative experiments (Boar 1 and Boar 2) from 8 replicates. (C) Concurrent Western blot of tubulin from the same boar semen samples analyzed in A and B, in order to compare the total protein content of each sample.

Immunocytochemistry of Mfn-2 in fresh sperm showed a specific expression at the midpiece. This expression was more intense at the apical area (Figs. 9A and 9E). The cooling phase of the cryopreservation induced an extension of the Mfn-2 signal to the rest of the midpiece (Figs. 9B and 9F). Finally, thawed spermatozoa showed an intense Mfn-2 signal that occupied the whole midpiece area (Figs. 9C and 9G).

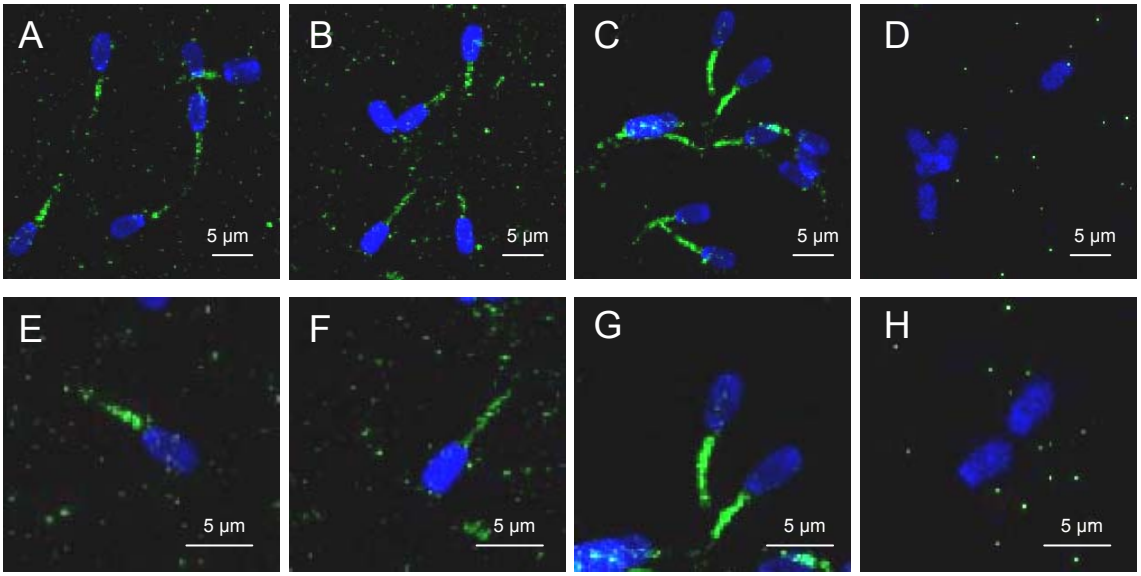

Fig. 9. Immunocytochemistry of mitofusin-2 in boar spermatozoa. The immunolocalization was performed as described in Materials and methods section. (A, E) A representative image of fresh sperm. (B, F) Sperm after the cooling phase to 5 ºC of the cryopreservation protocol. (C, G) Frozen-thawed sperm. (D, H) Negative control from frozen-thawed spermatozoa. Nuclei were counterstained with DAPI (blue). They are representative images from eight independent determinations.

Effects of cryopreservation on the expression and location of actin

Western blot analysis of total actin in fresh semen showed a band of 43 kDa, which was similar to that previously described (Colas *et al*, 2009; see Fig. 8B). There

was a clear decrease in the intensity of the signal after the cooling phase. This signal was subsequently decreased in thawed sperm (Fig. 8B).

Immunocytochemistry showed the presence of actin in the post-acrosomal region and the caudal zone of the midpiece in fresh semen, as well as irregulary distributed actin along the whole tail (Figs. 10A and 10E). The cooling phase increased the intensity of post-acrosomal signal, whereas the tail immunoreactivity reached the whole midpiece (Figs. 10B and 10F). Finally, thawed spermatozoa showed heterogeneity in the staining patterns, with a break up of the immunoreactivity in the post-acrosomal region (Figs. 10C and 10G). In contrast, the midpiece signal was faint and sometimes distributed throughout the whole area (Figs. 10C and 10G).

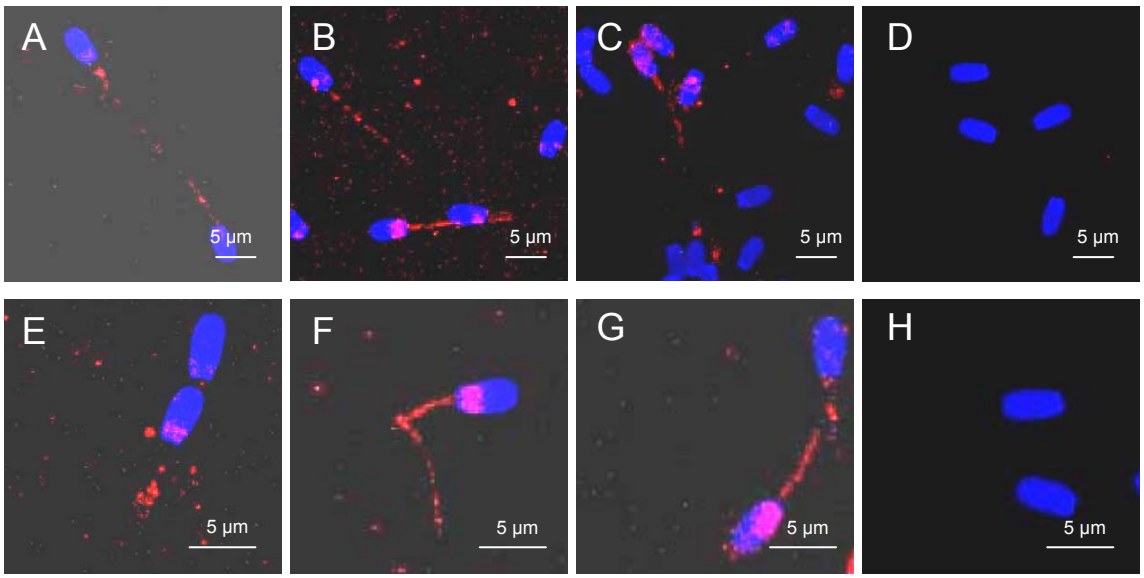

Fig. 10. Immunocytochemistry of actin in boar spermatozoa. The immunolocalization was performed as described in Materials and methods section. (A, E) A representative image of fresh sperm. (B, F) Sperm after the cooling phase to 5 ºC of the cryopreservation protocol. (C, G) Frozen-thawed sperm. (D, H) Negative control from frozen-thawed spermatozoa. Nuclei were counterstained with DAPI (blue). They are representative images from eight independent determinations.

Discussion

Our results indicate that cryopreservation caused alteration in mitochondrial function, which, in turn, are associated to changes in the expression and location of several mitochondria-controlling mechanisms, such as Mfn-2 and the midpiece actin network. The impairment of mitochondria function by cryopreservation has been previously described (Flores *et al*, 2009). Furthermore, our results reinforce others previously published studies that indicate that cryopreservation reduces the *de novo* formation of mitochondrial ROS, as a result of the decrease in the overall mitochondrial function (Cummins *et al*, 1994; Flores *et al*, 2009). Thus, we attribute our results to the association of the overall increase of ROS caused by freezing-thawing with a decrease in the activity of mitochondrial-linked mechanisms involving the elimination and destruction of ROS more than to an increase in the mitochondrial mechanisms that produce hydroxyl radicals. Furthermore, it is noteworthy that this impairment of mitochondrial function is already apparent after the cooling phase of the cryopreservation procedure. The impairing effect of cooling on boar sperm function has been previously described. Thus, cold shock causes plasma membrane disruption because the lipid bilayer becomes unstable at cool temperatures (Holt, 2000; Watson, 2000; Silva & Gadella, 2006). Furthermore, cytoskeletal elements are temperature-sensitive, and in other cell types cooling results in premature depolymerisation of actin filaments (Hall *et al*, 1993; Saunders & Parks, 1999; Watson, 2000). Thus, we propose that the impaired mitochondrial function caused by cooling is one of the mechanisms that modulate boar sperm resistance to the whole cryopreservation procedure.

To our knowledge, no previous studies have addressed the expression of Mfn-2 in spermatozoa. Mfn-2 is a dynamin family GTPase that is anchored to the mitochondrial outer membrane through the C-terminal membrane-binding domain, extruding the N-terminal GTPase domain to the cytoplasm (Ishihara *et al*, 2004). Mfn-2 is involved in the regulation of many mitochondria-modulated functions such as oxidative phosphorylation (Bach *et al*, 2003; Pich *et al*, 2005) and intracellular signaling (Chen *et al*, 2004). Moreover, Mfn-2 is also involved in embryonic development (Chen *et al*, 2003). These observations imply that changes in the expression and location of Mfn-2 are related to concomitant alteration in mitochondria function. In this regard, the changes observed in Mfn-2 expression and midpiece location could be due to a major accessibility of Mfn-2 to the antibody as a result of a structural alteration of the mitochondrial membrane during the whole freezing-thawing protocol. In this respect, it should be noted that sperm are highly differentiated and compartmentalized cells, in which mitochondria are tightly packed at the midpiece. Changes in the shape and volume of midpiece mitochondria directly induce concomitant changes in other aspects of sperm function (Woolley, 1970; Otani *et al*, 1988; Sadava, 1993). Similarly, Cardullo and Blatz (1991) observed a relationship between changes in tail beat frequency and concomitant variations of mitochondrial volume. In addition, boar spermatozoa lack a phosphorylcreatinine-creatinine kinase shuttle system to transport energy-rich phosphate from mitochondria to distal dynein ATPases (Kamp *et al*, 2003) and the changes in the expression and location of Mfn-2 induced by cooling and freezing-thawing would alter the energy transmission mechanisms to the entire tail in order to modulate flagellum contractibility in this area. This alteration in the tail-receiving energy would be instrumental in the well-known changes of sperm motility patterns

associated with cryopreservation (Watson, 2000; Cremades *et al*, 2005), thus explaining the presence and relevance of sperm Mfn-2 in the maintenance of the overall boar sperm function after cryopreservation.

Regarding the changes in actin expression and location, we must stress that, to our knowledge, this is the first study to address the effect of the cryopreservation on the actin cytoskeleton of boar spermatozoa. Our results indicate that the freezing-thawing protocol induced an increase in the actin polymerization, thereby making actin much more insoluble. Consequently, the polymerized actin was not present in the supernatants used for Western blot analyses. This result is consistent with previous studies on the effect of cooling and cryopreservation on the actin network in ram and macaque sperm (Holt & North, 1991; de las Heras *et al*, 1997; Correa *et al*, 2007). However, opposite effects have been reported in other species (Watson, 1995 and 2000). These findings suggest a species-specific effect, in which the alterations of midpiece actin network caused by cooling and the freezing-thawing protocol are modulated by the species-specific native actin ultrastructure. Regarding species in which an increase in actin polymeration has been described in response to cryopreservation, Correa *et al* (2007) proposed that hyperosmotic-induced reorganization of actin filaments in macaque sperm contributes to the sublethal cell damage that occurs during cryopreservation. This reorganization may be the basis for the capacitation-like changes observed in frozen-thawed sperm. Furthermore, changes in actin polymerization are closely related to concomitant changes in the surrounding protein environment (Saxena *et al*, 1986; Breitbart *et al*, 2005). This suggests that our results are linked to concomitant variations in midpiece proteins which control mitochondria activity, modulating in this way cooling- and freezing-thawing-induced variation of mitochondrial function.

Interestingly, Petrunkina *et al* (2004) demonstrated that the midpiece actin network is instrumental in the control of boar sperm volume. This is relevant, since in most cell types cell shrinkage is associated with an increase and cell swelling with a decrease in F-actin content (Pedersen *et al*, 2001). Cryopreservation induces clear changes in the overall sperm volume (Watson, 1995 and 2000; Gilmore *et al*, 1995; Holt, 2000; Curry *et al*, 2000; Petrunkina *et al*, 2005). We must remind that changes in the midpiece mitochondria form and volume directly induce concomitant changes in other aspects of sperm function (Woolley, 1970; Otani *et al*, 1988; Sadava, 1993). However, although these changes are not specific, they are strongly related to the changes observed in the expression and location of two of the key regulators of mitochondrial shape and volume, namely actin and Mfn-2.

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DISCUSSIÓ GENERAL / GENERAL DISCUSSION

Discussió general / General discussion

Aquesta tesi pretén aprofundir en el coneixement dels mecanismes íntims lligats a les alteracions produïdes pel procés de congelació-descongelació en l'espermatozoide porcí. Aquest aprofundiment, així com una millor comprensió de les fases del procés de congelació-descongelació més perjudicials per a l'espermatozoide porcí, poden ser d'utilitat per a obtenir un major rendiment en els resultats obtinguts rere la congelació espermàtica porcina.

Es ben conegut que el procés de congelació-descongelació és perjudicial per a l'espermatozoide porcí (Watson, 2000) i que aquest procés provoca danys a diferents estructures i orgànuls espermàtics (Guthrie & Welch, 2005). Incidint en aquest aspecte, els resultats d'aquest treball demostren que el procés de criopreservació també provoca canvis estructurals tant en el nucli com en els mitocondris espermàtics porcins i que aquests canvis estructurals es tradueixen en canvis en aspectes fonamentals de la funcionalitat espermàtica com ara alteracions en l'estructura subpoblacional mòtil dels espermatozoides i en l'activitat mitocondrial.

La implicació del procés de congelació-descongelació en la fragmentació de l'ADN en l'espermatozoide porcí és un tema molt debatut, ja que mentre alguns autors afirmen que la criopreservació causa la fragmentació de l'ADN (Fraser & Strezeżek, 2005), d'altres indiquen el contrari (Evenson et al, 1994; Hernández et al, 2006). En el nostre estudi s'ha observat que el procés de congelació-descongelació realitzat en les nostres condicions si bé no provoca una fragmentació evident de l'ADN sí que s'associa a una alteració en l'estructura protamina-1−ADN. Aquesta alteració es detecta ja durant

la fase de refrigeració a 5 ºC fent d'aquesta fase una de les més perjudicials en el procés de congelació-descongelació, tal i com ja van apuntar altres autors (Holt, 2000; Watson, 2000; Silva & Gadella, 2006). Desconeixem quina és la base d'aquesta alteració estructural, però sembla que la seva importància pot ser gran, donat que provocarà l'aparició d'espermatozoides que si bé mantindran la seva capacitat de penetració de l'oòcit, presentaran una estructura nuclear que donarà lloc a estructures aberrants durant el procés de singàmia. Aquestes possibles estructures aberrants podrien ser a la base de posteriors problemes en el repartiment cromosòmic en les primeres divisions cel·lulars rere la fecundació, originant per tant embrions no viables. El problema és encara més greu si tenim en compte que aquesta alteració de l'estructura protamina-1−ADN no és detectable mitjançant les tècniques habituals incorporades en l'anàlisi seminal. A nivell pràctic, aquest fet implicaria que una part de la caiguda de fertilitat associada a la congelació-descongelació en semen porcí no pot determinar-se mitjançant les tècniques clàssiques d'anàlisi, explicant-ne així, al menys de manera parcial, el conegut fenomen de caiguda de fertilitat no associada amb canvis importants en els paràmetres habituals de qualitat seminal.

D'altra banda, estudis anteriors del nostre laboratori ja havien observat que el procés de congelació-descongelació provocava canvis en l'estructura subpoblacional mòtil (Flores et al, 2008). En aquest estudi però es va pretendre fer un pas més enllà i observar si aquests canvis estaven relacionats amb la capacitat de congelació de cada ejaculat. Així, tenint en compte la congelabilitat de l'ejaculat, es van observar canvis tant en les estructures de les subpoblacions mòtils com en les característiques de motilitat de cada subpoblació. Aquests canvis suggereixen que la congelabilitat pot estar relacionada a un estat inicial específic de l'estructura subpoblacional mòtil espermàtica,

i aquesta estructura estaria relacionada amb els nivells de funcionalitat específica de l'ejaculat, on l'activitat mitocondrial seria un indicador important. Des d'un punt de vista pràctic, aquests resultats obren la porta al disseny de noves proves analítiques, basades en l'estudi de l'estructura subpoblacional de l'ejaculat, que permetin millorar la capacitat predictiva pel que fa referència a la congelabilitat del semen porcí dins de l'anàlisi de qualitat seminal.

La disminució de l'activitat mitocondrial deguda al procés de congelació-descongelació ja havia estat descrita amb anterioritat (Cummins et al, 1994). Aquest estudi ha permès reforçar aquest fet. Ara bé, és important remarcar que aquesta caiguda de l'activitat mitocondrial està associada amb una disminució en la formació *de novo* de ROS d'origen mitocondrial. Aquest aspecte és important, donat que diversos autors han mantingut que un dels principals problemes associats amb la congelació-descongelació és l'increment en els nivells de ROS, que, en principi, sempre s'han associat a un increment en el ritme de la seva síntesi (Alvarez & Storey, 1992; De Lamirande & Gagnon 1992; O'Flaherty et al, 1997; Mazur et al, 2000; Chatterjee & Gagnon, 2001). Per contra, els nostres resultats semblen indicar que l'increment en els nivells totals de ROS durant la congelació s'associen no a un increment en la seva síntesi, sinó més aviat a una disminució en el seu ritme de destrucció, donat que el principal orgànul productor de ROS, el mitocondri, disminueix notablement la seva capacitat de síntesi. Aquest resultat obre, per tant, una nova via en la comprensió dels mecanismes involucrats en l'aparició de dany oxidatiu en espermatozoides sotmesos a congelació-descongelació.

Un altre aspecte important d'aquest estudi ha sigut la demostració de que els canvis observats en la funció mitocondrial estan associats a alteracions en la expressió i localització de determinats mecanismes de control mitocondrial com són la Mfn-2 i

l'actina de la peça mitocondrial. Aquests canvis funcionals i estructurals en els mitocondris ja es comencen a detectar a la fase de refrigeració a 5 ºC, fet que reforça la idea que aquesta fase és una de les més perjudicials en el procés de criopreservació (Holt, 2000; Watson, 2000; Silva & Gadella, 2006). D'altra banda, aquests canvis també indiquen que l'alteració en la funció mitocondrial associada a la congelació-descongelació no es produeix únicament per efectes mecànics, sinó també per canvis en elements reguladors de la funció, introduint així un nou factor d'estudi en la comprensió de les alteracions mitocondrials lligades a aquest procés.

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CONCLUSIONS GENERALS / GENERAL CONCLUSIONS

Conclusions generals / General conclusions

The results of this PhD thesis let conclude that:

1. Standard freezing-thawing protocol induces a significant alteration of the protamine-1–DNA boar sperm-head structure without a significant increase in DNA fragmentation.

2. Resistance to freezing-thawing in boar sperm induces specific changes in both the structures of motile-sperm subpopulations and the motion characteristics of each subpopulation.

3. The existence of small but significant differences in the overall structures of motile-sperm subpopulations strongly suggests that freezability can be related to a specific, initial status of the precise motile-sperm subpopulations structure in an ejaculate.

4. This initial status of the presice motile-sperm subpopulations structure in an ejaculate would be closely related to the specific, overall functional levels of the whole ejaculate, of which the mitochondrial activity would be a very important indicator.

5. The cryopreservation process caused a decreased in mitochondrial activity and in the formation of mitochondrial reactive oxygen species (mROS), and this decrease is already apparent in the cooling phase of the cryopreservation process.

6. The freezing-thawing process caused changes in mitochondrial function, which, in turn, are associated to alterations in the expression and location of several mitochondria-controlling mechanisms, such as Mfn-2 and the midpiece actin network.

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