Universitat Autònoma de Barcelona

Dinàmica de la motilitat, la fosforil·lació de proteïnes i l'activitat mitocondrial de l'espermatozoide porcí durant la capacitació i reacció acrosòmica "in vitro".

Dynamics of motility, protein phosphorylation and mitochondrial activity in boar spermatozoa during "in vitro" capacitation and further acrosome reaction.

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CERTIFICA

Que la tesi titulada "**Dinàmica de la motilitat, la fosforil·lació i activitat mitocondrial de l'espermatozoide porcí durant la capacitació i reacció acrosòmica "in vitro"** presentada per la Laura Ramió i Lluch per optar al grau de Doctor en Veterinària s'ha realitzat sota la meva direcció i, considerant aquesta acabada, autoritzo la seva presentació per què sigui jutjada per la comissió corresponent.

I per tal que així consti als efectes que correspongui, firmo la present a Bellaterra (Cerdanyola del Vallès), el 18 de Juny del 2009.

Joan Enric Rodríguez Gil

A la meva família

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I. REVISIÓ BIBLIOGRÀFICA/ BIBLIOGRAPHY REVIEW

1. L'espermatozoide

L'espermatozoide és una cèl·lula haploide produïda en els testicles i altament especialitzada, amb una funció molt determinada: el transport del material genètic. Aquest objectiu el portarà a travessar el tracte genital femení fins a arribar a l'oòcit i fecundar-lo. Per aquesta raó, el procés de formació de l'espermatozoide (l'espermatogènesi) no només requereix d'una divisió meiòtica per tal de reduir el seu contingut genòmic a la meitat (espermacitogènesi), sinó que també farà falta un procés de maduració citoplasmàtica que li configurarà les característiques anatòmiques ideals per dur a terme la seva funció (espermiogènesi). L'espermatozoide és, doncs, una cèl·lula que requereix de processos de generació molt especialitzats que comportaran una elevada diferenciació cel·lular.

2. Parts de l'espermatozoide

L'espermatozoide de *Sus scrofa domesticus* és un gàmeta de 45 µm de longitud en què es distingeixen tres parts: el cap, la peça de connexió o coll i la cua (Figura 1A).

Al cap de l'espermatozoïde hi podem distingir entre d'altres estructures l'acrosoma i el nucli. L'acrosoma és una gran vesícula secretora derivada de l'aparell de Golgi, engloba la part anterior del cap (Figura 2 A i B) i està cobert per la membrana citoplasmàtica. El segment apical de l'acrosoma és la zona més dilatada de la vesícula acrosòmica i hi podem distingir la membrana acrosòmica interna i la externa. Si bé la membrana acrosòmica interna es disposa per sobre de l'embolcall nuclear, la membrana acrosòmica externa se situa directament per sota de la membrana plasmàtica (Bonet *et al.*, 2000). La matriu de l'acrosoma conté enzims hidrolítics que permetran la digestió de les membranes que rodegen l'oòcit facilitant així la fecundació. Des del punt de vista funcional l'acrosina i la hialuronidasa són els dos enzims hidrolítics més importants (Eddy, 1988). Amb aquest objectiu, el procés inicial de la unió

espermatozoide-òvul, l'acrosoma patirà un procés de fusió entre la membrana acrosomal externa i la membrana citoplasmàtica que comportarà l'alliberament d'aquests enzims hidrolítics a l'espai extracel·lular que participaran en la despolimerització del *cummulus oophorus* i de la zona pel·lúcida (ZP) de l'oòcit. Aquest procés de fusió de membranes s'anomena reacció acrosòmica.

El nucli de l'espermatozoide conté una cromatina altament estabilitzada. Alhora, és únic tant en la quantitat de ADN com en la composició de les nucleoproteïnes. Les dues divisions meiòtiques que transcorren durant l'espermatogènesi donen lloc a un genoma haploide, amb una sola còpia de cada parell de cromosomes. De la mateixa manera, els espermatozoides ténen com a nota característica el fet que el seu material genètic està altament condensat i lligat a unes nucleoproteïnes pròpies de l'espermatozoide, com són les protamines, tot i que també hi podem trobar en menys proporció les nucleoproteïnes comunes de la resta de cèl·lules somàtiques, les histones.

La peça de connexió o coll és la regió que uneix el cap amb la cua. En aquesta peça hi trobem el centríol, a la base del qual parteixen les estructures que conformaran l'axonema a partir del que s'organitza la cua o flagel. També hi ha els cossos laminars, que procedeixen d'evaginacions molt pronunciades de l'embolcall nuclear que contenen un espai nuclear lliure de cromatina (Bonet *et al.*, 2000).

La cua o flagel és una part de l'espermatozoide funcionalment destacada. En ella hi resideixen tant la capacitat mòbil pròpia i característica d'aquesta cèl·lula com la maquinaria encarregada de la obtenció d'energia. Al llarg del flagell s'hi perllonga l'axonema que presenta la fórmula microtubular de 9+2, amb 9 microtúbuls en disposició perifèrica al centre dels quals hi trobem els 2 restants. La cua està formada per tres peces: la intermèdia o mitocondrial, la principal i la terminal (Bonet *et al.*, 2000).

La peça intermèdia de la cua de l'espermatozoide s'estén des de la part distal de la peça de connexió fins a l'anell de Jensen. La característica pròpia d'aquesta part resideix en que és el lloc a on s'hi troba la beina mitocondrial,

que en un espermatozoide de mamífer aproximadament conté uns 100 mitocondris (Hallap *et al.*, 2005). A la part central de la peça intermèdia hi trobem l'axonema.

La peça principal és la part més llarga del flagel i s'estén des de l'anell de Jensen fins a la peça terminal. Es caracteritza per una beina fibrosa que envolta l'axonema i que alhora està rodejada per una estructura citoesquelètica que confereixen la rigidesa requerida pel moviment espermàtic. També s'ha descobert altres papers funcionals com en són l'ancoratge de proteïnes implicades en la transmissió de senyals i el de generació d'energia, ja que s'ha descrit com a lloc propi de localització d'enzims característics de la glucòlisi (Vadnais *et al.*, 2007).

La peça terminal és l'últim i més curt segment de la cua de l'espermatozoide. En aquesta part, l'axonema està cobert directament per la membrana plasmàtica i es va desorganitzant progressivament fins al seu final.

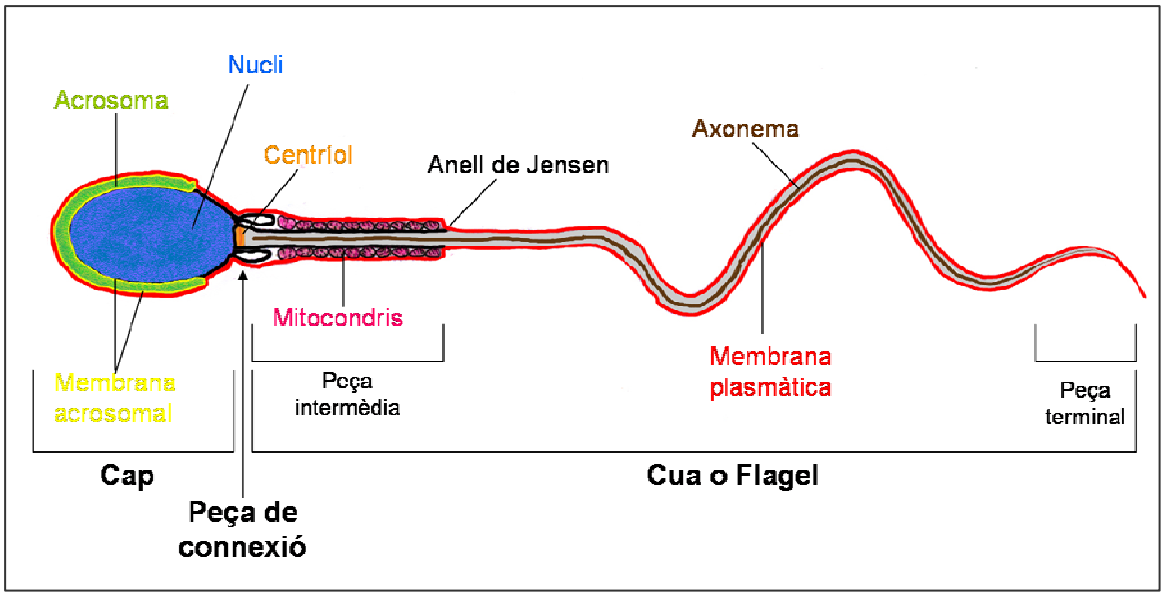

Figura 1A: Parts de l'espermatozoide porcí.

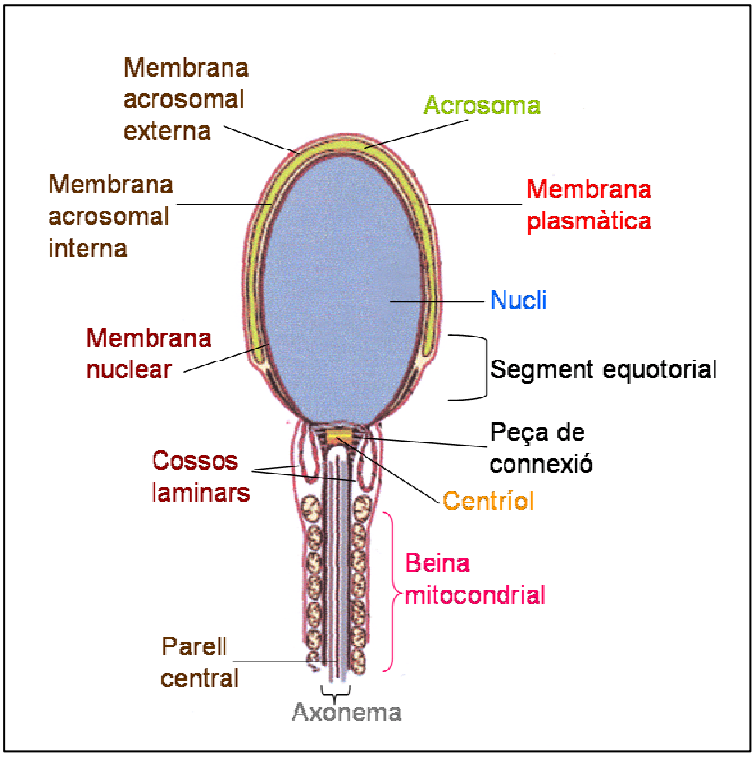

Figura 1B: Parts cranials de l'espermatozoide porcí.

3. L'Espermatogènesi:

Tal com s'ha esmentat prèviament, l'espermatogènesi o procés de formació de l'espermatozoide, engloba dues fases d'acord amb els canvis que es produeixen a nivell nuclear i els canvis a nivell citoplasmàtic anomenades espermatocitogènesi i espermiogèniesis respectivament.

L'espermatogènesi és el procés mitjançant el qual les cèl·lules germinals o espermatogònies (diploides) pateixen dues divisions meiòtiques que comportaran la formació de les espermàtides (haploides). Les espermàtides requeriran d'un procés de maduració citoplasmàtica que comportarà la condensació nuclear, la pèrdua de gran part del citoplasma i la formació de les parts diferenciades de l'espermatozoide a partir dels orgànuls cel·lulars (Figura 2). Aquest procés s'anomena espermiogènesi. En el porcí, el procés de l'espermatogènesi dura uns 39 dies (França *et al.*, 2005).

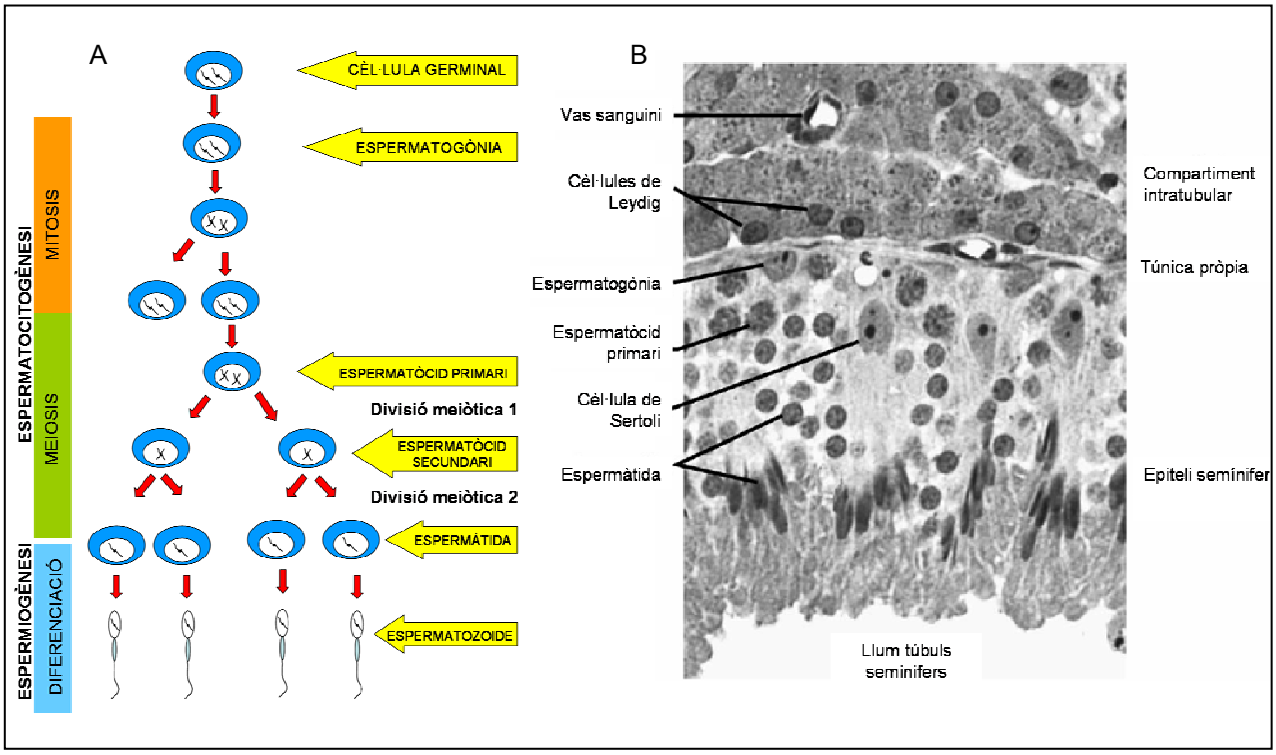

Figura 2: Espermatogènesi. Figura B: modificada de França et al., (2005).

El procés de diferenciació cel·lular que comporta la producció d'espermatozoides té lloc als túbuls seminífers del testicle. Tal com ja s'ha esmentat anteriorment, durant l'espermiogènesi, les espermàtides pateixen una sèrie de canvis morfològics que les portaran a adquirir les característiques morfològiques pròpies de l'espermatozoide. Amb aquesta finalitat, es dóna una notable reducció del citoplasma i el nucli s'allarga formant el cap de l'espermatozoide. Alhora, es forma l'acrosoma a partir de l'Aparell de Golgi, els centríols originen el flagel i les mitocondries s'ajunten i es disposen helicoïdalment, englobant la part més anterior de l'axonema. Així doncs, un cop alliberats a la llum de l'epidídim, l'espermatozoide és una cèl·lula altament especialitzada i amb una exquisida compartimentalització subcel·lular i funcional. L'espermatozoide ha sofert gran part dels canvis morfològics però li resta encara diferents processos que el portaran a adquirir la capacitat fecundant. Aquests processos tenen lloc tant a l'aparell reproductor masculí (maduració epididimària) com al femení (capacitació) i culminaran amb la reacció acrosòmica.

4. Maduració espermàtica epididimària

La maduració epididimària, té lloc durant el trànsit al llarg de tot l'epidídim i li conferirà bàsicament la capacitat mòbil, a més de canvis a la membrana plasmàtica. El trànsit normal d'un espermatozoide porcí a través dels 50-100 metres de l'epidídim dura uns 10 dies (França *et al.*, 2005) tot i que varia en funció de la freqüència d'ejaculació. Morfològicament l'epidídim està dividit en tres parts: cap, cos i cua. Els espermatozoides es van formant en el teixit testicular en cicles ondulars, maduren al llarg del cap, cos i part proximal de la cua de l'epidídim i resten dins de la part més distal de la cua l'epididimària. El trànsit epididimari és degut a la simple pressió de fluid i per contraccions de la musculatura llisa de l'epidídim i transporta els espermatozoides fins al conducte deferent. Així doncs, a l'epidídim els espermatozoides s'acumulen durant unes dues setmanes. Això dóna lloc a una població espermàtica molt heterogènia dins d'un mateix ejaculat, on hi podem trobar espermatozoides que tot just surten de la cos de l'epidídim que es barregen amb d'altres acumulats a la cua epididimària des de fa dies.

El trànsit a través de l'epidídim comporta diferents modificacions. En primer lloc es dóna una major condensació de la cromatina nuclear, resultant de la formació de nous ponts disulfur (Bedford i Hoskins, 1990). Alhora, hi ha un augment del potencial de producció d'ATP que motivarà l'activació de diferents vies metabòliques lligades a la motilitat del flagel (Bedford i Hoskins, 1990).

L'espermatozoïde immadur presenta una característica molt típica, respecte al madur: la presència de la gota citoplasmàtica. La gota citoplasmàtica és un residu del citoplasma eliminat en les darreres etapes de l'espermiogènesi. Els espermatozoides amb la gota citoplasmàtica a nivell de la peça de connexió (gota citoplasmàtica proximal) s'originen al testicle i, al llarg del seu trajecte per l'epidídim la gota citoplasmàtica es desplaça fins l'anell de Jensen (gota citoplasmàtica distal). Una vegada els espermatozoides immadurs amb gota citoplasmàtica distal arriben a la cua epididimària, perden la gota citoplasmàtica i adquireixen l'aspecte d'un espermatozoide madur. Així doncs, la presència d'espermatozoides amb gotes citoplasmàtiques indica el grau de maduració epididimària d'un ejaculat. En un ejaculat normal de *Sus domesticus* hom pot

trobar un percentatge d'entre el 5% i el 15% d'espermatozoides immadurs (Bonet *et al.*, 2000). Hi ha una clara evidència del paper importantíssim que juga la maduració epididimària per tal que l'espermatozoide sigui capaç de desenvolupar els mecanismes d'acció que el portaran a adquirir la capacitat fecundant. De fet, s'ha vist que hi ha una relació directe entre la incapacitat d'induir els canvis propis de la fecundació a nivell de membrana plasmàtica amb l'existència de la gota citoplasmàtica (Harrison i Gadella, 2005).

La maduració epididimària també comporta altres modificacions a nivell de la membrana citoplasmàtica. A mesura que els espermatozoides transiten al llarg de l'epidídim van interactuant amb els diferents fluids luminals. Les característiques d'aquests fluids van canviant alhora que indueixen canvis en els diferents dominis de la cèl·lula espermàtica. L'epiteli epididimal secreta una gran varietat de proteïnes i altres substàncies que interactuen amb la membrana espermàtica i alhora n'absorbeix d'altres procedents de les cèl·lules de testiculars. Així, durant el transport epididimari des del cap fins a la cua de l'epidídim es donen processos d'addició, pèrdua o modificació de proteïnes i lípids de membrana (Vadnais *et al.*, 2007). Un exemple en el porc n'és la secreció a nivell de cos de l'epidídim d'una proteïna de 25 kDa, anomenada anti-aglutinina, que s'uneix a l'acrosoma de l'espermatozoide i tal com deixa entreveure el seu nom, evita l'aglutinació entre espermatozoides (Harayama *et al.*, 1999). En porcí també s'ha identificat una altra proteïna, la Crisp-1, segregada a la regió caudal de l'epidídim i que s'uneix a la membrana plasmàtica durant el trànsit epididimari (Vadnais *et al.*, 2007). El suposat paper d'aquestes proteïnes inclou la prevenció d'una capacitació i reacció acrosòmica prematures alhora que les promou un cop l'espermatozoide és dipositat en el tracte genital femení.

Aquest procés d'adhesió de proteïnes a la superfície cel·lular no només està reduït a la fase de maduració epididimària, ja que també en el plasma seminal hi ha gran quantitats de proteïnes, com les esperma-adhesines, que s'uniran a la membrana plasmàtica durant l'ejaculació (Dostalova *et al.*, 1994; veure la Figura 3). Aquestes proteïnes actuen com a factors que prevenen la reacció acrosòmica prematura, i la seva retirada provocarà la capacitació. De fet, en el

cas del verro més d'un 90% de les proteïnes del plasma seminal són d'aquest tipus i actuen com a factors descapacitants. Nogensmenys, s'ha observat que la seva re-addició una vegada l'espermatozoide les ha perdudes fisiològicament inhibeix la capacitació (Vadnais *et al.*, 2007).

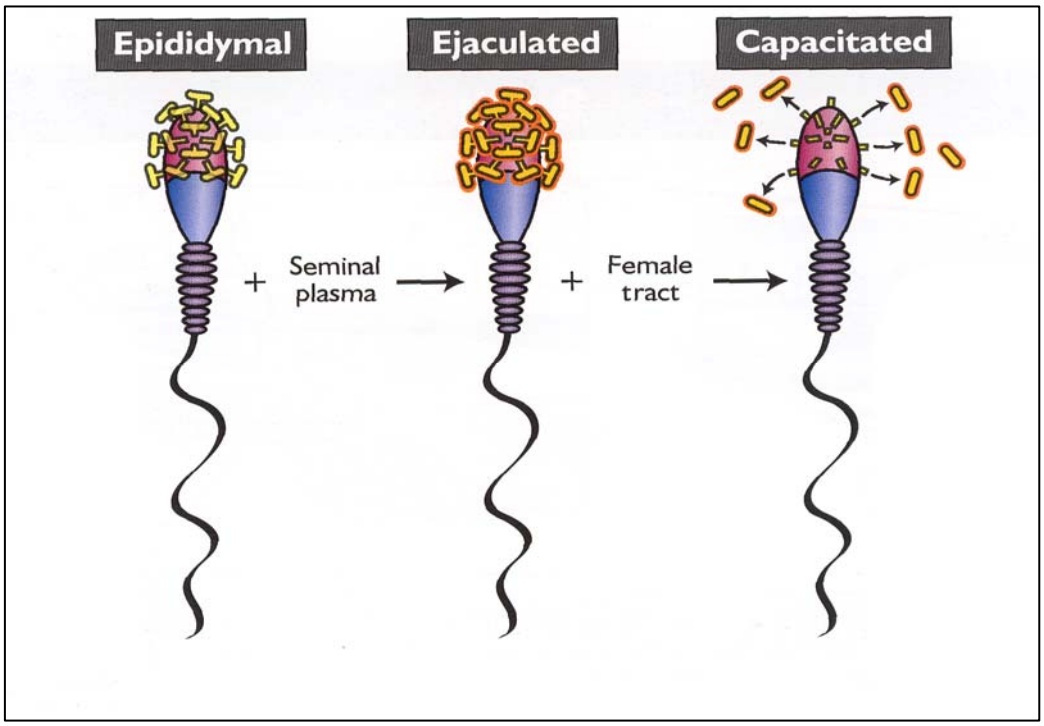

Figura 3: A: Durant el transport epididimal diferents tipus de molècules (carbohidrats i proteïnes) són afegits a la superfície de la membrana plasmàtica (T marcades de color groc). B: El plasma seminal conté proteïnes (halos taronges) que emmascaren les molècules epididimals. C: En el tracte reproductor femení tant les proteïnes emmascaradores (halos taronges) com les molècules epididimals (T grogues) són alliberades fent que la membrana plasmàtica quedi exposada en la seva totalitat, especialment, les zones d'unió amb la zona pelúcida de l'oòcit (Senger, 2003).

5. Capacitació

Un cop arribat la cua de l'epidídim, l'espermatozoide és funcionalment una cèl·lula madura si bé requereix d'un període de residència dins del tracte genital femení per a la plena adquisició de la capacitat fecundant. Austin (1951) i Chang (1951) van ser els primers en descriure aquest període, que més tard es va definir com a capacitació. La capacitació comença quan s'eliminen els factors estabilitzants de la membrana espermàtica adquirits del plasma seminal, dura al llarg de tot el trànsit pel tracte reproductor femení i es

considera completa quan l'espermatozoide és capaç d'unir-se a la zona pelúcida de l'oòcit i desencadenar la reacció acrosòmica (De Jonge, 2005). Tanmateix, la capacitació és un procés complex en el que s'hi donen diferents successos, com ara l'alteració de la fluïdesa de la membrana, fluxos de colesterol i de ions que provoquen alteracions en el potencial de la membrana, canvis en la fosforil·lació de residus tirosina de les proteïnes, inducció de la hipermotilitat entre d'altres i que tots junts acaben provocant la reacció acrosomal (Naz i Radesh, 2004). De fet, l'objectiu final de la capacitació és el de provocar la reacció acrosòmica una vegada estableix contacte amb la zona pel·lúcida de l'oòcit. Aquest és el motiu pel qual la capacitació es veu com un procés destinat a induir la "fusogenicitat" de la membrana plasmàtica que induirà el procés d'exocitosi acrosomal i provocarà la unió amb les membranes ovocitàries (Harrison i Gadella, 2005).

Tot i que la capacitació és un procés que es dóna fisiològicament dins del tracte reproductor femení, és un fenomen que es pot reproduir “in vitro” simulant les condicions uterines amb diferents tipus de solucions salines, sense requerir de l'addició dels fluids biològics uterins i/o oviductals. El fet que la capacitació es pot aconseguir espontàniament “in vitro” en un medi controlat sense requerir dels fluids biològics, suggereix que és un procés modulat intrínsecament per l'espermatozoide. Per tant, aquestes cèl·lules estan d'alguna manera pre-programades per patir la capacitació en un medi adequat (Visconti i Kopf, 1998). Això no s'oposa amb la influència positiva o negativa de factors reguladors del tracte reproductiu femení. De fet, és possible que la regulació de la capacitació recaigui més en l’eliminació de factors inhibitoris (alliberació dels agents descapacitants) que no pas en l'estimulació del procés en sí (Visconti i Kopf, 1998; veure Figura 3).

Així doncs, podem parlar de mecanismes extrínsecs i intrínsecs de regulació de la capacitació. Els mecanismes extrínsecs són els que exerceix el tracte genital femení i els intrínsecs són els produïts a partir dels canvis que pateix l'espermatozoide capacitat, ja esmentats anteriorment, i que sovint estan interrelacionats. Bàsicament, aquests canvis són fluxos de calci, canvis de la

membrana plasmàtica, hiperactivació de la motilitat, fosforil·lació en els residus tirosina de les proteïnes i canvis en el metabolisme energètic.

5.1. Paper del tracte reproductor femení en la capacitació:

Com ja s'ha esmentat anteriorment, la capacitació de l'espermatozoide es presenta com un succés activa i progressivament coordinat amb els diferents segments del tracte reproductor femení en relació al moment de la ovulació. Aquesta assumpció està basada en estudis fets *in vivo,* en concret en el verro, a on els espermatozoides es capacitaven en 2-3 hores al ser exposats primer en fluid uterí i després en l'oviductal. En canvi, els espermatozoides en fluid provinent de la unió útero-tubàrica requerien el doble de temps per a capacitar-se (Hunter i Dziuk, 1968; Hunter i Hall, 1974). De fet, la primera part de l'oviducte presenta unes condicions ambientals que el determinen com a reservori seminal (RS), facilitant així la disponibilitat d'espermatozoïdes capacitats en el moment del pas de l'oòcit per aquella zona (Rodríguez-Martínez, 2007). Els espermatozoides s'uneixen a les cèl·lules oviductals a nivell de l'istme mitjançant la interacció entre lligants específics. No només en porcs, sinó també en altres mamífers estudiats, el RS de l'oviducte sembla ser que, en comptes de promoure la capacitació, la retarda, allargant així el seu període vital (Petrunkina *et al.*, 2001; Rodríguez-Martínez *et al.*, 2005). També s'ha vist que l'ovulació no provoca cap canvi determinant en l'alliberament d'espermatozoides des del RS. Per tant, es creu que hi ha un alliberament progressiu d'espermatozoides durant el període periovulatori, des de la pre- fins a la post-ovulació (Rodríguez-Martínez, 2007). Així doncs, el paper del tracte reproductor femení en la capacitació no només recau en la dilució i alliberament dels components del plasma seminal, sinó que també exerceix un paper actiu actuant directament en la superfície espermàtica minvant o accelerant la capacitació segons estiguin o no units a l'epiteli oviductal.

5.2 Fluxos de calci

En l'espermatozoide porcí hi ha un influx de calci durant la capacitació que activa o modula diferents vies enzimàtiques o metabòliques (Harrison *et al.*,

1993). S'han descrit diferents tipus de canals de calci en la membrana plasmàtica de l'espermatozoide, com ara canals dependents de voltatge, Ca^{+2}-ATPases i bescanviadors Na^{+2}/Ca^{+2} entre d'altres (Flesh i Gadella, 2000). La capacitació finalitza amb la reacció acrosòmica i aquesta, a l'igual que altres processos de fusió de membranes, és depenent d'una entrada massiva de calci a nivell intracel·lular. Així, la capacitació es presenta com un procés de preparació de la cèl·lula espermàtica per a aquest flux massiu de calci. De fet, l'acrosoma és un gran reservori intracel·lular de calci, ja que l'espermatozoide manca de reticle endoplasmàtic i l'acrosoma presenta bombes d'entrada de Ca^{+2}. De fet, en porcí s'ha demostrat que la capacitació és un procés depenent de calci (Tardif *et al.*, 2003). L'entrada de Ca^{+2} activa l'adenilat ciclasa amb la conseqüent activació de la via de l'adenosin monofosfat cíclic (AMPc)/fosfoquinasa-A (PKA), que recau directament amb la potenciació de la capacitació (Visconti *et al.*, 1998). Endemés l'entrada de Ca^{+2} juga un paper molt important en la hiperpolarització i de la membrana i l'augment de pH intracitoplasmàtic detectats durant la capacitació (Vadnais *et al.*, 2007; veure Figura 4).

5.3 Canvis de la membrana plasmàtica

La membrana espermàtica de l'espermatozoide juga un paper molt important en el procés de la fecundació. La membrana plasmàtica no és només el límit de l'espermatozoide sinó que és una estructura molt dinàmica. De fet, com ja s'ha esmentat anteriorment, la capacitació es considera com una sèrie de canvis que permeten la unió de l'espermatozoide amb l'oòcit i per això són necessàries tot un seguit de modificacions a nivell de la membrana que la faran més "fusogènica" o hàbil per a patir processos de fusió. Aquesta "fusogenicitat" de la membrana no només és important per la unió entre les membranes espermàtica i ovocitària, sinó també en els processos d'unió de membranes que es donen en la reacció acrosòmica indispensables per a la posterior fecundació. Estudis biofísics de la membrana plasmàtica demostren que tant els lípids com les proteïnes membranoses estan organitzats en dominis molt concrets de l'espermatozoide. Durant el pas pel tracte reproductor femení, aquests dominis pateixen reorientacions i modificacions que provocaran una

major fluïdesa de la membrana plasmàtica de l'espermatozoide. En primer lloc, es dóna la pèrdua de components adquirits en l'epidídim i en el posterior contacte amb el plasma seminal (agents descapacitants) que induirà la capacitació. Per altra banda, una de les principals modificacions de la membrana és la pèrdua del colesterol membranós. De fet, les espècies amb un alt contingut de colesterol a la membrana (humana i bovina) requereixen temps elevats per la capacitació espermàtica (8 i 6 hores respectivament). En canvi en porcí o en oví, amb menys contingut de colesterol, aquest temps és sols de 3-4 hores. En els medis preparats per a induir la capacitació “in vitro” es sol afegir albúmina sèrica bovina (BSA), ja que actua com a segrestadora del colesterol de la membrana, provocant així la capacitació (Figura 4). De fet, el bicarbonat, que es considera un agent capacitant en moltes espècies, s'ha demostrat que en el porcí actua no només facilitant l'entrada de calci tant a la cua com al cap, ans provoca una notable desestabilització de la membrana plasmàtica que facilitarà el segrest del colesterol (Harrison *et al.*, 1993).

5.4 Fosforil·lació en els residus tirosina de les proteïnes

Donat que la capacitat de síntesi proteica en l'espermatozoide està molt limitada per la pràctica l'absència de ribosomes, la regulació de proteïnes a través de processos post-traduccionals és molt important. Una d'aquestes modificacions és la fosforil·lació/defosforil·lació en els residus de tirosina de les proteïnes, que en alguns casos indueix l'activació i en d'altres la inactivació de les proteïnes.

En molts espècies de mamífers, entre elles la porcina, s'ha observat una relació directa entre la fosforil·lació en els residus tirosina (PTyr) i la capacitació. De fet s'ha vist que la PTyr és un dels processos més importants que ocorre durant la capacitació (Naz i Radesh, 2004). L'activació de via de la AMPc/PKA és la principal responsable de la PTyr durant la capacitació (Figura 4) si bé d’altres quinases també són molt actives en l’espermatozoide. Diferents autors han demostrat la presència de PTyr específiques durant la capacitació, com és el cas de les proteïnes d'ancoratge de la fosfoquinasa-A (AKAPs; Carrera *et al.*, 1996) i de la proacrosina p32 (Dubé *et al.*, 2005) entre moltes d'altres (Arcelay *et al.*, 2008). Tal com el seu nom indica, les AKAPs fan d'ancoratge de la PKA a

la beina fibrosa del flagel espermàtic a on s'ha vist que la PTyr és responsable de la regulació de la motilitat (Vadnais *et al.*, 2007). La fosforil·lació de la proacrosina p32 és característica de l'espermatozoide porcí i ha estat suggerida com a indicador de l'estat de la capacitació (Bravo *et al.*, 2005). La PTyr no només està relacionada amb la capacitació, sinó també amb processos annexes a aquesta com ara l'afinitat per la ZP, amb la reacció acrosòmica i amb la hipermotilitat (Flesh i Gadella, 2000).

5.5 Hiperactivació de la motilitat

La capacitació està també correlacionada en diferents espècies animals amb canvis en el patró de motilitat que s'han definit en el seu conjunt com hiperactivació (Visconti *et al.*, 1998). En ejaculats frescos, els espermatozoides mostren un moviment simètric de baixa amplitud d'ona amb un moviment més o menys lineal. Durant la capacitació la cua de l'espermatozoide genera un moviment asimètric i d'elevada amplitud que li conferirà la força necessària per deslligar-se de l'epiteli oviductal i penetrar la zona pelúcida de l'oòcit (Suárez, 2008). Els mecanismes de la regulació de la motilitat està majoritàriament sota el control de la via AMPc/PKA (Harrison i Gadella, 2005). S'han fet diferents estudis en els que es demostra que la inhibició de la PKA resulta amb la pèrdua de la motilitat i l'addició de compostos anàlegs a l'AMPc la potencia (Holt i Harrison, 2002). De fet, la fosforil·lació de proteïnes, com ja s'ha esmentat anteriorment, és un dels punts de modulació més importants de la motilitat espermàtica durant la capacitació (Figura 4).

5.6 Canvis en el metabolisme energètic

La major part de l'energia necessària per l'espermatozoide la obté a través de la utilització de monosacàrids, des de la glucosa fins a altres glúcids menys comuns com la fructosa, el lactat, el piruvat, el sorbitol o el glicerol (Cao *et al*., 2009). L'equilibri o elecció pel metabolisme dels diferents tipus de monosacàrids depèn de diferents factors, entre ells la composició del medi extracellular. Així durant el pas a través del tracte reproductor masculí i femení, l'espermatozoide es veu envoltat de diferents medis extracel·lulars que van

canviant la composició i concentració dels diferents substrats energètics. D'aquesta manera l'espermatozoide ha d'anar adaptant les seves vies metabòliques a la disponibilitat de substrats en funció del medi extern en el que es troba per tal de satisfer les seves necessitats energètiques.

Els differents tipus de monosacàrids metabolitzables entren en l'espermatozoide mitjançant diversos tipus de transportadors específics, dels que els més coneguts són els de la familia GLUT. En l'espermatozoide porcí s'ha demostrat l'existència dels transportadors GLUT 1, 2 ,3 i 5 (Bucci *et al*. 2008). Cada una d'aquests manifesta diferent afinitat per cada monosacàrid. Així, el GLUT-3 té molta una gran afinitat per la glucosa. El GLUT-5, en canvi, presenta una gran afinitat per la fructosa i la manosa. Alhora la utilització dels diferents monosacàrids ve determinada no només per l'afinitat dels transportadors, sinó també per les hexoquinases, enzims que, mitjançant la fosforil.lació específica dels monosacàrids, els introdueixen dins de les vies catabòliques. Treballs anteriors han mostrat que en el espermatozoide porcí existeix una actividat hexoquinasa compatible amb l'enzim hexoquinasa-I, proteïna ubiqua general en cèl.lules eucariotes. Aquesta hexoquinasa en espermatozoides porcins té diferent afinitat per cada un dels monosacàrids, mostrant una afinitat màxima per la glucosa abans que la fructosa el sorbitol o la manosa. De fet, l'afinitat de la hexoquinasa-I per a la glucosa és tan elevada que la cèl·lula espermàtica té una resposta metabòlica rapidíssima i molt intensa, de les més ràpides entre les diverses cèl.lules eucariotes estudiades, en presència de baixes concentracions de glucosa (Rodríguez-Gil, 2006). Això dona a l'espermatozoide porcí una capacitat de resposta pràcticament instantània a canvis en l'entorn energètic durant el trànsit de la cèl.lula per tracte genital femení, sent-ne així un factor fonamental per a explicar la capacitat adaptativa i de supervivència de l'espermatozoide porcí "in situ".

L'equilibri entre el metabolisme glucídic via glucolisis o per transfrència cap el cicle de Krebs depèn de diferents factors com la pressió d'O_2, el pH, els nivells intracel·lulars d'ATP i l'acció de diferents factors de senyalització intracel·lular com ara els òxids nitrosilats (NO) (Stryer, 1995). Tots aquests factors permeten una fina regulació per tal de mantenir el nivell s'ATP

intracel·lular apropiat, i per tant l'energia requerida en cada precís moment. Així, durant la capacitació l'estructura mòbil de l'axonema necessitarà un requeriment superior d' ATP pel seu moviment. L'espermatozoide capacitat també requerirà més ATP per tal de satisfer l'increment de la demanda provocat per l'activació de l'adenilat ciclasa, que comportarà l'augment de l'AMPc indispensable per diferents processos intrínsecs a la capacitació. Així doncs, la cèl·lula espermàtica requereix un major aport energètic durant la capacitació, motiu pel qual es farà necessari una especial modulació del metabolisme energètic. La via catabòlica principal en l'espermatozoide porcí fresc és la glucòlisi (Marin *et al.*, 2003, Kamp *et al.*, 2003). Així doncs la fosforil·lació oxidativa, té un paper molt mins en la generació d'energia en aquestes condicions. De fet, s'ha vist que en l'espermatozoide porcí fresc, tan sols un 5% de la glucosa entra al cicle de Krebs (Marin *et al.*, 2003). Per altra banda, també s'ha demostrat que la font principal d'energia per la motilitat espermàtica en la major part de les espècies estudiades, deriva de la glucòlisi i no de la fosforil·lació oxidativa (Gravance *et al.*, 2000). El descobriment que la xarxa enzimàtica de la glucòlisi espermàtica està íntimament unida a la beina fibrosa del flagel de l'espermatozoide fa pensar que la glucòlisi té lloc en la part principal de la cua, que per altra banda manca de mitocondris. En canvi s'ha de recordar que l'eficiència energètica de la glucòlisi és superada amb escreix per la de la fosforil·lació oxidativa. Per aquest motiu s'ha hipotetizat que en moments de requeriments energètics tan importants com la capacitació, en el que tant la hiperactivació de la motilitat com l'activació de l'adenilat ciclasa requeriran grans aportacions d'ATP, la generació d'energia mitocondrial podria agafar protagonisme. Així doncs, aquesta hipòtesi implica que la activitat mitocondrial variarà en gran mesura durant la vida de l'espermatozoide, per tal d'adaptar-se als canvis en les necessitats energètiques que pateix aquesta cèl.lula durant el seu transcurs vital. De totes maneres, hi ha un clar dèficit de coneixement pel que respecta als mecanismes mitjançant els que l'espermatozoide porcí regula la seva despesa energètica en funció de l'ambient i, per tant, encara manca un coneixement clar del control de les vies energètiques espermàtiques durant la capacitació.

5.7 Producció d'espècies reactives d'oxigen

La producció d'espècies reactives d'oxigen (ROS) és fruit de l'activitat metabòlica aeròbica de la cèl·lula. A elevades concentracions, els ROS tenen efectes nocius per la cèl·lula, ja que afecten tant al DNA com a la membrana plasmàtica. Contràriament, baixos nivells de ROS en la cèl·lula són necessaris per molts processos cel·lulars, ja que els ROS funcionen també com a segons missatgers. En l'espermatozoide, la generació controlada i a baixos nivells de ROS com l'anió superòxid (O^{2-}), el peròxid d'hidrogen (H_2O_2) i el NO actua com a factor estimulant de la capacitació. S'ha demostrat la seva influència a diferents nivells com en l'activació de la via AMPc/PKA com en la fosforil·lació de determinades proteïnes lligades a la capacitació (de Lamirande i O'Flaherty, 2008; Figura 4).

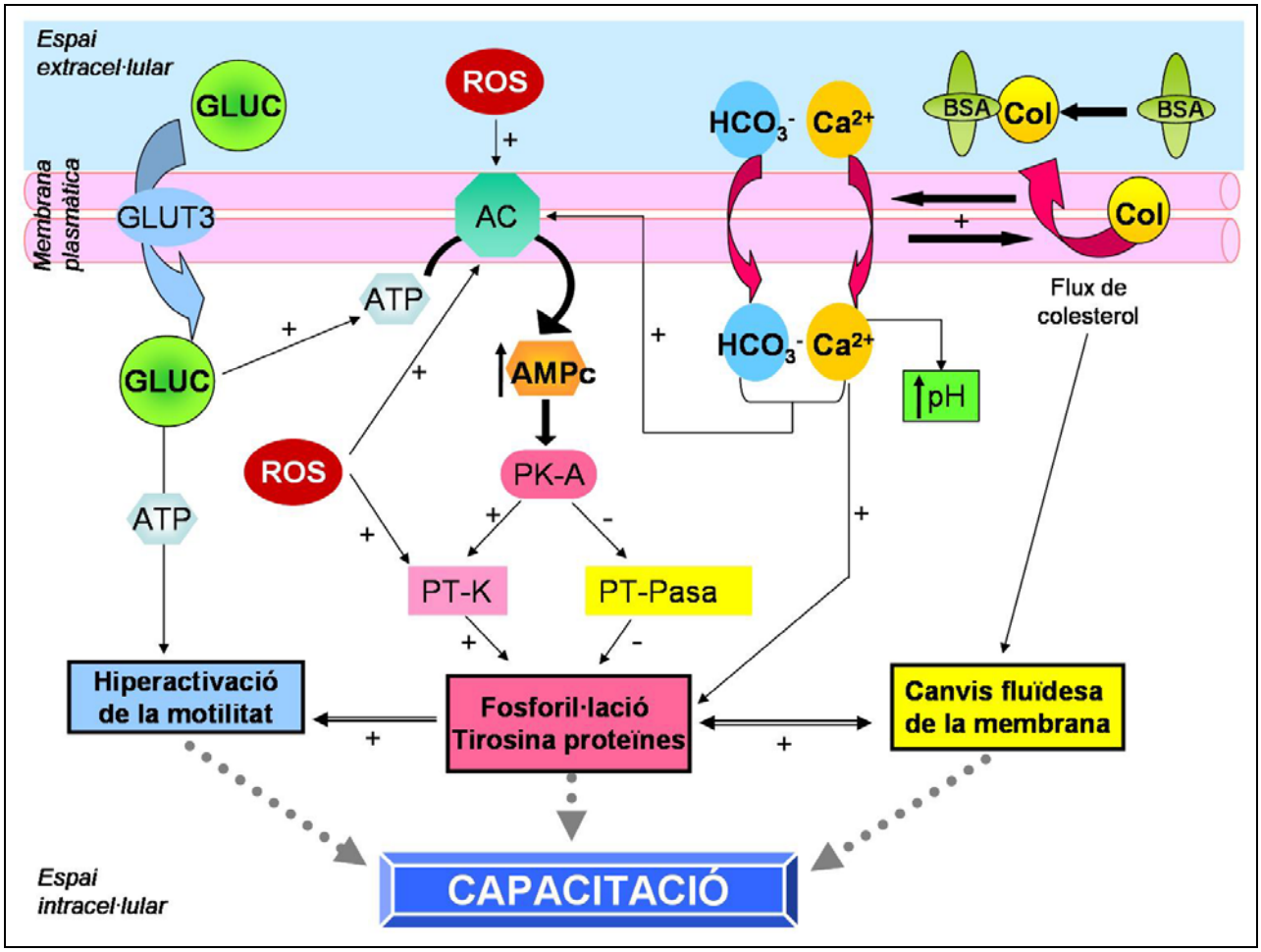

Figura 4. Esquema de les vies i processos transmembranosos i intracel·lulars que tenen lloc durant la capacitació de l'espermatozoide porcí. Abreviacions: GLUC: glucosa, AC: adenilat ciclasa, PT-K :proteïnes tirosina-quinasa, PT-Pasa: proteines tirosina-fosfatasa, Col: colesterol.

6. Reacció acrosòmica

La unió de l'espermatozoide amb la zona pel·lúcida de l'oòcit provoca la reacció acrosòmica. La reacció acrosòmica es caracteritza per la unió entre les membranes plasmàtica i acrosomal externa en forma de vacuolització. Aquesta fusió de membranes provocarà l'alliberament d'enzims hidrolítics i proteolítics de l'acrosoma (l'acrosina i la hialuronisasa en són els més destacats) que disoldran localment la zona pel·lúcida permetent l'entrada de l'espermatozoide a l'espai perivitel·lí. És un procés exocític irreversible, punt final de la capacitació. Com a conseqüència, la membrana acrosomal interna queda exposada a l'exterior, llevat de la zona equatorial, coberta encara per la membrana plasmàtica i que jugarà un paper molt important a l'hora de la fusió amb l'oòcit (Figures 5 i 6).

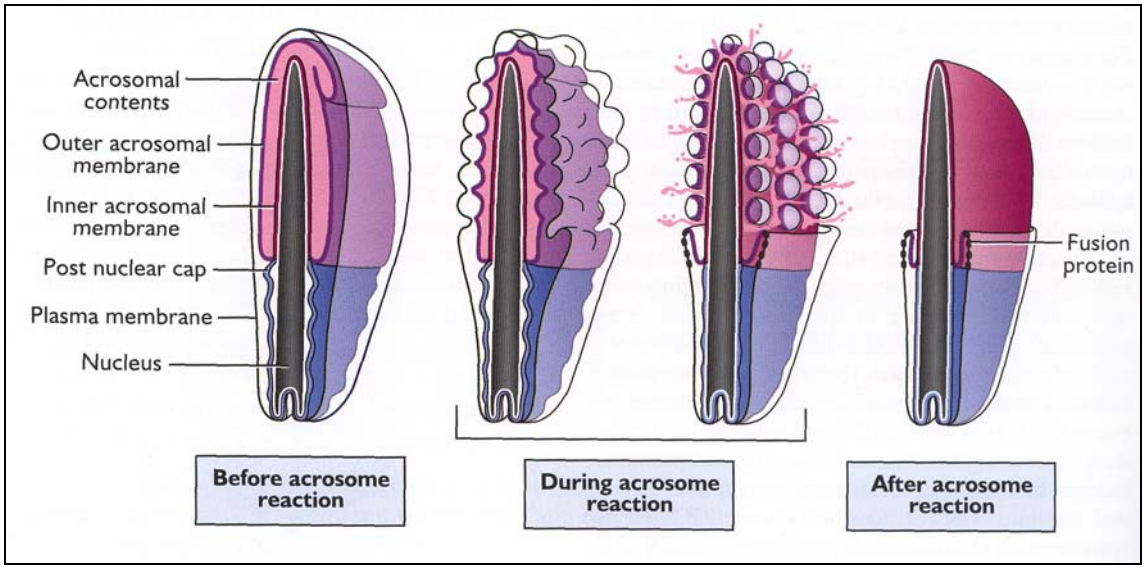

Figura 5: Abans de la reacció acrosòmica, totes les membranes espermàtiques estan intactes. Durant la reacció acrosòmica, la membrana plasmàtica es fusiona amb la membrana acrosomal externa. La fusió provoca la formació de petites vesícules, creant porus a través dels quals es vessa el contingut acrosomal. Els enzims hidrolítics permetran la penetració de l'espermatozoide a través de la zona pel·lúcida de l'oòcit. Després de la reacció acrosòmica la membrana acrosomal interna queda exposada a l'exterior (Senger, 2003).

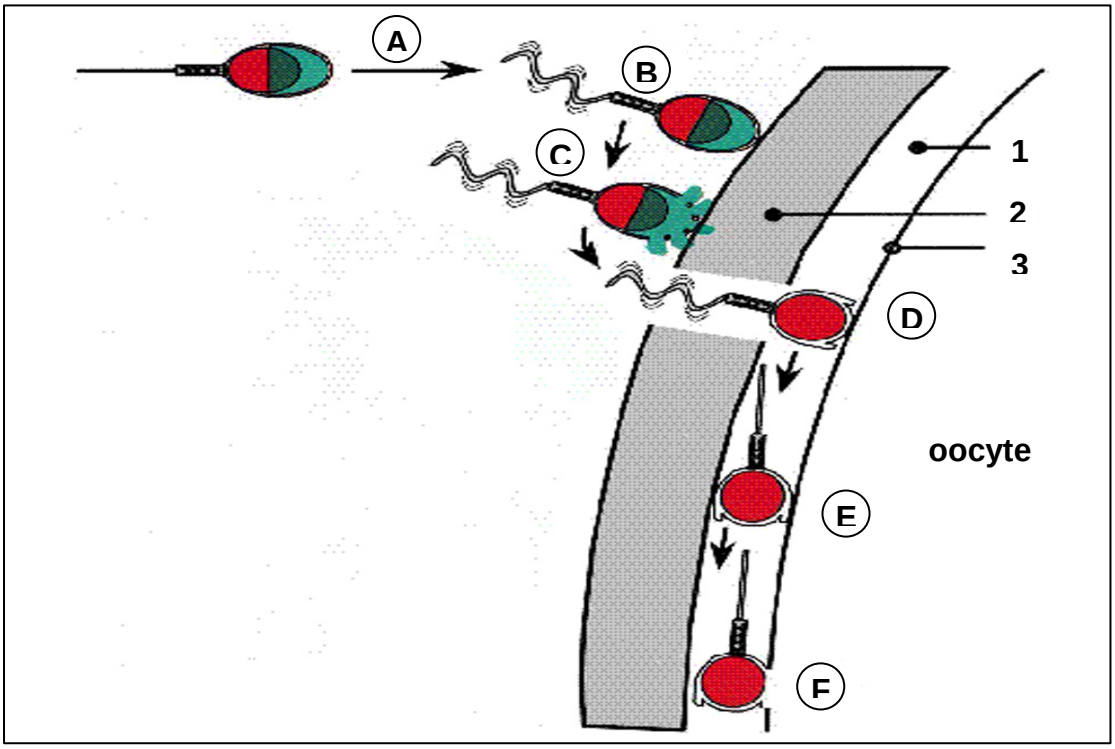

Figura 6. Seqüència dels successos que tenen lloc durant la fecundació. (A) Els espermatozoides ejaculats s'activen durant el transport a través del tracte genital femení: capacitació. (B) Els espermatozoides capacitats estan hiperactivats i són capaços d'unir-se a la ZP.(C) La unió dels espermatozoides a la ZP indueix la reacció acrosòmica, permetent el pas a través d'ella. Els espermatozoides entren a l'espai perivitel·lí i s'uneixen a la membrana plasmàtica ovocitària (oolema) pel seu extrem apical. (E) La unió a l'oolema passa de l'extrem apical a la zona ecuatorial. (F) L'espermatozoide es fusiona amb l'oòcit i s'incorpora dins seu. 1: espai perivitel·lí; 2: ZP: zona pelúcida; 3: oolema (Flesh i Gadella, 2000).

Durant la capacitació, els receptors de membrana de la ZP es van activant i exposant a la superfície espermàtica (Asquith *et al.*, 2004). La unió entre la ZP i l'espermatozoide activa els canals de calci, provocant així un important increment en el calci intracel·lular i de resultes en altres missatgers secundaris com l'AMPc que activaran determinades vies intracel·lulars, com ara l'activació de les proteïnes quinases tal com s'ha vist durant la capacitació.

La inducció fisiològica de la reacció acrosòmica prové de la unió amb la ZP. La reacció acrosòmica també es pot obtenir “in vitro” no només amb la utilització de ZP purificada sinó també amb altres substàncies fisiològiques que intervenen en la fecundació, com és el cas de la progesterona. S'ha suggerit que aquesta hormona interactua amb la membrana plasmàtica espermàtica mitjançant receptors específics de membrana, amb la posterior activació de

vies inductores de la reacció acrosòmica. Les prostaglandines, els glicosaminoglicans i altres substàncies presents en el fluid oviductal o en les secrecions de les cèl·lules del cúmulus també han demostrat ser inductors de la reacció acrosòmica (Abou-haila i Tulsiani, 2009). Hi ha un altre tipus de inductors, els no fisiològics, entre ells el ionòfor de calci A23187 que indueix la reacció acrosòmica obrint els canals de calci per permetre l'influx de calci en l'espermatozoide .

Els fluxos de calci són un dels principals successos que protagonitzen la reacció acrosòmica (Figura 7). En primer lloc, l'activació de la via AMPc/PKA durant la capacitació promou l'entrada de calci a través dels canals específics de la membrana acrosomal externa, provocant un increment de calci citosòlic. Aquest increment progressiu provoca, en segon lloc, l'activació de la fosfolipasa C (PLC) que al ser fosforil·lada hidrolitza el fosfatidilinositol-4,5-bifosfat (PIP2) produint diacilglicerol (DAG) i l'inositol-trifosfat (IP3). Tant el DAG com el IP3 promouen l'entrada de calci al citoplasma espermàtic a través de canals de la membrana acrosomal externa alhora que activen la proteïna quinasa C (PKC). La PKC juga un paper central durant la reacció acrosòmica, ja que activarà un altre tipus de canal de calci, situat a la membrana plasmàtica, que comportarà el tercer flux de calci que resultarà, juntament amb les altres dues entrades de calci al citoplasma, amb un elevat increment sobtat d'aquest ió a nivell citoplamàtic. Aquest increment en la concentració càlcica del citoplasma coordinat amb la pèrdua d'aquest ió a nivell acrosomal acabarà amb la unió entre la membrana acrosomal externa i la membrana plasmàtica (Breitbart i Naor, 1999).

Així doncs, la fosforil·lació de proteïnes també juga un paper important durant la reacció acrosòmica. La PLC no és la única proteïna fosforil·lada. Així, també durant la capacitació i reacció acrosòmica té lloc la fosforil·lació del receptor de la ZP que provocarà l'externalització d'aquest a l'espai extracel·lular (Asquith *et al.*, 2004) i que promourà la fusió entre ambdós gàmetes (Figura 7) .

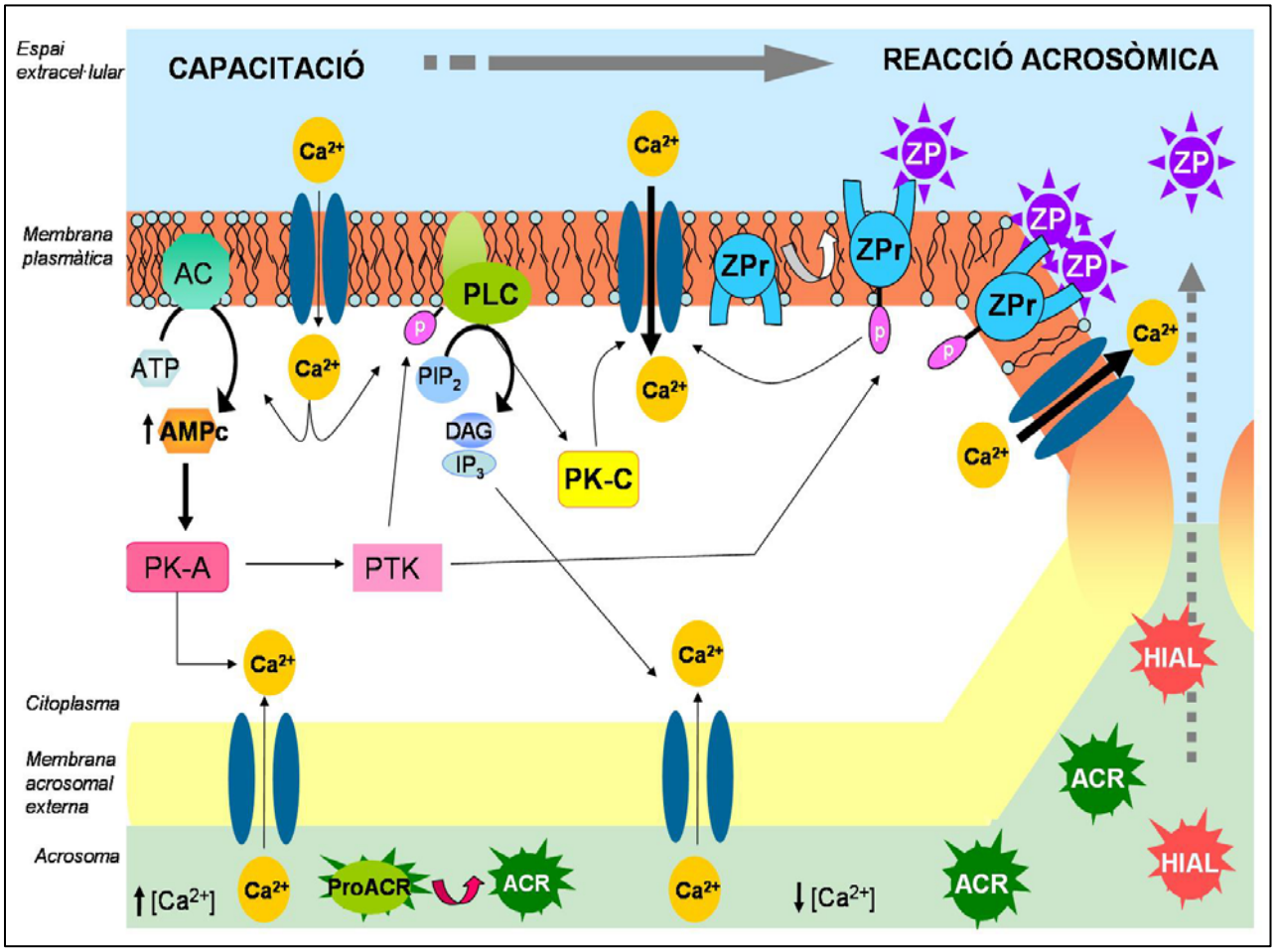

Figura 7. Esquema de les vies i processos transmembranosos i intracel·lulars que tenen lloc durant la reacció acrosòmica de l'espermatozoide porcí. Abreviacions: AC: adenilat ciclasa, PK-A: proteïna quinasa A, PTK: proteïnes tirosina-quinasa, PLC: fosfolipasa C, PIP_2: fosfatidilinositol-4,5-bifosfat, DAG: diacilglicerol, IP_3: inositol-trifosfat, PK-C: proteïna quinasa C, ZPr: receptor de ZP, ZP: zona pel·lúcida, ProACR: proacrosina, ACR: Acrosina, HIAL: hialuronidasa.

7. Heterogenicitat funcional de la població espermàtica.

En mamífers, diferents estudis han demostrat una resposta heterogènia entre els espermatozoides d'un mateix ejaculat. El desenvolupament de sistemes computeritzats d'anàlisi de semen (Computer assisted semen analysis: CASA) van facilitar l'estudi de la motilitat permetent així el descobriment de l'existència de subpoblacions espermàtiques caracteritzades pel seu patró de moviment (Abaigar *et al.*, 1999; Hirai *et al.*, 2001; Quintero *et al.*, 2004). Nogensmenys, s'ha observat que aquesta estructuració poblacional no es manifesta tan sols en els valors de la motilitat, sinó que es pot comprovar

en la majoria d'estudis amb espermatozoides. Tanmateix, en estudis fets induint la capacitació “in vitro” s'ha observat que no tots els espermatozoides reaccionen de la mateixa manera. Així, Harrison *et al.* (1996) van descriure 2 subpoblacions en l'ejaculat porcí, amb resposta diferent a la tinció de merocianina-540 (tinció que permet detectar canvis en la fluïdesa de la membrana) davant la inducció de la capacitació “in vitro”. Endemés, la proporció de cèl·lules espermàtiques que adquirien els canvis propis de la capacitació variava en funció del mascle. També s'han descrit diferent resposta en quant a la fosforil·lació de proteïnes espermàtiques durant la capacitació. Així, Carrera *et al.* (1996) va descriure que entre un 10 i un 15% d'espermatozoides no presenten la ja esmentada fosforil·lació de les AKAP. De fet Urner i Sakkas (2003) afirmaren que en l'home i el ratolí, només un 50% dels espermatozoides presenta la fosforil·lació en els residus tirosina de les proteïnes.

Per una banda s'ha suggerit que el que diferencia a una subpoblació de l'altre és la velocitat de resposta. Així doncs, tots els espermatozoides, tard o d'hora presenten un mateix patró (Rodríguez-Martínez, 2007). Per altra banda, altres autors descriuen que fins i tot perllongant els temps d'incubació hi ha espermatozoides que no desencadenen els canvis propis de la capacitació (Harrison i Gadella, 2005). La causa d'aquesta heterogenicitat ha estat atribuïda a variacions individuals durant l'espermatogènesi (Abaigar *et al.*, 1999) així com als diversos estats de maduració en el que es troben els diferents espermatozoides d'un mateix ejaculat (Abaigar *et al.*, 1999; Harrison i Gadella, 2005), fet que ja s'ha comentat anteriorment en referència a la maduració epididimària. Aquesta diversitat de resposta facilitaria la disponibilitat d'espermatozoides en diferents estats de capacitació al llarg del període periovulatori, assegurant així que en el moment de l'ovulació hi hagi espermatozoides viables i capaços de fecundar l'oòcit (Rodríguez-Martínez, 2007).

Altres autors han hipotetizat sobre la possibilitat que els espermatozoides d'una determinada subpoblació siguin els potencialment fèrtils (Quintero-Moreno *et al.*, 2003). De fet, Druart *et al.* (2009) assenyalen l'existència d'una

subpoblació espermàtica més resistent a canvis hipotònics en comparació amb la resta de l'ejaculat. Alhora demostraren que la resistència osmòtica estava directament relacionada amb la maduració epididimària i amb la fertilitat. Tant és així que l'estudi de l'estructura subpoblacional d'un ejaculat ha estat suggerida com a mètode d'anàlisi seminal.

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II. OBJECTIUS / AIMS

OBJECTIUS / AIMS

L'objectiu principal d'aquesta tesi és contribuir a un millor coneixement dels mecanismes moleculars implicats en el correcte assoliment de la capacitació "in vitro" i posterior reacció acrosòmica "in vitro" dels espermatozoides porcins. Alhora, aquest objectiu bàsic s'ha subdividit en els següents objectius concrets:

1.- L'estudi de l'evolució temporal de l'estructura subpoblacional en els espermatozoides porcins durant la capacitació "in vitro" i la posterior reacció acrosòmica, per tal de determinar si aquests processos es relacionen amb canvis específics i temporals en l'estructura subpoblacional dels espermatozoides mòbils de la mostra de semen.

2.- Oferir una visió general de la dinàmica de la fosforil·lació en residus tirosina, serina i treonina de les proteïnes durant la capacitació i reacció acrosòmica "in vitro". Això ens permetrà aprofundir en el coneixement del paper de la fosforil·lació/desfosforil·lació de proteïnes com a mecanisme regulador durant aquests processos espermàtics en el porcí.

3.- Avaluar els canvis en l'activitat mitocondrial de l'espermatozoide porcí concomitants a la capacitació i la reacció acrosòmica en l'espermatozoide porcí, cercant alguna possible relació entre canvis en l'activitat mitocondrial, capacitació i reacció acrosòmica i possibles mecanismes moleculars implicats en aquests canvis.

III. CAPÍTOLS / CHAPTERS

CAPITOL / CHAPTER 1:

Dynamics of motile sperm subpopulations structure in boar ejaculates subjected to "in vitro" capacitation and further "in vitro" acrosome reaction.

Dynamics of motile sperm subpopulations structure in boar ejaculates subjected to "in vitro" capacitation and further "in vitro" acrosome reaction

1.1 Abstract

Incubation of diluted boar sperm from fresh ejaculates in a previously established "in vitro" capacitation medium induced significant, time-dependent increase in several mean parameters of sperm motility, such as curvilinear motility (VCL), linear velocity (VSL), mean velocity (VAP), linearity coefficient (LIN), straightness coefficient (STR) and wobble coefficient (WOB). Furthermore, motile boar-sperm semen samples were structured in four definite subpopulations. Subpopulation 1 showed the lowest values of VCL, VSL and VAP and also low values of linearity. Subpopulation 2 showed the second lowest values of VCL and VAP and higher values of LIN and STR. Subpopulation 3 was characterized by high values of velocity and low values of linearity. Finally, Subpopulation 4 was characterized by high values of velocity and linearity. "In vitro" capacitation and further acrosome reaction induced changes in the motility characteristics of each subpopulation as well as in their percentage distribution, Subpopulations 3 and 4 being those that showed the most significant changes. However, despite these changes, the observed, overall four-subpopulations structure was firmly maintained during the whole "in vitro" capacitation and acrosome-reaction process. Our results suggest that capacitation-induced motility changes are related to specific changes in the percentage of each motile-sperm subpopulation in the ejaculate without losing the overall, specific four-subpopulation structure. In this way, the maintenance of a four-subpopulation structure seems to be important in the control of the whole ejaculate physiology.

1.2 Introduction

In recent years, it has been reported that ejaculates for an increasing number of mammals are structured in well-defined subpopulations, which are

characterized by precise values of the motion parameters obtained after a computer-assisted motility analysis (CASA; Holt 1996; Abaigar *et al.*, 1999; Rigau *et al.*, 2001; Quintero-Moreno *et al.*, 2003; Martínez-Pastor *et al.*, 2005; Miró *et al.*, 2005; Quintero-Moreno *et al.*, 2007). Notwithstanding, although the presence of such a structure seems to be practically universal in mammals, there is not a consensus about the physiological role of a motile-sperm subpopulations structure in ejaculates. In this way, the presence of a concrete motile sperm subpopulations structure has been related to semen characteristics, such as cryosurvival and "in vivo" fertilizing ability in species like boar and horse (Quintero-Moreno *et al.*, 2003; Hirai *et al.*, 2001; Quintero-Moreno *et al.*, 2004). Moreover, the specific sperm selection and competition that is observed after sperm colonization of oviducts in pigs is modulated by the existence of a specific pattern of the motile-sperm subpopulation structure, based on the sperm's response to bicarbonate, in boar semen (Satake *et al.*, 2006). These results indicate that oviduct colonization can depend on the specific subpopulations structure of the ejaculate. However, the physiological role of sperm subpopulations on other important points like the selection of sperm to survive the first stages after ejaculation are not well documented, thus leaving many unknown questions in this area.

One of the most important modifications that mammalian sperm undergoes during the capacitation process is the change in the mean motility pattern of capacitated cells. Mammalian sperm undergoing capacitation shows a very characteristic motion pattern, called "hyperactivation", characterized by rapid changes of linearity patterns together with high velocity and strength (Yanagimachi, 1994). This hyperactivated motility pattern is the final result of a complex cascade of molecular and subcellular events, including calcium and bicarbonate fluxes and changes in the phosphorylation status of a wide array of proteins, which yields sperm able to penetrate oocytes (Visconti *et al.*, 1998). Nevertheless, although the exact processes by which sperm undergo capacitation are well studied, there are very few reports regarding how the separate sperm subpopulations from an ejaculate has different sensitivities to undergo capacitation in the appropriate circumstances. In this sense, it is noteworthy that motile-sperm subpopulations from an ejaculate have different

sensitivities to respond to external stimuli such as sugars (Rigau *et al.*, 2001), caffeine and bicarbonate (Holt, 1996). This latter agent is of importance, since in its presence boar sperm acts as a capacitating, responding cell with a rapid activation of motility in different incubation conditions (Holt, 1996; Holt and Harrison, 2002; Harrison *et al.*, 1996). Thus, the different responsiveness to agents like bicarbonate would indicate that the separate sperm subpopulations in an ejaculate are characterized by their different abilities to change their function and, hence, to attain capacitation. This point is of the utmost importance, since the ability of an ejaculate to attain a correct percentage of capacitated spermatozoa after ejaculation would be dependent on the exact distribution of the motile-sperm subpopulations.

Taking into account the facts described above, the main aim of this manuscript is to study the temporal evolution of the motile-sperm subpopulations structure of boar spermatozoa subjected to "in vitro" capacitation (IVC) and further "in vitro" acrosome reaction (IVAR). This analysis has as the main purpose to determine how the attainment of the capacitated status is, or not, accompanied by a specific, temporal sequence of changes in the overall motile-sperm subpopulation structure of the semen sample. For this purpose, diluted boar sperm from fresh ejaculates was incubated for 4 h at 38.5ºC in a specific medium for IVC and, after this period, they were further incubated in the presence of progesterone as an activating agent for acrosome reaction. During these incubations, motility patterns were analyzed by means of a CASA system, which allowed for a precise quantification of the motility characteristics of the sperm included in the samples.

1.3 Material and Methods

1.3.1 Boar Semen Collection

Boar semen was collected from eight healthy, mature boars from a commercial farm. Boars were three Large White, three Pietrain and two Pietrain x Large White, which were 2-3 years old. The ejaculates were manually collected and immediately placed in a water bath at 37ºC. The semen was

immediately diluted to a concentration of $2x10^7$ sperm/mL in a commercial extender for refrigerated semen (MR-A Extender; Kubus, S.A.; Majadahonda, Spain) and 400 mL of the diluted semen, distributed in four 100-mL commercial artificial insemination (AI) recipients which were sent to the laboratory of the Autonomous University of Barcelona (UAB). The time-lapse between semen extraction and receiving semen samples at the UAB laboratory was, in no case, more than 3 h. After receiving semen samples, they were immediately processed for experiments.

1.3.2 "In Vitro" Capacitation Procedure

"In vitro" capacitation was performed taking as a basis the procedure described in Tardif *et al.* (2001). For this, the four 100-mL commercial AI doses described above were pooled. At this time, an aliquot was taken to carry out the appropriate analysis. Immediately, pooled doses were washed three times by centrifugation at 200 x *g* for 10 min at 16ºC and re-dilution in MR-A extender. After the last washing, the sperm was resuspended in the capacitation medium (IVC medium) at a final concentration of (50-70) x 10^6 sperm/mL. Incubation in the IVC medium was maintained for 4 h at 38,5ºC in a 5% CO_2 atmosphere. At the indicated times, aliquots were taken to perform the required analyses. The IVC medium was a 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), containing 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 4.5mM $CaCl_2$ and 5 mg/mL bovine serum albumin (BSA). The osmolarity of the IVC medium was 304 $\pm$ 5 mOsm.

1.3.3 "In Vitro" Acrosome Reaction Procedure

The induction of IVAR was carried out through incubation in the presence of progesterone, as described in (Jiménez *et al.*, 2003; Wu *et al.*, 2006). For this purpose, 10 μg/mL progesterone was added to boar sperm previously incubated in the IVC medium for 4 h at 38,5ºC in a 5% CO_2 atmosphere. After thorough mixing, the sperm was further incubated for an additional 1h at 38,5ºC

in a 5% CO_2 atmosphere. At the indicated times, aliquots were taken to perform the required analyses.

1.3.4 Analytical Procedures

Percentages of viability, altered acrosomes and true acrosome reaction 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 is firstly incubated with a solution of 15 µM bis-benzamidine (proportion 1:1000, v/v) for 10 min at 37ºC. Afterwards, a 2mM propidium iodide solution is added (proportion 6:1000, v/v) and the sperm is subjected for further incubation for 10 min at 37ºC. After this incubation, the sperm suspensionis centrifuged at 1500 x *g* for 10 min and the supernatant is discarded. The obtained sperm pellet is resuspended in 1mL of a solution of 100nM 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 37ºC and then was immediately centrifuged at 1500 x *g* for 12 min. The resultant supernatant was discarded, whereas the sperm pellet was again 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 (Karl Zeiss GmbH; Jena, Germany) with the appropriate filters. Viability and altered acrosome percentages were determined after counting 200-300 spermatozoa per slide at 1000X. Unaltered acrosomes were considered to be those which showed a faint-to-moderate and uniform STBI lectin stain, whereas altered acrosomes showed a very faint and non-uniform stain. Sperm subjected to a true acrosome reaction were considered to be those which showed, after the stimulation of IVAR, positive viability (blue stain of the sperm head) and an increase and non uniform STBI lectin stain. Finally, non-viable sperm showed an intense red stain of the head.

The determination of tyrosine phosphorylation (Tyr-Phos) patterns was carried out through a Western blot analysis. For this purpose, sperm samples

were homogenized by sonication in ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 1% (w/v) dodecyl sodium sulfate (SDS) and 1 mM Na_2VO_4 (proportion 1:5, v/v) to avoid changes in the overall phosphorylation of the homogenates. Samples were briefly boiled and were then centrifuged at 10,000 x *g* for 15 min at 4ºC. Western blot analyses were performed on the supernatants obtained after this centrifugation. The analysis was based on SDS gel electrophoresis (Laemmli, 1970) followed by transfer to nitrocellulose (Burnette, 1981). The transferred samples were tested with an anti-phosphotyrosine antibody (PY-20; Chemicon International; Temecula, CA, USA) at a dilution of 1:1000 (v/v). Immunoreactive proteins were tested using peroxidase-conjugated anti-rabbit secondary antibody (Amersham; Buckinghamshire, UK) and the reaction was developed with an ECL-Plus detection system (Amersham).

1.3.5 Computer-Assisted Motility Analysis

The CASA analysis was performed by using a commercial system (Integrated Sperm Analysis System V1.0; Proiser; Valencia, Spain). In this procedure, samples were previously warmed at 37ºC for 5 min in a water bath and 5 μL aliquots of these samples were then placed on a warmed (37ºC) slide and covered with a 25-mm x 25-mm coverslip. Our CASA system was based upon the analysis of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 100X in a positive phase-contrast field. These 25 consecutive photographs were taken in a time-lapse of 1 s, which implied a velocity of image capturing of one photograph every 40 ms. Two to three separate fields were taken for each sample. The obtained sperm motility descriptors were described following Quintero-Moreno *et al.* (2003). Motility descriptors obtained after CASA analysis were:

- Curvilinear velocity (VCL): the mean path velocity of the sperm head along its actual trajectory (μm/s).
- Linear velocity (VSL): the mean path velocity of the sperm head along a straight line from its first to its last position (μm/s).
- Mean velocity (VAP): the mean velocity of the sperm head along its average trajectory (μm/s).

- Linearity coefficient (LIN): (VSL/VCL) x 100 (%).
- Straightness coefficient (STR): (VSL/VAP) x 100 (%).
- Wobble coefficient (WOB): (VAP/VCL) x 100 (%).
- 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 (μm).
- Frequency of head displacement (BCF): the frequency with which the actual sperm trajectory crosses the average path trajectory (Hz).

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

1.3.6 Statistical Analysis

The complete statistical analysis was performed by applying the SAS statistical package (SAS, 1982). The first step was to apply a PROC NORM PLOT procedure to determine if the motility parameters followed a normal distribution or not. Those parameters that did not follow a normal distribution were normalized before applying the different statistical procedures. The data shown in the different tables correspond to the real values in both, normal and not normal distributed data, but all the statistical differences are performed with normalized distributed data.

A FASTCLUS procedure was then applied to distribute every individual and motile spermatozöon into a specific sperm subpopulation. The FASTCLUS procedure performs a disjointed cluster analysis based on Euclidean distances computed from one or more quantitative parameter variables. In this case, these variables were 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 PROC GLM procedure was applied to evaluate significant differences ($P<0.05$) and the LSMEANS procedure was applied to list these differences. Finally, a Chi-square

procedure was applied to determine the subpopulational distribution percentage of every single experiment. Once the percentage distribution per experiment was determined, new PROC GLM and LSMEANS procedures were applied to determine and list, respectively, the differences among the different treatments. The total number of spermatozoa analyzed following this protocol was 11,465 in the experiments regarding "in vitro" capacitation and 6,441 in those regarding "in vitro" acrosome reaction. These cells were from a total of 16 separate experiments.

1.3.7 Suppliers

All of the suppliers utilized in this work were of analytical grade and came from Sigma (St. Louis, MO), Boehringer Mannheim (Mannheim, Germany), and Merck (Darmstadt, Germany).

1.4 Results

1.4.1 Effects of incubation in the IVC medium on "in vitro" capacitation markers

The incubation in the IVC medium induced a progressive decrease in the percentage of viability of boar sperm. Thus, this percentage ranged from 72.7 ± 2.1% at 0 h of incubation to 52.9 ± 4.5% after 4 h of incubation (Table 1). Concomitantly, the percentage of altered acrosomes underwent a progressive increase, which went from 22.3 ± 2.5% at 0 h of incubation to 42.5 ± 3.7% after 4 h of incubation (Table 1). Further incubation in the presence of progesterone did not change these evolutions, so, thus, the percentage of viability continued to decrease, reaching values of 44.6 ± 4.6% after 60 min of incubation with progesterone, whereas the percentage of altered acrosomes increased to 72.3 ± 4.6% after 60 min of incubation with progesterone (Table 1).

Incubation in the IVC medium also induced significant changes in the STBI lectin/viability dtain. Thus, in samples at 0 h of incubation, the percentage of

spermatozoa which presented a stain compatible with a true acrosome reaction was of only 2.3 ± 0.9%. This percentage slightly increased, reaching values of 4.9 ± 2.0% after 4 h of incubation in IVC (Table 1). Further incubation with progestereone had a much more dramatic effect on the percentage of these viable sperm with an intense and non-uniform STBI lectin stain, which increased to 34.8 ± 3.9% after 60 min of incubation with progesterone (Table 1). This indicates that progesterone induced the acrosome reaction in sperm previously incubated in IVC for 4 h.

Additionally, incubation of boar sperm in the IVC medium induced significant changes in the Tyr-Phos pattern. Hence, as shown in Figure 1, protein extracts from samples at 0 h of incubation showed a specific pattern with two majority bands of about 45 kDa and 60 kDa. Incubation in the IVC medium induced a significant change in this pattern, with the appearance of a specific band of about 32 kDa, which was clearly evident after 1-2 h of incubation (Figure 1). These changes are compatible with the previously published changes in Tyr-Phos patterns after "in vitro" capacitation of boar sperm (Tardif *et al.*, 2001; Bravo *et al.*, 2005). On the other hand, further incubation in the presence of progesterone for 60 min did not modify the "in vitro" capacitation-induced Tyr-Phos pattern (data not shown).

1.4.2 Effects of "in vitro" capacitation and further acrosome reaction on mean values of motility parameters of boar sperm

The incubation of boar sperm in the IVC medium induced several time-dependent changes on the mean motility parameters studied. Thus, total motility showed a progressive decrease, which fell from 81.2 ± 1.1% at 0 h of incubation to 69.4 ± 0.9% after 4 h of incubation (Table 1). This decrease was maintained when sperm was further incubated in the presence of progesterone, reaching total motility values of 53.9 ± 0.9% after 1 h of incubation with the hormone (Table 1). Moreover, a progressive increase in the mean values of VCL (from 88.3 ± 0.6 μm/s at time 0 to 98.3 ± 0.5 μm/s after 4 h of incubation), VSL (from 35.0 ± 2.4 μm/s at time 0 to 49.1 ± 1.3 μm/s after 4 h of incubation)

and VAP (from 48.5 ± 2.5 μm/s at time 0 to 62.6 ± 2.4 μm/s after 4 h of incubation) was observed (Table 1). Similar increases were observed in other parameters, such as LIN (from 34.3 ± 2.4% at time 0 to 47.4 ± 2.4% after 4 h of incubation), STR (from 63.1 ± 2.5% at time 0 to 74.9 ± 2.4% after 4 h of incubation) and WOB (from 51.6 ± 2.4% at time 0 to 62.2 ± 2.3% after 4 h of incubation, see Table 1). These results are compatible with the progressive achievement of IVC in the samples studied. Further incubation with progesterone did not have great effects on mean motility parameters, which, in general, showed similar values to those observed in samples incubated in IVC medium for 4 h.

Table 1. Mean values of semen quality and motility parameters of boar spermatozoa subjected to "in vitro" capacitation and further "in vitro" acrosomes reaction

	Capacitation medium					Capacitation medium+Progesterone			
Incubation time	0h	1h	2h	3h	4h	5 min	15 min	30 min	60 min
Viability (%)	72.7 ± 2.1^{a}	69.4 ± 2.0^{a}	54.7 ± 4.2^{b}	54.7 ± 4.4^{b}	52.9 ± 4.5^{b}	51.0 ± 5.0^{b}	47.8 ± 6.4^{b}	45.3 ± 4.7^{b}	44.6 ± 4.6^{b}
Altered acrosomes (%)	22.3 ± 2.5^{a}	29.6 ± 2.9^{b}	41.7 ± 3.5^{c}	41.7 ± 3.7^{c}	42.5 ± 3.7^{c}	46.7 ± 4.2^{c}	60.4 ± 6.7^{d}	68.4 ± 6.2^{d}	72.3 ± 6.2^{d}
True acrosome reaction (%)	2.3 ± 0.9^{a}	3.4 ± 1.1^{a}	3.6 ± 1.2^{a}	4.8 ± 2.0^{a}	4.9 ± 2.0^{a}	9.0 ± 2.1^{b}	23.2 ± 3.0^{c}	30.9 ± 3.5^{d}	34.8 ± 3.9^{d}
Total motility (%)	81.2 ± 1.1^{a}	78.4 ± 1.1^{a}	75.9 ± 1.9^{a}	70.5 ± 1.2^{b}	69.4 ± 0.9^{b}	69.9 ± 1.1^{b}	66.1 ± 1.2^{c}	63.8 ± 1.2^{c}	53.9 ± 0.9^{d}
VCL (μm/s)	88.3 ± 0.6^{a}	87.6 ± 0.9^{a}	87.7 ± 0.3^{a}	90.0 ± 0.8^{a}	98.3 ± 0.5^{b}	101.5 ± 0.7^{b}	106.1 ± 0.6^{b}	106.5 ± 1.0^{b}	107.0 ± 0.9^{b}
VSL (μm/s)	35.0 ± 2.4^{a}	39.4 ± 2.6^{a}	43.5 ± 1.3^{b}	46.5 ± 2.7^{bc}	49.1 ± 1.3^{c}	52.2 ± 2.4^{c}	55.0 ± 1.4^{c}	53.9 ± 1.6^{c}	54.5 ± 1.6^{c}
VAP (μm/s)	48.5 ± 2.5^{a}	51.8 ± 2.5^{a}	57.8 ± 2.2^{b}	59.3 ± 2.5^{bc}	62.6 ± 2.4^{c}	64.8 ± 2.5^{c}	66.8 ± 2.5^{c}	66.0 ± 2.7^{c}	65.7 ± 2.6^{c}
LIN (%)	34.3 ± 2.4^{a}	40.3 ± 2.6^{b}	46.9 ± 2.3^{c}	47.0 ± 2.3^{c}	47.4 ± 2.4^{c}	48.2 ± 2.4^{c}	50.5 ± 2.4^{c}	50.2 ± 2.4^{c}	50.1 ± 2.5^{c}
STR (%)	63.1 ± 2.5^{a}	70.8 ± 2.4^{b}	73.9 ± 2.2^{b}	72.7 ± 2.2^{b}	74.9 ± 2.4^{b}	76.3 ± 2.3^{bc}	79.0 ± 2.5^{c}	79.2 ± 2.6^{c}	79.3 ± 2.7^{c}
WOB (%)	51.6 ± 2.4^{a}	58.4 ± 2.5^{b}	63.9 ± 2.2^{b}	63.6 ± 2.4^{b}	62.2 ± 2.3^{b}	62.0 ± 2.4^{b}	62.2 ± 2.4^{b}	62.0 ± 2.7^{b}	61.2 ± 2.6^{b}
Mean ALH (μm)	4.2 ± 0.1^{a}	3.8 ± 0.1^{b}	3.9 ± 0.1^{b}	$3,8 \pm 0.1^{b}$	4.0 ± 0.1^{ab}	4.3 ± 0.1^{a}	4.4 ± 0.1^{c}	4.4 ± 0.1^{c}	4.4 ± 0.1^{c}
BCF (Hz)	6.5 ± 0.3^{a}	6.4 ± 0.3^{a}	6.2 ± 0.3^{a}	6.4 ± 0.3^{a}	6.7 ± 0.4^{a}	6.7 ± 0.5^{a}	6.8 ± 0.4^{a}	6.7 ± 0.3^{a}	6.9 ± 0.4^{a}

Semen quality, true acrosome reaction and motility parameters have been defined in the Material and Methods section. Results are expressed as means ± S.E.M. for 16 separate experiments, which yielded a total, final number of 11,465 analyzed spermatozoa. Different superscripts in a row indicate significant ($P<0.05$) differences among groups.

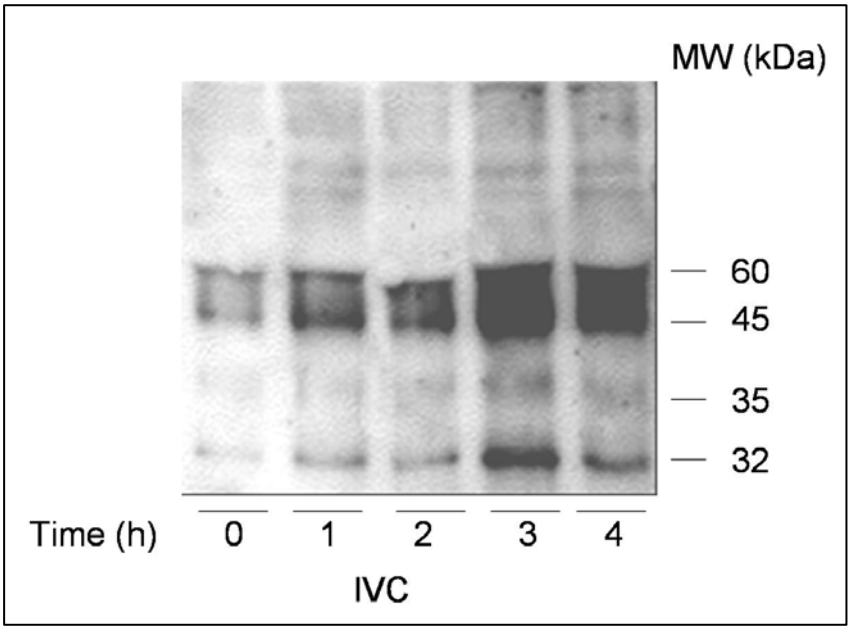

Figure 1. Western blot against the tyrosine-phosphorylated proteins pattern in boar spermatozoa subjected to "in vitro" capacitation. Boar sperm was subjected to "in vitro" capacitation by means of incubation for 4 h in the IVC medium as described in the Material and Methods section. IVC: sperm incubated in the specific IVC medium for the indicated times. MW: Molecular-weight markers. Figure shows a representative image for 16 separate experiments.

1.4.3 Motile sperm subpopulations structure of boar-semen samples subjected to "in vitro" capacitation and further, progesterone-induced acrosome reaction

Our study identified four separate, specific subpopulations of motile sperm in samples from fresh, diluted boar ejaculates. These results were similar to those published before by several authors. In our experimental design, subpopulations were classified taking VCL as the marker point. Under this point of view, subpopulations are characterized as follows (see Table 2):

Subpopulation 1: This subpopulation showed the lowest values of VCL (53.0 ± 1.4 μm/s). Subpopulation 1 was defined by overall low values of velocity, based on the results of VCL, VSL and VAP, low values of linearity, as indicated by values of LIN and STR and low values of oscillatory movement, as indicated by WOB, mean ALH and BCF values (Table 2). Subpopulation 1 was formed by an important percentage of cells, since it included 25.3 ± 1.8% of all motile sperm (Table 2).

Subpopulation 2: This ubpopulation was characterized by the second lowest values of VCL (82.6 ± 1.6 μm/s). Sperm included in Subpopulation 2 showed medium velocity, as indicated by VCL, VSL and VAP, high linearity, as indicated by LIN and STR and high values of oscillatory movement, as indicated by WOB, mean ALH and BCF. The percentage of spermatozoa included in this subpopulation was the highest, reaching 29.8 ± 2.8% of all of motile sperm, although there was not a significant difference between this percentage and that of Subpopulations 1 and 3 (Table 2).

Subpopulation 3. This Subpopulation had high values of VCL (116.6 ± 1.4 μm/s). Subpopulation 3 was constituted by sperm with high velocity and relatively low linearity, as indicated by VCL, VSL, VAP, LIN and STR. Additionally, sperm included in this subpopulation also had a relatively high oscillatory movement, as values of WOB, mean ALH and BCF indicated. The cells included in this subpopulation accounted for 25.9 ± 2.2% of all motile sperm, which was a percentage similar to that of Subpopulations 1 and 2 (Table 2).

Subpopulation 4. Finally, this subpopulation included those sperm with the highest VCL (121.1 ± 1.4 μm/s). Cells included in Subpopulation 4 showed the highest velocity and linearity characteristics, as values of VCL, VSL, VAP, LIN and STR indicated. Furthermore, the overall oscillatory movement of these spermatozoa was also very high, as WOB, mean ALH and BCF values indicated. The percentage of motile sperm included in this subpopulation was the lowest, including only 19.0 ± 2.4% of the total motile sperm (Table 2).

Table 2. Motile-sperm subpopulations structure of fresh, diluted boar semen samples.

	Subpopulation 1	Subpopulation 2	Subpopulation 3	Subpopulation 4
VCL (μm/s)	53.0 ± 1.4^{a}	82.6 ± 1.6^{b}	116.6 ± 1.2^{c}	121.1 ± 1.8^{d}
VSL (μm/s)	10.2 ± 0.9^{a}	37.3 ± 1.1^{b}	26.7 ± 1.0^{c}	65.0 ± 1.2^{d}
VAP (μm/s)	21.3 ± 2.0^{a}	46.1 ± 1.2^{b}	51.2 ± 1.0^{c}	75.4 ± 1.3^{d}
LIN (%)	20.2 ± 1.9^{a}	47.7 ± 1.0^{b}	23.0 ± 1.0^{a}	54.9 ± 1.2^{c}
STR (%)	49.5 ± 1.1^{a}	82.3 ± 1.2^{b}	53.9 ± 1.1^{a}	$86,7 \pm 1.4^{b}$
WOB (%)	41.1 ± 1.9^{a}	57.6 ± 1.1^{b}	44.5 ± 1.1^{a}	63.4 ± 1.2^{c}
mean ALH (μm)	2.76 ± 0.17^{a}	3.78 ± 0.18^{b}	5.01 ± 0.17^{c}	4.87 ± 0.12^{c}
BCF (Hz)	4.3 ± 0.2^{a}	7.3 ± 0.2^{b}	6.6 ± 0.2^{c}	7.9 ± 0.3^{b}
Frequency (%)	25.3 ± 1.8^{a}	29.8 ± 2.8^{a}	25.9 ± 2.2^{a}	19.0 ± 2.4^{b}

Motility parameters have been defined in the Material and Methods section. The parameter “Frequency” indicates the percentage of spermatozoa that are in each subpopulation from the total number of analyzed cells. Results are means ± S.E.M. for 16 separate experiments, which yielded a final number of analized spermatozoa of 322 (Subpopulation 1), 380 (Subpopulation 2), 329 (Subpopulation 3) and 242 (Subpopulation 4). Different superscripts in a row indicate significant ($P<0.05$) differences among groups.

Table 3. Effects of "in vitro" capacitation and further progesterone-induced acrosome reaction on motility parameters of Subpopulations 1 and 2 from boar semen samples.

Subpopulation 1	Capacitation medium					Capacitation medium + progesterone			
Incubation time	0h	1h	2h	3h	4h	5 min	15 min	30 min	60 min
VCL (μm/s)	53.0 ± 1.4^{a}	55.7 ± 0.8^{a}	50.5 ± 0.8^{a}	50.0 ± 0.9^{a}	53.1 ± 1.0^{a}	57.9 ± 1.2^{b}	58.0 ± 1.2^{b}	58.3 ± 1.1^{b}	61.4 ± 1.5^{b}
VSL (μm/s)	10.2 ± 0.9^{a}	13.0 ± 2.6^{ab}	12.8 ± 1.7^{a}	11.1 ± 1.7^{a}	14.9 ± 1.6^{b}	18.6 ± 1.7^{c}	19.2 ± 1.9^{c}	21.1 ± 1.9^{c}	17.8 ± 2.1^{bc}
VAP (μm/s)	21.3 ± 2.0^{a}	24.7 ± 1.5^{a}	23.6 ± 1.5^{a}	21.2 ± 1.6^{a}	24.7 ± 1.7^{a}	30.6 ± 1.8^{b}	25.3 ± 1.8^{a}	27.0 ± 1.8^{ab}	25.6 ± 2.0^{a}
LIN (%)	20.2 ± 1.9^{a}	24.3 ± 1.3^{ab}	26.4 ± 1.3^{b}	22.7 ± 1.4^{a}	28.6 ± 1.6^{b}	30.8 ± 1.7^{b}	24.9 ± 1.7^{a}	26.2 ± 1.8^{ab}	21.0 ± 1.9^{a}
STR (%)	49.5 ± 1.1^{a}	52.4 ± 1.5^{a}	52.3 ± 1.5^{a}	51.5 ± 1.6^{a}	58.2 ± 0.8^{b}	61.0 ± 2.0^{b}	58.7 ± 2.1^{b}	60.0 ± 2.0^{b}	54.5 ± 2.4^{ab}
WOB (%)	41.1 ± 1.9^{a}	45.7 ± 1.4^{a}	44.6 ± 1.4^{a}	43.7 ± 1.5^{a}	48.6 ± 1.7^{b}	54.7 ± 1.9^{c}	48.4 ± 1.9^{b}	49.9 ± 1.8^{b}	44.6 ± 2.1^{a}
Mean ALH(μm)	2.76 ± 0.2^{a}	2.88 ± 0.1^{a}	2.75 ± 0.1^{a}	2.67 ± 0.2^{a}	2.57 ± 0.2^{a}	2.66 ± 0.2^{a}	2.75 ± 0.2^{a}	2.79 ± 0.2^{a}	2.98 ± 0.2^{a}
BCF (Hz)	4.3 ± 0.2^{a}	4.7 ± 0.1^{a}	4.0 ± 0.1^{a}	4.2 ± 0.1^{a}	4.3 ± 0.2^{a}	4.3 ± 0.1^{a}	4.3 ± 0.1^{a}	4.2 ± 0.1^{a}	4.0 ± 0.2^{a}
Subpopulation 2	Capacitation medium					Capacitation medium + progesterone			
Incubation time	0h	1h	2h	3h	4h	5 min	15 min	30 min	60 min
VCL (μm/s)	82.6 ± 1.6^{a}	87.4 ± 1.7^{b}	90.5 ± 1.8^{b}	90.7 ± 1.9^{b}	89.8 ± 1.9^{b}	105.0 ± 2.1^{c}	85.2 ± 2.1^{ab}	95.8 ± 2.0^{d}	77.0 ± 1.6^{e}
VSL (μm/s)	37.3 ± 1.1^{a}	46.0 ± 1.4^{b}	52.8 ± 1.4^{c}	51.4 ± 1.5^{c}	53.1 ± 1.6^{c}	57.7 ± 1.7^{d}	53.5 ± 1.7^{c}	53.5 ± 1.6^{c}	47.3 ± 1.1^{b}
VAP (μm/s)	46.1 ± 1.2^{a}	53.8 ± 1.4^{b}	58.6 ± 1.5^{c}	57.6 ± 1.6^{c}	60.6 ± 1.7^{c}	68.1 ± 1.8^{d}	60.1 ± 1.8^{c}	62.0 ± 1.7^{c}	53.5 ± 1.2^{b}
LIN (%)	47.7 ± 1.0^{a}	53.5 ± 1.4^{b}	59.2 ± 1.4^{c}	57.6 ± 1.4^{c}	55.9 ± 1.6^{b}	85.5 ± 2.2^{c}	86.8 ± 2.2^{c}	86.5 ± 2.1^{c}	84.6 ± 2.7^{c}
STR (%)	82.3 ± 1.2^{a}	84.5 ± 1.5^{a}	88.8 ± 1.6^{b}	87.7 ± 1.6^{b}	87.8 ± 1.0^{b}	85.4 ± 2.0^{ab}	89.1 ± 1.9^{b}	86.6 ± 1.8^{ab}	88.1 ± 1.4^{b}
WOB (%)	57.6 ± 1.1^{a}	62.9 ± 1.4^{b}	66.2 ± 1.4^{b}	65.3 ± 1.4^{b}	69.0 ± 1.8^{b}	66.3 ± 1.8^{b}	71.6 ± 1.8^{c}	66.1 ± 1.7^{b}	70.6 ± 1.5^{bc}
Mean ALH(μm)	3.78 ± 0.2^{a}	3.88 ± 0.2^{a}	3.87 ± 0.1^{a}	3.81 ± 0.1^{a}	3.68 ± 0.2^{a}	4.32 ± 0.2^{b}	3.88 ± 0.2^{a}	3.99 ± 0.1^{a}	3.25 ± 0.2^{c}
BCF (Hz)	7.3 ± 0.2^{a}	7.0 ± 0.1^{a}	7.6 ± 0.1^{a}	7.6 ± 0.1^{a}	7.4 ± 0.2^{a}	7.5 ± 0.2^{a}	6.8 ± 0.2^{a}	7.7 ± 0.2^{a}	6.7 ± 0.3^{a}

Motility parameters have been defined in the Material and Methods section. Results are means±S.E.M. for 16 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences among groups.

Table 4. Effects of "in vitro" capacitation and further progesterone-induced acrosome reaction on motility parameters of Subpopulations 3 and 4 from boar semen samples.

Subpopulation 3	Capacitation medium					Capacitation medium + progesterone			
Time	0h	1h	2h	3h	4h	5 min	15 min	30 min	60 min
VCL (μm/s)	116.6 ± 1.2^{a}	114.6 ± 1.7^{a}	114.3 ± 1.7^{a}	116.0 ± 1.0^{a}	109.5 ± 1.2^{b}	127.9 ± 1.5^{c}	134.8 ± 1.3^{d}	135.7 ± 1.4^{d}	140.9 ± 1.7^{e}
VSL (μm/s)	26.7 ± 1.0^{a}	30.0 ± 1.4^{ab}	35.3 ± 1.5^{b}	31.1 ± 1.6^{ab}	32.0 ± 0.8^{ab}	29.7 ± 1.8^{a}	43.0 ± 1.7^{c}	33.1 ± 1.8^{ab}	48.1 ± 2.0^{c}
VAP (μm/s)	51.2 ± 1.0^{a}	50.2 ± 1.5^{a}	52.7 ± 1.6^{a}	51.2 ± 1.7^{a}	53.2 ± 0.8^{a}	49.1 ± 1.9^{a}	61.6 ± 1.8^{b}	59.5 ± 1.9^{b}	62.6 ± 2.1^{b}
LIN (%)	23.0 ± 1.0^{a}	26.3 ± 1.5^{ab}	30.9 ± 1.5^{b}	27.0 ± 1.6^{ab}	33.8 ± 1.7^{b}	21.9 ± 1.7^{a}	27.2 ± 1.6^{ab}	20.8 ± 1.6^{a}	29.4 ± 1.7^{b}
STR (%)	53.9 ± 1.1^{a}	51.7 ± 1.5^{a}	56.9 ± 1.6^{b}	54.6 ± 1.8^{ab}	61.6 ± 1.0^{b}	61.7 ± 1.1^{b}	70.4 ± 1.9^{c}	58.3 ± 2.0^{b}	77.1 ± 1.1^{d}
WOB (%)	44.5 ± 1.1^{a}	46.6 ± 1.4^{a}	52.9 ± 1.5^{b}	52.9 ± 1.6^{b}	48.9 ± 1.8^{b}	43.7 ± 1.9^{a}	51.7 ± 1.8^{b}	50.0 ± 1.8^{b}	50.6 ± 2.1^{b}
Mean ALH(μm)	5.01 ± 0.2^{a}	5.06 ± 0.1^{a}	5.02 ± 0.1^{a}	5.02 ± 0.1^{a}	5.08 ± 0.2^{a}	5.36 ± 0.2^{b}	5.50 ± 0.2^{b}	5.39 ± 0.2^{b}	5.54 ± 0.2^{b}
BCF (Hz)	6.6 ± 0.2^{a}	6.0 ± 0.1^{a}	6.0 ± 0.1^{a}	6.1 ± 0.2^{a}	6.6 ± 0.2^{a}	6.6 ± 0.2^{a}	6.7 ± 0.2^{a}	6.4 ± 0.2^{a}	7.3 ± 0.2^{b}
Subpopulation 4	Capacitation medium					Capacitation medium + progesterone			
Time	0h	1h	2h	3h	4h	5 min	15 min	30 min	60 min
VCL (μm/s)	121.1 ± 1.8^{a}	123.9 ± 1.7^{a}	127.7 ± 1.8^{b}	128.9 ± 1.8^{b}	129.6 ± 1.3^{b}	137.0 ± 1.7^{c}	127.2 ± 1.9^{b}	125.8 ± 1.5^{ab}	125.8 ± 1.5^{ab}
VSL (μm/s)	65.0 ± 1.2^{a}	79.1 ± 1.5^{b}	85.9 ± 1.5^{c}	83.9 ± 1.5^{bc}	89.6 ± 1.8^{c}	101.6 ± 1.2^{d}	91.5 ± 1.7^{cd}	88.2 ± 1.1^{c}	92.6 ± 1.0^{cd}
VAP (μm/s)	75.4 ± 1.3^{a}	88.5 ± 1.5^{b}	91.5 ± 1.6^{b}	90.7 ± 1.6^{b}	97.1 ± 1.1^{c}	101.5 ± 1.3^{c}	97.5 ± 1.8^{c}	97.3 ± 1.2^{c}	98.7 ± 1.2^{c}
LIN (%)	54.9 ± 1.2^{a}	64.4 ± 1.5^{b}	67.8 ± 1.5^{b}	65.6 ± 1.5^{b}	69.9 ± 1.0^{b}	74.6 ± 1.1^{c}	72.3 ± 1.7^{c}	70.8 ± 1.0^{bc}	74.4 ± 1.0^{c}
STR (%)	$86,7 \pm 1.4^{a}$	89.6 ± 1.6^{a}	93.8 ± 1.6^{b}	92.4 ± 1.6^{b}	92.0 ± 0.9^{b}	92.3 ± 1.4^{b}	93.7 ± 1.8^{b}	90.8 ± 1.2^{ab}	93.9 ± 1.2^{b}
WOB (%)	63.4 ± 1.2^{a}	72.0 ± 1.4^{b}	72.3 ± 1.4^{b}	71.0 ± 1.5^{b}	75.7 ± 1.8^{b}	80.6 ± 1.2^{c}	76.7 ± 1.7^{bc}	77.8 ± 1.0^{c}	78.8 ± 1.0^{c}
Mean ALH(μm)	4.87 ± 0.12^{a}	4.49 ± 0.14^{a}	4.66 ± 0.14^{a}	4.72 ± 0.14^{a}	4.57 ± 0.17^{a}	4.46 ± 0.19^{ab}	4.42 ± 0.16^{ab}	4.26 ± 0.18^{b}	4.22 ± 0.18^{b}
BCF (Hz)	7.9 ± 0.3^{a}	7.6 ± 0.1^{a}	8.2 ± 0.2^{a}	8.4 ± 0.3^{a}	8.3 ± 0.2^{a}	7.9 ± 0.3^{a}	8.2 ± 0.2^{a}	7.8 ± 0.2^{a}	8.0 ± 0.2^{a}

Motility parameters have been defined in the Material and Methods section. Results are means±S.E.M. for 16 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences among groups.

Incubation for 4 h in the IVC had different effects on the values of the separated motion parameters depending on each subpopulation. As example, whereas only LIN, STR and WOB saw a slight, significant ($P<0.05$) increase of their values in Subpopulation 1 only after 4 h of incubation, VCL, VSL, LIN, STR and WOB showed a time-dependent increase in Subpopulations 2 and 4, which started to be significant after 1 h of incubation and reached maximal values after 4 h (Tables 3 and 4). Another example can be showed regarding Subpopulation 3 which showed a clearly appreciable increase of VSL, LIN, STR and WOB after 4 h of incubation, whereas VCL decreased in this subpopulation after the same time of treatment (Table 4). Notwithstanding, all of the determined significant modifications as are shown in Tables 3 and 4 were not great, since the maximal difference was that observed for LIN of Subpopulation 3, which went from 23.0 ± 1.0% at time 0 to 33.8 ± 1.7% after 4 h of incubation, which represented a percentage increase of 46.9% (Table 4).

The addition of progesterone to the IVC medium after 4 h of incubation induced further changes on motility parameters of each subpopulation, although again the intensity of these changes varied with the subpopulation. Thus, Subpopulation 1 showed an increase of VCL, VSL, VAP and WOB when compared with the results obtained after 4 h of incubation that was already significant after 5 min of incubation with progesterone and, after this, motion values progressively decreased (Table 3). Mixed, more complex effects were observed in the other subpopulations although, in general, there were increases of separate motion parameters which were generally appreciable after only 5 min of incubation (Tables 3 and 4). It is noteworthy that incubation with progesterone also induced changes in mean ALH, which saw a significant ($P<0.05$) increase after 5 min of incubation with progesterone followed by a decrease in Subpopulation 2, an increase in Subpopulation 3 after 5 min of incubation, that was maintained after 1 h (mean ALH values of 5.54 ± 0.18 μm after 1 h with progesterone when compared with 5.01 ± 0.16 μm at time 0 and 5.08 ± 0.16 μm after 4 h of incubation, see Table 4) and a decrease in Subpopulation 4 after 30 min and 1 h of incubation with progesterone (Table 4). Furthermore, BCF values significantly ($P<0.05$) increased in Subpopulation 1

from 6.6 ± 0.2 Hz in both fresh samples and after 4 h of incubation in IVC medium to 7.3 ± 0.2 Hz after additional incubation with progesterone for 1 h (Table 3). Nevertheless, the observed changes were not very intense, since the maximal difference was observed in LIN of Subpopulation 2, which rose from 55.9 ± 1.6% after 4 h of incubation in the IVC medium to 86.8 ± 2.2% after 15 min of further incubation with progesterone, which represents a percentage increase of 54.7% (Table 3).

The percentage of sperm included in each subpopulation underwent important and significant changes that were related to incubation in the IVC medium. Thus, as shown in Figure 2A, Subpopulation 1 showed a slight, significant ($P<0.05$) increase in its population percentage, since it rose from 25.3 ± 1.8% of total motile sperm at time 0 to 31.0 ± 1.6% after 4 h of incubation. A greater, significant increase ($P<0.05$) was observed in the percentage of motile sperm included in Subpopulation 4, which rose from 19.0 ± 2.4% of total motile sperm at time 0 to 30.1 ± 0.6% after 4 h of incubation (Figure 2A). Concomitantly, Subpopulations 2 and 3 showed a time-dependent, slight and significant ($P<0.05$) decrease in their percentages. In this way, Subpopulation 2 fell from 29.8 ± 2.8% at time 0 to 21.6 ± 0.8% after 4 h of incubation, whereas Subpopulation 3 decreased from 25.9 ± 2.2% at time 0 to 17.3 ± 1.1% after 4 h of incubation (Figure 2A).

The addition of progesterone to the IVC medium after 4 h of incubation induced further changes in the percentage of motile sperm included in each subpopulation. Hence, as shown in Figure 2B, progesterone induced a rapid increase in sperm included in Subpopulation 3, which was concomitant to a parallel decrease of sperm from Subpopulation 4. These changes were evident after only 5 min of incubation, and Subpopulation 3 reached 30.0 ± 2.2% of the total motile-sperm population after 60 min of incubation with progesterone, whereas Subpopulation 4 included 20.6 ± 3.2% of the total motile-sperm population after 60 min of incubation with the hormone (Figure 2B). Furthermore, Subpopulation 1 also underwent a gradual decrease in the percentage of sperm included in it, which dropped from 31.0 ± 1.6%

immediately before the addition of progesterone to 23.1 ± 1.0% after 1 h of incubation with the hormone (Figure 2B).

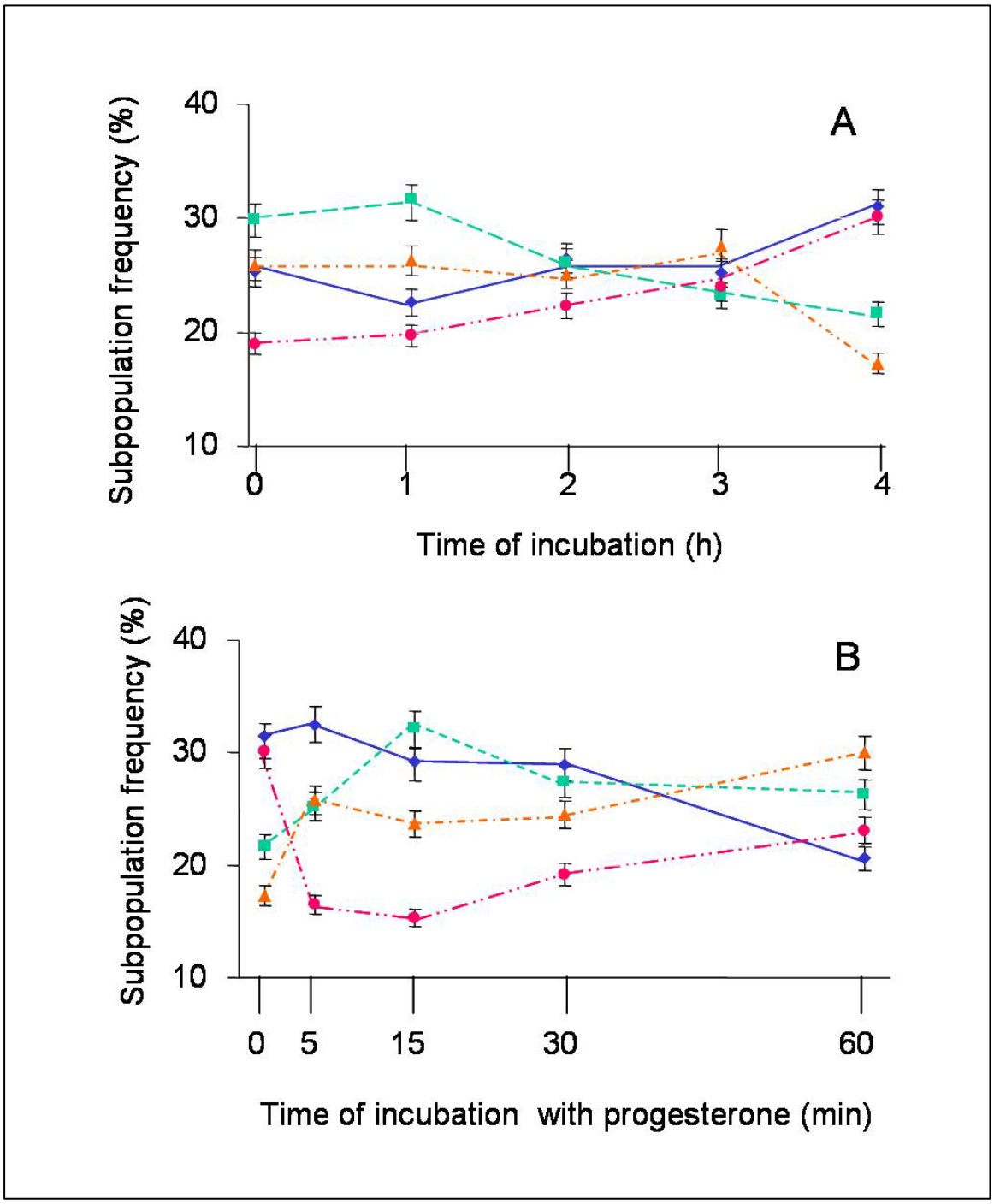

Figure 2. Boar motile-sperm subpopulations distribution after "in vitro" capacitation and further "in vitro" acrosome reaction. Boar sperm was subjected to "in vitro" capacitation through incubation for 4 h in the IVC medium and were then further subjected to progesterone-stimulated "in vitro" acrosome reaction (marked with arrows) as described in the Material and Methods section. Motile sperm subpopulations have been defined in the Material and Methods section. ◆: Subpopulation 1. ■: Subpopulation 2. ▲: Subpopulation 3. ●: Subpopulation 4. Results shown are means±S.E.M. for 16 separate experiments.

1.5 Discussion

Our results suggest that the motility changes associated with the IVC process in boar sperm would be related to specific effects on each motile-sperm subpopulation of the boar ejaculate, which responds with different sensitivities to the IVC stimulus. In this sense, the main subpopulation responsible for the increase of the mean motion parameters after IVC was Subpopulation 4. This subpopulation contained sperm cells with the maximal values of both velocity and linearity, and, after 4 h of incubation in the IVC medium, we observed both an increase in the values of the main motion parameters of this subpopulation and an increase in the percentage of motile sperm included in it. We suggest that this double effect can be induced by two ways, the first an activating effect that the incubation in the IVC medium causes on sperm, and the second, the loss of motility of sperm that was not included in Subpopulation 4, specially those from Subpopulation 1. Regarding the activating effect caused by incubation in the IVC medium by itself, our results indicate that this incubation has a different effect on each subpopulation. In this way, sperm from Subpopulation 3 did not practically modify its velocity characteristics, although linearity increased, whereas that from Subpopulations 2 and 4 showed significant increases in practically all of the motion parameters analyzed. This different sensitivity to incubation in IVC medium is not surprising, and, in this way, it has been described that boar sperm from separate subpopulations have different sensitivities in reacting against the addition of activators like caffeine and bicarbonate to the medium (Holt, 1996). In the same sense, it has been described that the separation through a Percoll gradient of human semen renders separate sperm subpopulations with different abilities to undergo capacitation depending on their overall Tyr-Phos status (Buffone *et al.*, 2004). A similar relationship between semen subpopulations and sperm functional differences has been reported in dog ejaculates, in which the addition of both glucose and fructose to a plain incubation medium induces the formation of a specific subpopulation with motion characteristics that reminds us of hyperactivation (Rigau *et al.*, 2001). Thus, all of these reports clearly suggest that sperm included in separate subpopulations shows specific differences in its

ability of response to stimulus such as IVC and incubation with energy substrates.

One of the most important consequences that was derived from the above-commented sensitivity differences of each subpopulation to IVC is that this process and the further, associated progesterone-induced IVAR induces a progressive change in several motion characteristics and, more importantly, in the percentage of motile spermatozoa included in each subpopulation. In this sense, although total motility progressively decreased during the IVC and additional IVAR processes, an increase in most of the motility parameters is present in all subpopulations during IVC. In fact, the observed changes in the overall motility parameters are concordant with the typical hyperactivated movement characterisitics of capacitated cells (Yanagimachi, 1994). However, although the increases in motility parameters in each subpopulation after IVC are evident, these increases are not intense enough to completely explain the mean, overall changes on motility observed in capacitated sperm when the whole sample was analyzed without considering subpopulations. This indicates that the changes in motility parameters in each subpopulation are not the only factor that explains the results obtained in mean motility values. The other factor that significantly contributes to the mean results is the observed changes in the percentage distribution of motile sperm in each subpopulation. Thus, it is important to emphasize that at the end of capacitation process, the subpopulation that showed the highest percentage was Subpopulation 4, which is precisely the subpopulation with the highest velocity and linearity parameters. Nevertheless, the addition of progesterone induced a strong and fast decrease of Subpopulation 4 that was concomitant with an increase in Subpopulations 2 and 3. The interpretation of these results is difficult, although they indicate that the sperm included in each subpopulation has different sensitivities in its response to the acrosome-reaction agent. Despite this, the results suggest that the further increase in mean velocity observed in sperm after the addition of progesterone would be related to the increase of sperm from Subpopulations 2 and 3. Since Subpopulation 4 was the most affected after progesterone addition, we would suggest that the majority of sperm that was incorporated in Subpopulations 2 and 3 at the start of IVAR were from Subpopulation 4,

although this suggestion needs more experimental support to be totally accepted. On the whole, the results would suggest that the IVC processwould stimulate the incorporation of sperm to Subpopulation 4, with motion characteristics similar to those classically described for capacitated sperm (Yanagimachi, 1994). However, after the achievement of IVC, the further activation of IVAR would stimulate the transfer of sperm from Subpopulation 4 to Subpopulations 2 and 3, this change marking the achievement of IVAR. This indicates that cells from these subpopulations would be prone to being subjected to IVAR. Since IVAR is completely specific of previously capacitated sperm (Zaneveld *et al.*, 1991; Breitbart, 2003), our results would indicate that a high percentage of cells that were subjected to IVAR would be those that were included in Subpopulation 4 after incubation for 4 h in the IVC medium. These results would be in accordance with the described existence of an initial pre-selection of boar sperm to undergo capacitation which has been suggested by other authors. In this sense, Satake *et al.* (2006) indicates that the interaction of sperm with oviductal proteins is related to the sensitivity of this sperm to respond to bicarbonate stimulation, thus relating the specific sensitivity of each sperm to undergoing capacitation to its ability to reach oocytes. In this way, the existence of a certain pre-determination to undergo capacitation in boar sperm from fresh samples should be considered when evaluating the fertilizing ability of boar ejaculates.

It is noteworthy that the overall, four-subpopulations structure defined in fresh samples was not essentially changed throughout all of the IVC and further "in vitro" acrosome reaction processes. In fact, the observed motility changes were caused in such a manner that the four-subpopulations structure was maintained at every moment. The constant existence of a four-subpopulations structure in boar sperm has already been defined in previous reports (Rivera *et al.*, 2005; Rivera *et al.*, 2006), whereas references of a three-subpopulations structure could be related to differences in the statistical model applied in each case (Abaigar *et al.*, 1999; Quintero-Moreno *et al.*, 2004). In any case, our results seem to indicate that boar ejaculates tend to have a constant, specific motile-sperm subpopulation structure that could be considered as an inherent characteristic of these ejaculates. Moreover, boar ejaculates tend to maintain

this inherent structure in all conditions, since variations in sperm characteristics like motility seem to be related more to a change in characteristics and percentages of each subpopulation than to the appearance or disappearance of one or more subpopulations. It is not known what the exact explanation is for this behavior. However, results seem to indicate that the existence of a specific four-subpopulations structure is an important characteristic of boar ejaculates, and the maintenance of this structure has to be important for the full development of the boar-sperm physiology.

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CAPITOL / CHAPTER 2:

"In vitro" capacitation and further "in vitro", progesterone-induced acrosome reaction are linked to specific changes in the expression and acrosome location of protein phosphorylation in serine and threonine residues of boar spermatozoa.

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2.1 Abstract

The aim of this study is the establishment of a general overview of the amount and specific location of protein phosphorylation during "in vitro" capacitation (IVC) and further progesterone-induced "in vitro" acrosome reaction (IVAR) in boar spermatozoa. With this purpose, boar sperm was incubated for 4 hours in an IVC medium, and afterwards progesterone-induced IVAR was performed. Samples were taken at 0 h and 4 h of incubation in the IVC medium and also after 5 min and 60 min of progesterone induction. The mono-dimensional (1D) analysis of protein phosphorylation in tyrosine (pTyr), serine (pSer) and threonine (pThre) residues indicated the presence of specific, IVC- and IVAR-associated changes. Among them, the appearance of a specific, approximately 32-kDa band of pTyr after the attainment of IVC that was maintained during IVAR which is similar to that previously described elsewhere. Furthermore, there was an increase in the overall intensity of pTyr, pSer and pThre patterns during the attainment of IVC. However, whereas the overall pTyr and pThre intensity decreased during IVAR, this intensity was gradually increased after progesterone addition. Bi-dimensional (2D) analysis showed separate effects depending on the protein phosphorylation studied. Thus, the pTyr pattern underwent changes that were in accordance with the appearance of the specific 32-kDa protein described above, as well as with structural changes in other proteins that caused an increase in different protein structures with a broader range of pI. The induction of IVAR decreased the pTyr phosphorylation of the majority of the detected proteins, whereas the most intense pTyr signal observed at 0 h and 4 h of incubation in the capacitation medium was separated into 3 recognizable marks of about 40-45 kDa and pI 7.5, 35 kDa and pI 7.5 and

34 kDa and pI 8. The pSer pattern showed a very complex structure, which was greatly modified by IVC by the disappearance of several specific marks, accompanied by the new appearance of specific pSer signals, specially one of about 75 kDa and a pI of about 9.2, and another of about 80 kDa and a pI of about 9.6. Subsequent IVAR induction caused an overall decrease, and even disappearance, of pSer signals, which were accompanied by the appearance of a new, IVAR-linked pSer spot of about 75 kDa and a pI of 6-7. Regarding pThre, the induction of IVC caused great changes in the observed patterns, with a general decrease in the intensity signal and an increase in different protein structures with a broader range of pI, similarly to that observed in pTyr. The induction of IVAR caused a new increase in the intensity of the observed marks, with an increase in the pI ranges of the most intense signals. Regarding the specific location of protein phosphorylation, pTyr, pSer and pThre in cells before IVC were mainly located at the whole tail (pTyr and pThre) or at the midpiece (pSer), as well as at the equatorial zone of the head in the case of pTyr. The attainment of IVC displaced the main pTyr signal to the principal and terminal pieces of the tail. However, IVC induced the appearance of a clear, specific signal of pSer at the acrosome, which was greatly increased in its intensity after the induction of IVAR. The appearance of a specific acrosomal signal was also apparent for pThre, but only after 5 min of the progesterone induction of IVAR. Both tail pSer and pThre increased their intensity after IVAR, although this increase was only maintained for pSer, but not for pThre, after 60 min of incubation with progesterone. Our results indicate that the changes in protein phosphorylation associated with IVC and subsequent IVAR affected not only pTyr, but also pSer and pThre, and they comprise not only the appearance of specific phosphorylated proteins, like pTyr 32-kDa, pSer-75 kDa and pSer 80-kDa proteins, but also structural changes that induce changes in pI and displacements in the sperm location of the phosphorylated proteins, such as the appearence of specific IVC- and IVAR-linked pSer and pThre signals in the acrosome.

2.2 Introduction

Sperm capacitation has been defined as a gradual, essential event that acts as a prerequisite for fertilization (Austin, 1951; Chang, 1951; Yanagimachi, 1994). "In vivo" capacitation takes place during the sequential exposure of spermatozoa to the different compartments of the female genital tract that occurs during sperm transport. This definition has changed from a mere temporal event of the spermatozoon in the female reproductive tract to a more complex process reflecting all of the physiological, biochemical and biophysical changes that spermatozoa undergoes in the female reproductive tract in order to become fertilization-competent (Jha and Shivaji, 2002).

In a general sense, capacitation results in altered plasma-membrane architecture and permeability, which ultimately modulates flagellar activity and renders the sperm apical head plasma-membrane fusogenic (Rodríguez-Martínez, 2007). This would permit the exocytotic process called acrosome reaction (AR), which enables the fusion of both gametes. Furthermore, capacitation includes structural changes of protein-lipid organization, plasma membrane fluidification, activation of ion channels, calcium uptake, generation of cAMP and production of reactive oxygen species (ROS) (Tardif *et al.*, 2001). The knowledge of all of these events has allowed for the ability to sustain both "in vitro" capacitation (IVC) and "in vitro" acrosome reaction (IVAR) by using defined media with a composition that attempts to mimic the female reproductive tract environment (Visconti and Kopf, 1998).

One of the most important mechanisms involving the modulation of the structural and functional changes that sperm undergo during capacitation is linked to post-translational modifications through phosphorylation of serine (pSer), threonine (pThre) and tyrosine residues (pTyr) of specific sperm proteins (Jha *et al.*, 2006). This is a general mechanism that is present in all cells, playing a major role in many cellular processes including the transduction of extracellular signals, intracellular transport and cell-cycle progression (Jha *et al.*, 2006). The phosphorylation state of phosphoproteins is controlled by the activity of a complex system of specific protein kinases (PK) and phosphatases.

Regarding mature mammalian spermatozoa, it must be stressed that they are highly specialized cells, with a very high degree of cellular compartmentalization, besides being transcriptionally inactive and unable to synthesize new proteins. Therefore, that the reliance of a mature spermatozoon on protein phosphorylation as a general mechanism of modulation of cellular function is greater than in many other types of cell (Urner and Sakkas, 2003). In this way, protein phosphorylation is involved in the regulation of sperm functions like motility (Vijayaraghavan *et al.*, 1997; Tash and Means, 1983) and zona pellucida (ZP) recognition, affinity and AR (Leyton and Saling, 1989; Naz and Ahmad, 1994; Flesh and Gadella, 2000; Asquith *et al.*, 2004). Even more, pTyr has just been hypothesized as being able to regulate glycolysis in sperm (Arcelay *et al.*, 2008). In this way, it is not surprising that in species such as mouse (Visconti *et al.*, 1995), human (Leclerc *et al.*, 1996), bovine (Galantino-Homer *et al.*, 1997), pig (Kalab *et al.*, 1998) and stallion (Pommer *et al.*, 2003) capacitation is directly linked to the pTyr of sperm proteins in a protein kinase A (PKA)-dependent manner (Tardif *et al.*, 2001).

The discovery that sperm capacitation in all mammalian species studied to-date is associated with the appearance of pTyr proteins has been paramount in proving that capacitation is a regulated, signal transduction-mediated event (Dubé *et al.*, 2005). Identification of these tyrosine-phosphorylated substrates had been one of the principal aims in the study of the process of phosphorylation during capacitation. Some of the most important proteins that are tyrosine-phosphorylated during capacitation are the calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein (CABYR; Naaby-Hansen *et al.*, 2002), the A-kinase anchoring protein 3 (AKAP 3) and its pre-A-kinase anchoring proteins AKAP 82 and AKAP 83 (Carrera *et al.*, 1994; Carrera *et al.*, 1996; Jha and Shivaji, 2002), endoplasmin and heath-shock protein 60 (Asquith *et al.*, 2004), aldolase 1 and 2, pyruvate dehydrogenase and voltage-dependent anion channel 2 among others (Arcelay *et al.*, 2008). Focusing on boar sperm, Green and Watson (2001) described an increase in the number and expression of tyrosine-phosphorylated proteins ranging from 33 kDa to 220 kDa after incubation in a capacitation medium. More specifically, capacitation in boar sperm is associated with a specific increase of pTyr of a 32-kDa protein,

which was identified as proacrosin-binding protein (Tardiff *et al.*, 2001; Dubé *et al.*, 2005). This is important, since a positive correlation has been described between levels of p32 pTyr and the percentage of capacitated or acrosome-reacted boar spermatozoa (Bravo *et al.*, 2005), thus indicating that this is an essential phenomenon for boar-sperm capacitation.

There is less information regarding both pSer and pThre changes during capacitation and AR. Despite this, it has been shown that both PKA and PKC activities increase during IVC and subsequent IVAR (Visconti and Kopf, 1998; Breitbart and Naor, 1999). This could be linked with the existence of changes in both the overall pSer and pThre levels in human sperm, which are also related to an increase in the phosphorylation of the fertilization antigen-1 (FA-1) during IVC (Naz, 1999). Some relationship between Ser and pThre and the development of a feasible AR would exist, however, since a 100 kDa, acrosomal protein in hamster sperm is phosphorylated in specific Ser and Thre residues during IVC (Jha and Shivaji, 2002). At the same time, a decrease in the pSer and pThre phosphorylation levels of an unidentified, postacrosomal protein in boar sperm during IVC has been described (Adachi *et al.*, 2008). It has been suggested that these changes in acrosomal and post-acrosomal proteins would be involved in the suppression of premature AR before and immediately after ejaculation. However, further knowledge is needed in order to show a more overall vision about the importance of pSer and pThre in the modulation of sperm IVC and IVAR.

The main aim of this work is to display an overall vision of the dynamics of pTyr, pSer and pThre during IVC and subsequent, progesterone-induced IVAR, in order to deepen our knowledge in the dynamics of the overall protein activation mechanisms that are modulated during these processes. For this purpose, we have analyzed pTyr, pSer and pThre through Western blot analyses, in order to determine the intensity of protein phosphorylation. The Western blot analyses were performed after both mono- and bi-dimensional electrophoresis, to obtain a more in-depth display of the changes in the boar-sperm protein phosphorylation status during IVC and IVAR. Finally, changes in pTyr, pSer and pThre were also analyzed through immunocytochemistry to

determine changes not only in the intensity, but also in the location of phosphorylated proteins, which would be strongly linked to local changes of protein activity related to the IVC and IVAR processes.

2.3 Material and Methods

2.3.1 Boar-semen obtainment

All procedures described within were approved, when needed, by the Autonomous University of Barcelona Animal Care and Use Committee and were performed in accordance with the Animal Welfare Law issued by the Catalan Government (Generalitat de Catalunya, Spain) .

Semen was obtained from boars of proven fertility ranging in age of 1-3 years, which were from a regularly managed commercial farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Spain). Ejaculates were manually collected, and they were immediately diluted at a final sperm concentration of 2 x10 ^{7} sperm/mL in a commercial dose extender for refrigerated semen (MR-A Extender; Kubus, S.A.; Majadahonda, Spain). Diluted semen was then distributed in 100-mL commercial, artifical insemination (AI) doses. Six of the 100-mL doses obtained, chosen at random and coming from different boars from the same farm, were placed in portable refrigerator at 16 ºC for approximately 45 minutes, which was the time required to arrive at the laboratory as previously reported (Ramió *et al.*, 2009).

2.3.2 "In vitro" capacitation and acrosome reaction procedures.

Fifty mL of each AI dose were washed three times by centrifugation at 600 x g for 5 min at 16ºC and re-diluted in a 20-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), containing 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (NCM). After the last wash, the sperm was resuspended in a specific capacitation medium (CM) at a final concentration of 50-70x10^{6} sperm/mL. Incubation in CM was

maintained for 4 h at 38.5ºC in a 5% CO_2 atmosphere, as described in Ramió *et al.* (2008). The CM was the same NCM added to 5 mg/mL bovine serum albumin (BSA). The osmolarity of the CM was 304 ± 5 mOsm and the pH was adjusted at 7.4. Sperm aliquots were taken at 0, 1, 2, 3 and 4 hours of incubation in order to perform the appropriate analysis.

The induction of IVAR was carried out through incubation in the presence of progesterone, as described before (Jimenez *et al.*, 2003; Wu *et al.* 2006). For this purpose, after the incubation of boar sperm in CM for 4 h at 38.5ºC in a 5% CO_2 atmosphere, progesterone was added and thoroughly mixed to a final concentration of 10 µg/mL. Boar sperm was further incubated for an additional 1 h at 38.5ºC in a 5% CO_2 atmosphere and aliquots were taken after 5 min and 60 min after progesterone addition.

Mono- (1D) and bi-dimensional (2D) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed from 1.5-mL aliquots of boar sperm. Cells were first centrifugated at 10,000 x g for 30 s and the resultant pellet was immediately frozen at -196 ºC in liquid N_2 and stored at -80ºC before use. Furthermore, other 100-µL aliquots of sperm were fixed with 300 µL of 2% paraformaldehyde for 1 hour and stored at 4ºC before performing indirect immunofluorescence procedures.

2.3.3 Boar-Semen Quality-Analysis Procedures

Percentages of viability, altered acrosomes and true acrosomal reaction were determined by using the double-staining fluorescein isothiocyanate-conjugated Arachis hypogaea (PNA) lectin and propidium iodide stain (PNA-FITC/IP stain) as described in Bussalleu *et al.* (2005). These percentages were determined after counting 200-300 spermatozoa per slide at 1000x. Altered acrosomes were considered to be those which did not show a clear and uniform PNA lectin staining, whereas those which were considered as being subjected to a true acrosomal reaction showed, after the stimulation of IVAR, positive viability and disruption of the acrosome PNA, stain as referred to by Ramió *et al.* (2008).

2.3.4 Application in Boar-Sperm Samples of both 1D- and 2D-SDS Polyacrylamide Electrophoresis

For 1D SDS-PAGE, sperm pellets were homogenized by sonication in 200 µl of ice-cold 50-mM Tris-HC buffer (pH 7.4) containing 1 mM EDTA, 10 mM EGTA, 25 mM DTT, 1.5% (w/v) Triton X-100, 1mM phenylmethyl sulfonyl fluoride (PMSF), 1mM benzamidin, 10 µg/mL leupeptin and 1 mM Na_2VO_4, this latter added to avoid changes in the overall phosphorylation of the homogenates. Samples were then centrifuged at 13,000 x g for 15 min at 4 ºC. Supernatants were recovered and total protein content of the samples was determined through the Bradford method (Bradford, 1976) by using a commercial kit (BioRad Laboratories; Hercules, CA, USA). Supernatants were immediately stored immediately at -80 ºC until their use. The sperm protein samples were boiled for 1 minute before being transferred to the SDS-gel, and SDS-PAGE was carried out following the standard protocol established by Laemmli (1970). The total amount of protein loaded in each lane was 15 µg.

For 2D SDS-PAGE, the first step was to eliminate lipids and DNA through a trichloroacetic acid/acetone washing. For this purpose, frozen pellets were resuspended in 250 µL of an ice-cold 8M urea buffer with 2% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) being added. This cell suspension was immediately mixed with 250 µL of ice-cold 10% (w/v) trichloroacetic acid (TCA). The mixture was gently mixed for 1h at 4ºC and afterwards it was centrifuged at 11,000 x g for 10 min at 4ºC and the resultant supernatant was discarded. The obtained pellets were washed with 500 µL of ice-cold 10% TCA and samples were again centrifuged at 11,000 x g for 10 min at 4ºC. Supernatants were again discarded and the resultant pellets were washed with 1 mL of freezing-cold (-20ºC) acetone. Suspensions were centrifuged at 11,000 x g for 10 min at 4ºC, supernatants were carefully discarded and the resultant pellets were left to air dry for 5 min.

After the TCA/acetone washing, the resultant pellets were resuspended in 250 μL of an ice-cold 8M urea buffer with 2% (v/v) CHAPS, 18 mM dithiotreitol (DTT) and 1% (v/v) of IPG ampholite buffer (GE Healthcare; Wikströms, Sweden) being added. The resuspended pellets were then sonicated in order to obtain an homogeneous protein dilution. Afterwards, a 2D-SDS-PAGE was carried out following a standard protocol established by O'Farrell (1975). The total amount of protein loaded in each lane was 15 μg.

2.3.5 Western Blot Analysis

The Western blot analysis was carried out on both 1D and 2D SDS-PAGE following the standard protocol of transferring the SDS-PAGE to nitrocellulose membranes (Burnette, 1981). Transference was tested through the staining of membranes with Red Poinceau stain (Bannur *et al.*, 1999), which also allowed for the determination that the presence of BSA in the medium did not interfere with the position of phosphorylated proteins in the Western blot following 2D-SDS PAGE (see Figure 1). Transferred samples were tested by applying an anti-pTyr antibody (PY-20; Chemicon International; Temecula, CA, USA) at a final dilution of 1:1,000 (v/v), an anti-pSer or an anti-pThreo antibody (Zymed Laboratories; Carlsbad, CA, USA), these latter two at a final dilution of 1:200 (v/v). Immunoreactive proteins were detected by using peroxidase-conjugated anti-mouse (for pTyr) or anti-rabbit (for pSer and pThreo) secondary antibody (Amersham; Buckinghamshire, UK). The reaction was developed with an ECL-Plus detection system (Amersham; Buckinghamshire, UK).

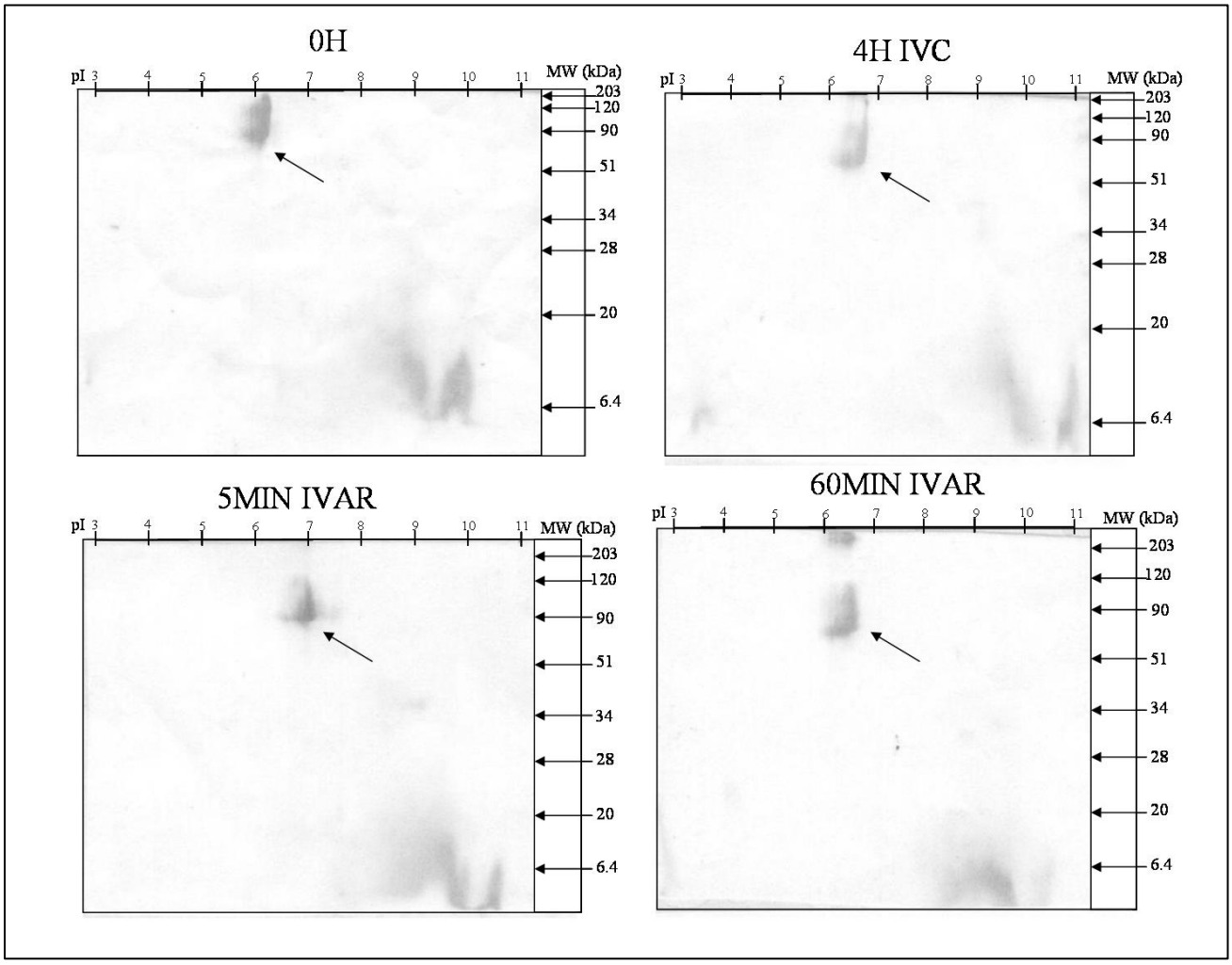

Figure 1. Detection of total protein pattern in bi-dimensional transferred samples from 2D-SDS-PAGE by Ponceau Red. Arrows indicate the location of bovine serum albumin, which was added to the capacitation medium and, hence, it is not a sperm protein.

2.3.6 Boar Sperm Immunocytochemistry following Indirect Immunofluorescence

Aliquots of 50 µL of the previously described paraformaldehyde-fixed sperm samples were spread onto poly-lysined (Poly-L-lysine solution 1 % w/v in H_2O; Sigma; Saint Louis, MO, USA) microscope slides and then were left to air dry. After this, samples were permeabilized by incubation for 10 min at room temperature in a standard phosphate-buffered solution (PBS; pH 7.4) added to 0.25% (v/v) Triton x-100. Afterwards, slides were washed three times with PBS

and they were then blocked through incubation with PBS including 0.1% (v/v) Tween-20 and 1% (w/v) BSA for 30 min at room temperature. Incubation with the respective primary antibody (pTyr at a final dilution of 1:100 or pSer or pThreo, at a final dilutiom of 1:50) diluted in blocking buffer was carried out at 4ºC overnight. After incubation, the sperm was washed thoroughly with PBS and incubated with the corresponding Alexa-conjugated secondary antibody (pTyr: Alexa-Fluor® 488 goat anti-mouse igG at a final dilution of 1:2000; pSer and pThreo: Alexa Fluor® 488 donkey anti-rabbit igG at a final dilution of 1:5000). As negative controls, samples without primary antibody and incubated with the respective secondary antibody were run in parallel. Slides were gently washed with PBS and were then incubated with 10 µl of a commercial solution of 4.6-diamidino-2-phenylindole hydrochloride (DAPI; 125 ng/mL. Vysis Inc.; Downers Grove, USA) as both a nuclear stain and an antifade mounting solution. After being covered, the slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed with colorless nail polish, and slides were stored at 4ºC in the dark until their microscope observation. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) adapted to an inverted Leitz DMIRBE microscope and a 63 x (NA 1.4 oil Leitz Plan-Apo lens (Leitz, Stuttgart, Germany). The light source was an argon/krypton laser. Successive confocal slices of images (image thickness: from 0.5µm) were integrated in order to perform three-dimensional spermatozoa images, which were further stored as TIFF-format images.

2.3.7 Suppliers

All of the supplies utilized in this work were of analytical grade and came from Sigma (Saint Louis, MO) Boehringer Mannheim (Mannheim, Germany), GE Healthcare and Merck (Darmstadt, Germany).

2.4 Results

2.4.1 Changes in the percentages of viability and reacted acrosomes during "in vitro" capacitation and subsequent acrosomal reaction.

Incubation in the CM induced a time-dependent, progressive decrease in viability which went from 72.7 ± 2.1% at 0 h to 52.9 ± 4.5% after 4 h of incubation (Table 1). The induction of IVAR was accompanied by a further decrease in viability, which reached values of 44.6 ± 4.6% after 1 h of progesterone addition (Table 1). This effect on viability was accompanied by a concomitant increase in the percentage of altered acrosomes. In this way, this percentage rose from 22.3 ± 2.5% at 0 h to 42.5 ± 3.7% after 4 h of incubation. Furthermore, the percentage of spermatozoa with altered acrosomes greatly increased after IVAR induction, reaching values of 72.3 ± 6.2% after 60 min of IVAR induction (Table 1). That the increase in the percentage of altered acrosomes associated with IVAR was induced by a true acrosomee reaction was indicated by the observed changes in the PNA lectin distribution. In this sense, as shown in Table 1, the percentage of sperm that showed a regular, non-capacitated PNA label at the acrosomal area at 0 h of incubation was of 51.7 ± 4.6%. This percentage decreased to 22.7 ± 3.0% after 4 h of incubation in CM. This decrease was further accentuated after progesterone addition, reaching values of 5.2 ± 0.5% after 60 min of incubation with progesterone (Table 1).

Table 1. Mean values of viability and acrosome reacted spermatozoa of boar spermatozoa subjected to “in vitro” capacitation and further progesterone-induced “in vitro” acrosome reaction.

	IVC					IVAR			
Incubation time	0h	1h	2h	3h	4h	5 min	15 min	30 min	60 min
Viability (%)	72.7 ± 2.1^{a}	69.4 ± 2.0^{a}	54.7 ± 4.2^{b}	54.7 ± 4.4^{b}	52.9 ± 4.5^{b}	51.0 ± 5.0^{b}	47.8 ± 6.4^{b}	45.3 ± 4.7^{b}	44.6 ± 4.6^{b}
Altered acrosomes (%)	22.3 ± 2.5^{a}	29.6 ± 2.9^{b}	41.7 ± 3.5^{c}	41.7 ± 3.7^{c}	42.5 ± 3.7^{c}	46.7 ± 4.2^{c}	60.4 ± 6.7^{d}	68.4 ± 6.2^{d}	72.3 ± 6.2^{d}
PNA-marked acrosomal area (%)	51.7 ± 4.6^{a}	39.2 ± 4.9^{b}	28.4 ± 2.5^{c}	27.2 ± 2.8^{c}	22.7 ± 3.0^{c}	20.7 ± 1.8^{d}	18.4 ± 1.6^{d}	9.5 ± 1.0^{e}	5.2 ± 0.5^{f}

Semen quality parameters have been defined in the Material and Methods section. Results are expressed as means±S.E.M. for 16 separate experiments, Different superscripts in a row indicate significant ($P<0.05$) differences among groups after an Students-Neumann-Keuls statistical analysis.

2.4.2 Changes in boar-sperm tyrosine, serine and threonine phosphorylation expression patterns after "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosome reaction

The Western blot of pTyr performed after 1D SDS-PAGE showed an overall increase in pTyr intensity of sperm proteins. Namely, there were 4 main bands of about 27 kDa, 32 kDa, 40 kDa and 50 kDa that progressively increased their intensity during the incubation in the CM medium (Figure 2A). Moreover, there was also the appearance and increase of a pTyr signal of about 30 kDa-35 kDa. This band, which would correspond to the previously described p32 protein associated with capacitation (Ramírez *et al.*, 2009) appeared after 1-2 h of incubation, reaching a maximum level after 4 h of incubation in the CM (Figure 2A). Progesterone-induced IVAR caused a slight decrease in the overall pTyr phosphorylation intensity, which was especially evident in the 30 kDa-35 kDa band. There were not significant changes in this pattern during all of the time of IVAR induction (Figure 2A).

The 2D analysis of pTyr showed concomitant results. In this way, cells at time 0 h showed a main pTyr signal that was of about 40 kDa and with a pI of about 7.5. This main signal was accompanied by less intense signals, in which one of about 45 kDa and a pI of 8 and another of about 15 kDa and a pI of about 7.5 were the most important (Figure 2). The incubation of boar sperm for 4 h in the CM induced the expansion of the main pTyr signal observed at 0 h, in this way a broad signal appearing, distributed between 34 kDa and 50 kDa of molecular weight and with a pI ranging from 7 to 8. This main signal was accompanied by the appearance of a specific mark of about 30 kDa and a pI from 7.5 to 8, which would correspond to the capacitation-marker protein p32 (Bravo *et al.*, 2005). Additionally, the loss of the signal of about 15 kDa and pI of about 7.5 that was evident in 0 h samples is noteworthy (Figure 2). The induction of IVAR induced further changes in the 2D pattern and, after 5 min of incubation with progesterone, the most important change was the loss of the pTyr signal corresponding to the p32 protein. This was maintained after 60 min of incubation with progesterone, in which a separation of the main pTyr signal in

3 recognizable marks of about 40 kDa-45 kDa and pI 7.5, 35 kDa and pI 7.5 and 34 kDa and pI 8 were detected (Figure 2).

Concerning pSer, the 1D Western blot analysis showed the presence of 3 major bands of about 40 kDa, 45 kDa and 50 kDa. The incubation for 4 h in the CM did not modify this pattern, although a slight increase in the intensity of the bands, specially that of the 50 kDa, was evident (Figure 3A). The induction of IVAR did not clearly affect the results, although a slight, incubation time-dependent decrease in the intensity of the bands was observed, with a maximum decrease after 60 min of progesterone incubation (Figure 3A).

The 2D Western blot analysis for pSer showed a phosphorylation pattern much more complex than that observed after the 1D analysis. In this way, as shown in Figure 3 (PSER OH) sperm at 0 h had a minimum of 5 protein clusters that were phosphorylated in Ser residues. The first cluster (Cluster I) included several proteins of high molecular weight (minimum 200 kDa) with a pI about 6-7. The second cluster (Cluster II) included 4 or 5 single proteins with a molecular weight from about 60 kDa to about 90 kDa and with a pI of from 7.5 to 8.5. In an area below Cluster I and with a more acidic pI of Cluster II, an unspecific mark corresponding to BSA was detected. The third cluster (Cluster III) is shown as a very intense, unique mark which would include separate proteins with an extension of 40 kDa-60 kDa of molecular weight and 8.5-10 of pI. The fourth cluster (Cluster IV) was formed by 4 separate proteins with a molecular weight of 35-40 kDa and a pI of 8-8.5. The last cluster (Cluster V) is formed by a faint group of proteins with low molecular weight (15-to-20 kDa) and a basic pI (8.5-9.5; see Figure 3). The incubation for 4 h in the CM induced dramatic changes in this structure. Thus, Cluster I practically disappeared, whereas the two proteins of Cluster IV with the more acidic pI also suffered a great decrease in their pSer status (Figure 3). On the other hand, the attainment of the IVC was accompanied by the new appearance of pSer markings, specially in the form of 2 marks, one of about 75 kDa and a pI of about 9.2, and the other of about 80 kDa and a pI of about 9.6. The addition of progesterone after 4 h of incubation in the CM induced further changes in this pattern. In this way, as shown in Figure 3, there was a clear decrease in pSer markings of

proteins included in Cluster II, which were practically reduced to one spot of about 60 kDa and a pI of 7.8. Similarly, Cluster IV showed a further decrease in pSer, only one protein appearing of about 40 kDa and a pI of 8.5 with an intensity similar to that observed at 0 h and 4 h of incubation in the CM. Additionally, the two pSer marks that newly appeared after 4 h of incubation were reduced to a single spot of about 75 kDa and a pI of 9.5, and a new, intense pSer spot appeared, with a molecular weight of about 75 kDa and a pI of 6-7. Further incubation of sperm for 60 min induced only slight changes, when compared with those observed after 5 min of the addition of progesterone (Figure 3).

The pThre pattern obtained after 0 h of incubation in CM after the 1D analysis showed the presence of 4 main bands with a molecular weight of about 28 kDa, 30 kDa, 45 kDa, and the most intense of about 70 kDa. The incubation in the CM did not modify this overall pattern, although there was a time-dependent increase of the intensity of the bands, which reached a maximum level after 3-4 h of incubation (Figure 4A). The addition of progesterone did not modify the overall pattern again, although several specific changes in the intensity of the signal could be observed. Thus, there was a progressive decrease in the intensity of the bands, specially of that of about 70 kDa, which reached the maximum intensity after 60 min of incubation with progesterone (Figure 4A).

The Western blot applied to bi-dimensional electrophoresis showed a phosphorylation pattern that was less complex than that obtained with pSer. In this sense, as shown in Figure 4, sperm at 0 h of incubation showed 3 main markings. The first (Cluster A), a faint broad area, which would include several proteins, with a molecular weight ranging from about 60 kDa and about 120 kDa and a pI ranging from 6.5 to about 8.5. In this cluster, an unspecific signal for BSA can be appreciated. The second one (Cluster B), an intense area, which would also include several proteins, ranging from about 34 kDa to about 50 kDa and a pI of 8.5-9. The last main spot (Cluster C) was also intense and it seems to be formed by at least 2 proteins. This spot had a molecular weight of about 30 kDa-35 kDa and a pI of about 8.2-8.5. Incubation for 4 h in the CM had a

strong effect on the distribution of pThre spots. Thus, Cluster A was drastically reduced in its intensity, and several single spots appeared in a molecular-weight range of 60-70 kDa and with a pI of 6.5-8 (Cluster D, see Figure 4). Furthermore, Cluster B underwent an expansion in its pI, which in this case increased its range from about 6 to about 10.2. Finally, there was a decrease in the intensity of Cluster C, in which a single protein seemed to maintain its pThre intensity, with a molecular weight of about 30 kDa and a pI of about 8 (Figure 4). The induction of IVAR caused new changes. In this case, after 5 min of incubation with progesterone, proteins in Clusters C, D and B increased their pThre intensity mark (Figure 4). Further incubation for 60 min induced a general decrease in pThre intensity, with a displacement of the pI of Cluster B, which ranged from about 8 to about 11 (Figure 4).

2.4.3 Changes in boar-sperm tyrosine, serine and threonine phosphorylation location patterns after "in vitro" capacitation and further, progesterone-induced "in vitro acrosome reaction

The location of pTyr in boar sperm at 0 h of incubation was mainly observed at the equatorial zone of the head and on the whole tail (Figure 5). This distribution varied after 4 h of incubation in CM. In these cells, pTyr was mainly located at the main and terminal pieces of the tail. Further addition and incubation with progesterone for 5 min did not greatly vary the location observed after 4 h of incubation in CM, although after 60 min of the addition of progesterone, a clear decrease in the intensity of the pTyr was observed (Figure 5).

Regarding pSer location, this was mainly observed in sperm at 0 h of incubation as a faint signal at the midpiece. Remarkably, after 4 h of incubation in CM, an intense pSer signal was detected at the acrosome, which was accompanied by an increase in the intensity of the midpiece mark (Figure 5). The acrosome-related signal underwent a very remarkable increase in its intensity after the progesterone-induced IVAR, although no mark was detected in that sperm that had been subjected to an acrosomal loss. Moreover, the increase of the pSer acrosomal signal was dependent on the time of incubation

and, in this way, was more pronounced after 60 min of incubation with progesterone (Figure 5).

The IVC and further progesterone-induced IVAR also modified the location of the boar-sperm pThre signal. As shown in Figure 5, cells at 0 h of incubation showed a faint signal of pThre, mainly located at the whole tail. This location did not vary after 4 h of incubation with CM, although there was a noticeable increase in its intensity. The addition of progesterone induced a significant change in the pThre signal which, after 5 min of the addition, showed a clear increase in the intensity of the tail's signal, accompanied by the appearance of a signal at the acrosome in some cells that maintain this structure. After 60 min of the addition of progesterone, however, a clear decrease, and even disappearance, of both the tail and acrosomal pThre signal was observed (Figure 5).

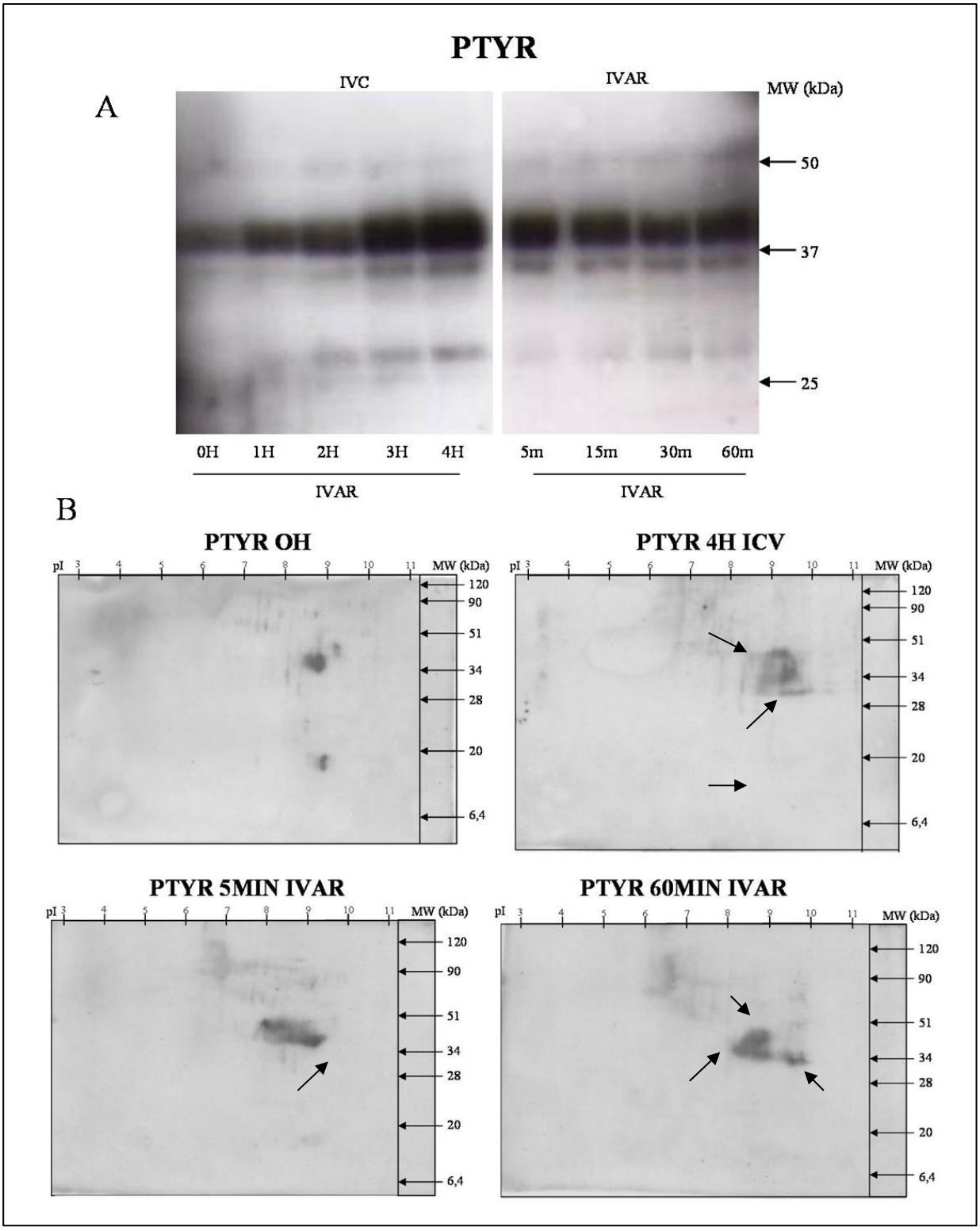

Figure 2. Protein tyrosine-phosphorylation expression in boar sperm after "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosomal reaction by Western blot analysis after both 1D and 2D electrophoresis. A: 1D analysis. PTYR OH, PTYR 4H ICV, PTYR 5MIN IVAR, PTYR 60 MIN IVAR: 1D analysis after 0 h (PTYR OH) and 4 h of incubation in the capacitation medium (PTYR 4H ICV), and after 5 min (PTYR 5MIN IVAR) and 60 min of progesterone addition (PTYR 60 MIN IVAR). MW: Molecular weight markers. In the 2D blots, the horizontal scale indicates the pI. Arrows in the 2D blots indicate the presence of specific signals. Figure shows representative images for 4 separate experiments.

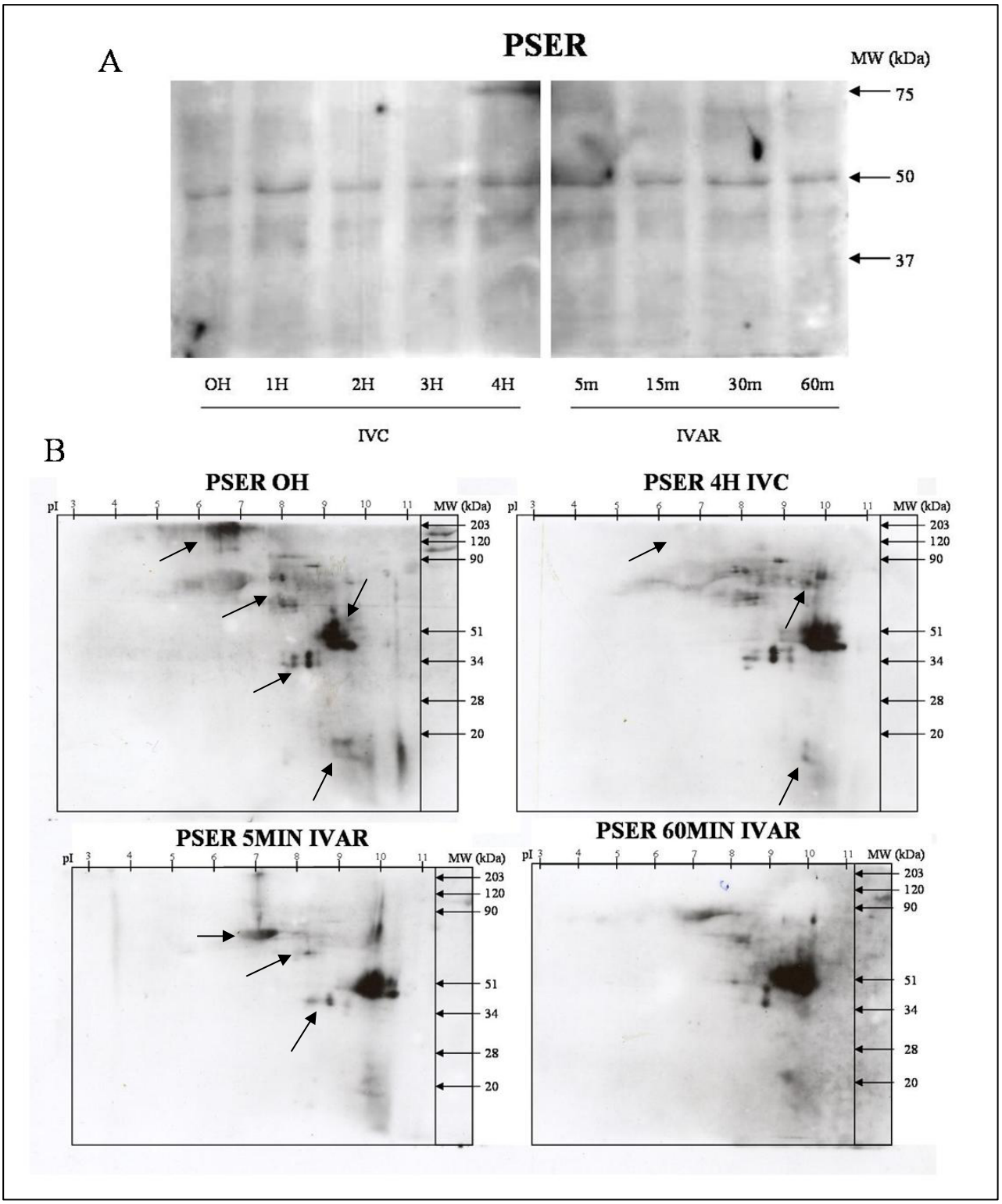

Figure 3. Protein serine-phosphorylation expression in boar sperm after "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosomal reaction by Western blot analysis after both 1D and 2Delectrophoresis. A: 1D analysis. PSER OH, PSER 4H ICV, PSER 5MIN IVAR, PSER 60 MIN IVAR: 1D analysis after 0 h (PSER OH) and 4 h of incubation in the capacitation medium (PSER 4H ICV), and after 5 min (PSER 5MIN IVAR) and 60 min of progesterone addition (PSER 60 MIN IVAR). MW: Molecular weight markers. In the 2D blots, the horizontal scale indicates the pI. Arrows in the 2D blots indicate the presence of specific signals. Figure shows representative images for 4 separate experiments.

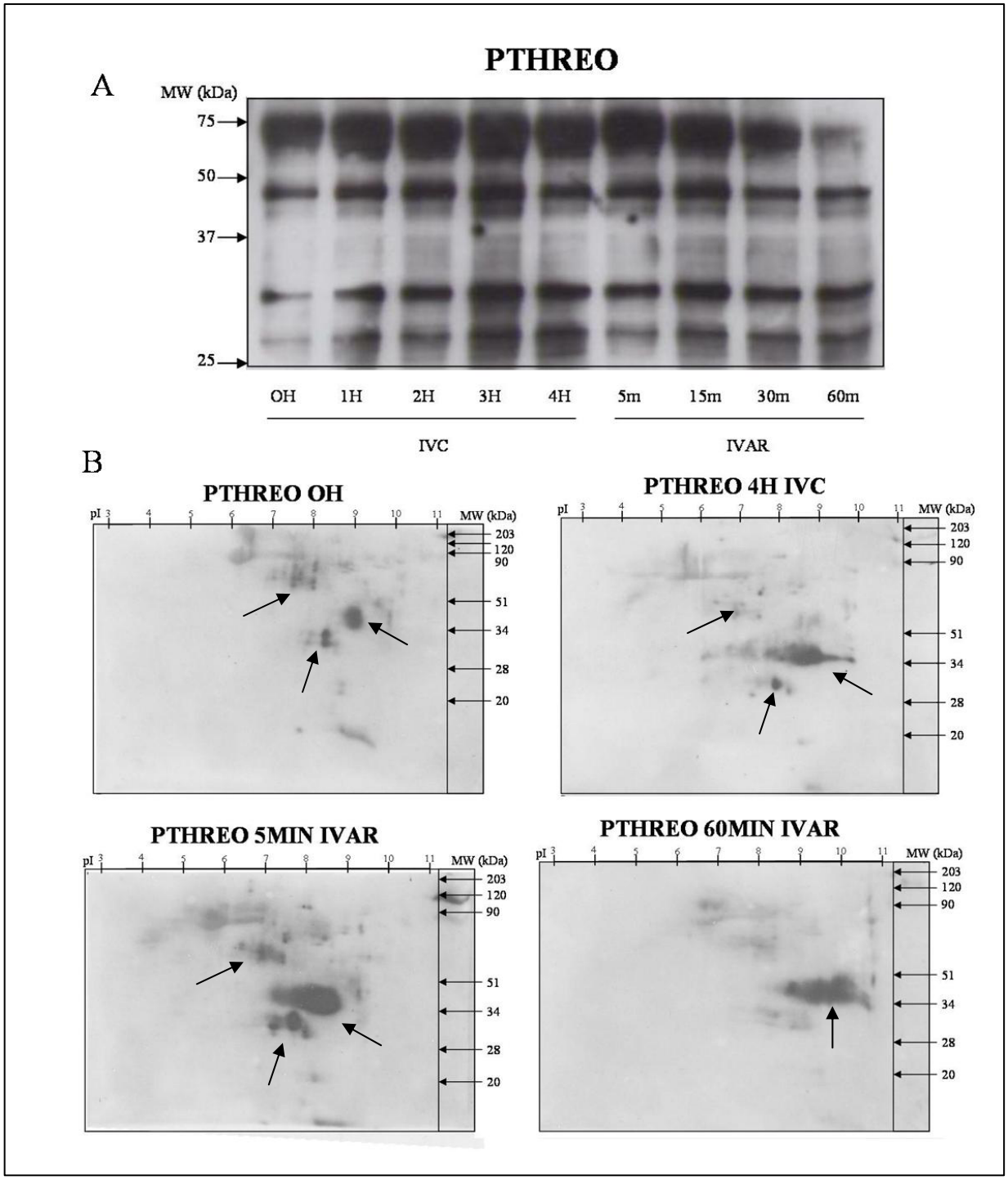

Figure 4. Protein threonine phosphorylation expression in boar sperm after "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosomal reaction by Western blot analysis after both 1D and 2D electrophoresis. A: 1D analysis. PTHREO OH, PTHREO 4H ICV, PTHREO 5MIN IVAR, PTHREO 60 MIN IVAR: 1D analysis after 0 h (PTHREO OH) and 4 h of incubation in the capacitation medium (PTHREO 4H ICV), and after 5 min (PTHREO 5MIN IVAR) and 60 min of the progesterone addition (PTHREO 60 MIN IVAR). MW: Molecular weight markers. In the 2D blots, the horizontal scale indicates the pI. Arrows in the 2D blots indicate the presence of specific signals. Figure shows representative images for 4 separate experiments.

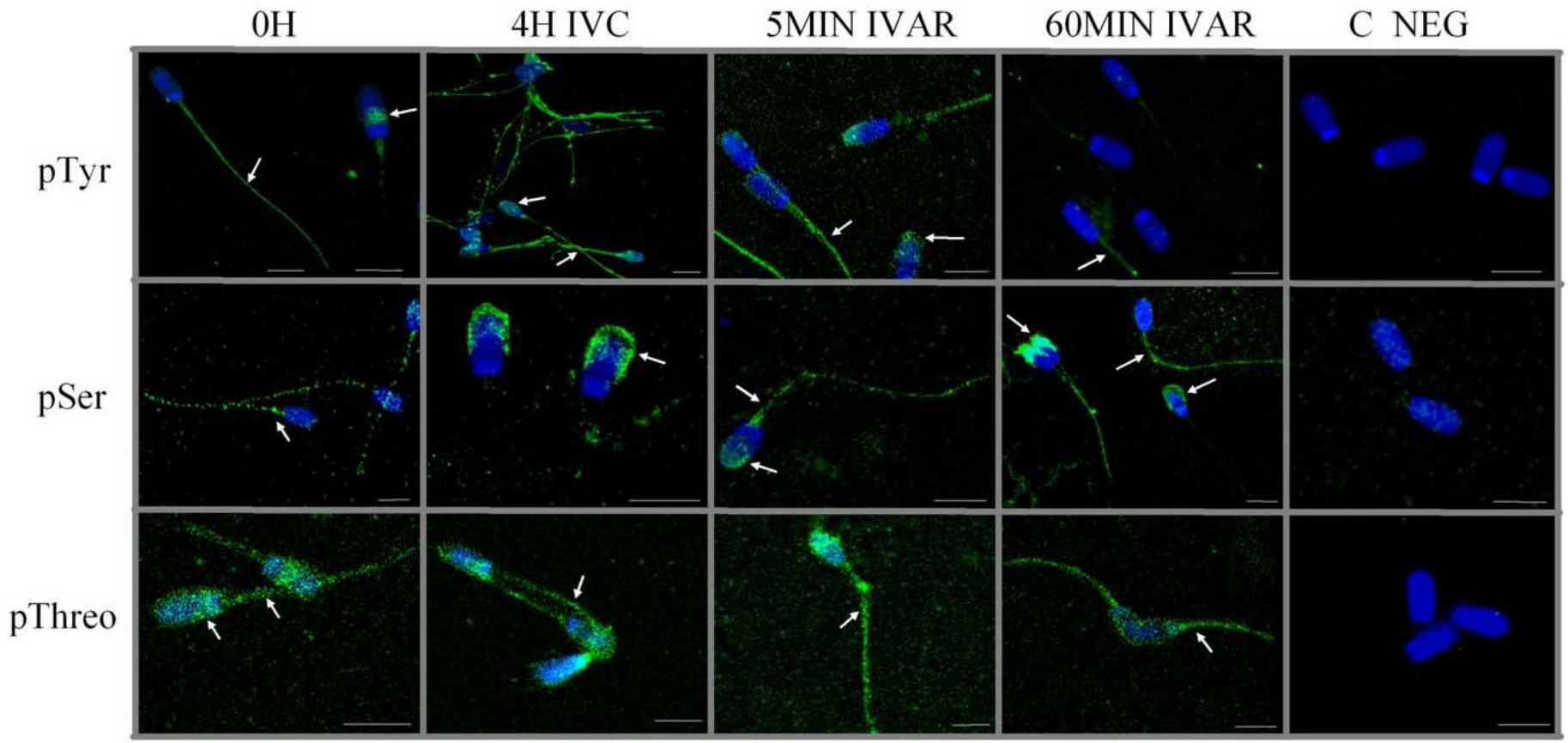

Figure 5: Immunocytochemistry for the presence of tyrosine, serine and threonine phosphorylation in boar spermatozoa subjected to "in vitro" capacitation and subsequent, progesterone-induced "in vitro" acrosomal reaction. Figure shows representative images for 6 separate experiments. Arrowheads indicate the location of the specific signals. 0H: Samples at the start of the incubation in the CM medium. 4H IVC: Sperm incubated for 4 h in the CM. 5MIN IVAR: Cells 5 min after the addition of progesterone after 4 h of incubation in the CM. 60MIN IVAR: Cells after 5 min of the addition of progesterone after 4 h of incubation in the CM. C NEG: Negative controls obtained through incubation in the absence of primary antibody, in order to observe unspecific fluorescence. Bars: 7μm. Arrowheads indicate the presence of positive signals at the acrosomal region.

2.5 Discussion

Our results clearly indicate that IVC and progesterone-induced IVAR are related to specific changes not only in the intensity and the appearance of protein phosphorylation in Tyr, Ser and Thre residues, but also in the exact location of these phosphorylated proteins in boar sperm. This is an important point to be addressed, since until now protein phosphorylation had been mainly related to IVC as the appearance of specific, phosphorylated proteins, such as the tyrosine-phosphorylated p32 (Kaneto *et al.*, 2002), subsequently identified as proacrosin (Bailey *et al.*, 2005).

Regarding IVC, it is worth noting that there were not only specific changes in phosphorylation of some proteins, but also a general increase in the intensity of overall Tyr, Ser and Thre protein phosphorylation, as shown through the 1D analysis. This is of interest since, whereas there has been a great number of studies concerning changes of pTyr during IVC, little is known about pSer/pThre despite observations such as that serine/threonine kinases like protein kinases A (PKA) and C (PKC) are involved in sperm capacitation (Jha and Shivaji, 2002). Moreover, another capacitation-related protein kinase which is regulated by changes in its serine/threonine phosphorylation status named tyrosine kinase 32 (TK 32), has been identified in pig sperm (Tardiff *et al.*, 2001). In a similar way, the presence of glycogen synthase kinase-3 (GSK3) has been recently described in boar sperm (Aparicio *et al.*, 2007). The presence of this kinase is important, since GSK3 activity is regulated by serine phosphorylation, establishing a close relation between the pSer of GSK3 and sperm motility. The overall change in the activity of all of the protein kinases could be related to the overall increase in protein phosphorylation during capacitation, which would increase pSer and pThre in proteins such as FA-1 and AKAP83 (Jha and Shivaji, 2002). Thus, our results strongly stress the importance of specific pSer and pThre changes, not only of pTyr, during capacitation. In this way, the identification of the proteins which undergo differential phosphorylation in serine/threonine residues would be very helpful in understanding the molecular basis of capacitation.

Notwithstanding, IVC not only induces an overall increase in protein phosphorylation, but there are also specific changes besides that regarding p32. Thus, the appearance of specific, IVC-related marks of pSer of 74 kDa/pI 9.2 and 80 kDa/pI 9.6 confirms the appearance of IVC-caused pSer in single proteins. But this is not the only interesting result that is highlighted by the 2D analysis. The loss of several specific signals, such as that of pTyr of 15k Da/pI 7.5 and the common expansion through their pI in the main spots in all of the analysis, is also noteworthy. This increase in the pI range would indicate the existence of capacitation-induced conformational changes in these phosphorylated proteins, mainly due to the presence of species with a different number of phosphorylated residues. Hence, it seems that capacitation increases the number of conformational species with different allosteric properties of a single protein, in this way causing a concomitant increase in the range of activity of this protein. (Naz and Radesh, 2004). This would increase the overall activity of this protein, although the precise purpose of this capacitation-related phenomenon is not completely clear to us, and further investigations are needed in order to define a more precise role for this increase in the pI range of phosphorylated proteins. In any case, our study has demonstrated that the 2D analysis is a much more potent tool to study capacitation, and also acrosomal reaction, than the common 1D analysis much more commonly performed in the literature.

IVC also causes changes in the precise location of pTyr, pSer and pThre in boar sperm. This is logical, and several authors have obtained similar results. In this way, the appearance of an acrosomal pTyr mark during capacitation, compatible not only with the phosphorylation of acrosine, but also with other important proteins like dopamine receptors, has recently been described in boar (Ramírez *et al.*, 2009). Additionally, Harayama (2003) observed that the stimulation of PKA, a phenomenon closely related to the achievement of capacitation, caused the appearance of new pSer/pThre proteins of 220 kDa, 180 kDa, 84 kDa and 54 kDa, which were mainly located at the connecting and principal pieces of capacitated spermatozoa. This could be related to a general increase in pSer and pThre in the whole flagellum during IVC and IVAR (Adachi *et al.*, 2008 and the results described here). Moreover, Harrison (2004) detected

bicarbonate-dependent phosphorylation changes in the postacrosomal region of the head and in the neck, midpiece, and anterior regions of the tail. There are more descriptions regarding an overall increase of protein phosphorylation of the flagellum during capacitation not only in boar sperm, but also in other species like human and mouse (Carrera *et al.*, 1994; Carrera *et al.*, 1996; Urner *et al.*, 2001; Moseley *et al.*, 2005; Asquith *et al.*, 2004), despite some contrary description (Tardiff *et al.*, 2001). This increase in protein phosphorylation is generally linked to the capacitation-linked, specific changes in sperm motility patterns that would be required for successful fertilization, including penetration through cumulus and ZP (Urner and Sakkas, 2003; Asquith *et al.*, 2004). However, it should be stressed here that capacitation-induced flagellum phosphorylation is not only centered on Tyr residues, but also on Ser and Thre ones, in concordance with other authors (Harayama, 2003; Adachi *et al.*, 2008), and the study of all of the phosphorylation changes are absolutely necessary in order to obtain a complete idea of protein changes associated with sperm capacitation.

Regarding protein phosphorylation in the head area during IVC, we must also mention some interesting results. First of all, our results concerning pTyr are similar to those previously published (Tardiff *et al.*, 2001; Petrunkina *et al.*, 2001; Bailey *et al.*, 2005; Ramírez *et al.*, 2009). In this sense, the appearance of a specific pTyr signal in the acrosome during IVC is also logical when considering that, as explained above, one of the specific p32 bands that shows an increase in its pTyr during IVC corresponds to proacrosin (Bailey *et al.*, 2005). However, the results observed in pSer and pThre are also of interest. Thus, the appearance of a clear, specific acrosomal mark of pSer and pThre after IVC is especially important. This clearly indicates that capacitation induces specific changes in the structural conformation and. hence, the activity of at least some of the pSer- and pThre-regulated acrosomal proteins. This again indicates that pTyr is not the only important process involving protein phosphorylation during capacitation. Furthermore, it must be remembered that the appearance of the acrosomal pSer mark is accompanied by the concomitant appearance of new, specific pSer signals of 74 kDa/pI 9.2 and 80 kDa/pI 9.6. The simultaneous appearance of the acrosomal pSer marking and the pSer

phosphorylated proteins of 74 kDa and 80 kDa leads us to hypothesize about the possibility that the 74-kDa and 80-kDa proteins were those responsible for the acrosomal mark. We can only speculate about this point, and further studies are needed in order to prove a possible relationship among these IVC-induced signals.

Progesterone-induced IVAR also caused several interesting results. Among them, the overall, time-dependent loss of protein phoshorylation, as well as that of several specific proteins, like pTyr of p32, are perhaps the most significant. These results are in accordance with others previously published, in which spermatozoa subjected to the acrosomal reaction lost their pTyr labeling, not only in the acrosome but also in the tail (Petrunkina *et al.* 2001). Notwithstanding, there are other important changes to report. The most interesting one would be the appearance of an IVAR-specific pSer signal of 75 kDa/pI 6-7. This could be related to the very remarkable increase in the intensity of the pSer acrosomal mark that is detected after IVAR. Thus, our results indicate that progesterone-induced IVAR is related with an increase on pSer phosphorylation of some acrosomal proteins, on of which could be that of 75 kDa/pI 6-7. The detection of a new, specific marker of IVAR related to pSer is of a great importance, since this opens new insights about the mechanisms involving protein conformational changes that induce not only capacitation, but also acrosomal reaction in mammalian sperm, clarifying the importance of not only pTyr, but also pSer and pThre in the regulation of these processes.

So, we can conclude that IVC and progesterone-induced IVAR of boar sperm are related to specific changes in the pattern and location not only of tyrosine phosphorylated proteins, but also in serine- and threonine-phosphorylated ones. These specific changes include the appearance of new pSer proteins, especially related to the achievement of IVAR. Other important changes include an increase in the range of conformational changes of phosphorylated proteins and the appearance of protein phosphorylation in new sperm locations, like the acrosome. Furthermore, an overall increase on protein pTyr, pSer and pThre during IVC, which was followed by an overall decrease after the induction of IVAR is also observed. All of these results highlight the

importance of not only the specific pTyr, but also of pSer and pThre in the regulation of mammalian sperm capacitation and acrosomal reaction processes.

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CAPITOL / CHAPTER 3:

“In vitro” capacitation and acrosome reaction are related to an increase of mitochondrial activity in boar seprm: putative control roles of actin and mitofusin-2.

"In vitro" capacitation and acrosome reaction are related to an increase of mitochondrial activity in boar seprm: putative control roles of actin and mitofusin-2

3.1 Abstract

The induction of "in vitro" capacitation (IVC) and subsequent, progesterone-induced "in vitro" acrosome reaction (IVAR) was concomitant to an increase of mitochondrial activity. This was evident through an increase in the O_2 consumption (from $4.83x10^{-7}$ nmol O_2 / viable sperm at 0 h of incubation to 7.76 x 10^{-7} nmol O_2 / viable sperm after 5 min of IVAR induction), and by concomitant changes in the mitochondria activity-specific JC-1 stain. Following JC-1, mitochondrial activation followed a nucleated pattern, with specific starting activation points at the midpiece from which mitochondrial activation was spread. Moreover, four separate sperm subpopulations were detected following JC-1, indicating thus the presence of sperm with separate sensitivity to IVC and IVAR. The IVC and IVAR also induced actin polimerization, showing also an increase in the actin presence at the apical area of midpiece. The presence of mitofusin-2, a protein involved in the regulation of the coordinated mitochondria function was found by the first time in sperm. Mitofusin-2 location expanded from midpiece to the principal piece after IVC and IVAR. All of these results indicate that the increase of boar sperm mitochondria activity during IVC and the first minutes of IVAR is instrumental to perform the sperm function changes associated with these processes. Furthermore, the increase in mitochondria activity is originated in concrete nucleation points at the midpiece. Finally, actin and mitofusin-2 play important roles in the modulation of boar sperm mitochondria function, both by originating changes in the protein membrane environment and by changes in the mitochondrial structure by itself.

3.2 Introduction

Capacitation has been defined as the overall changes that the sperm undergoes after being ejaculated that allow it to fertilize the oocyte (Austin, 1951; Chang, 1951; Yanagimachi, 1994). Capacitation results in altered plasma membrane architecture and permeability, which ultimately modulates flagellar activity and renders the sperm apical head plasma membrane fusogenic (Rodríguez-Martínez, 2007). Physiologically, spermatozoa acquire fertilization-competence in the female reproductive tract but capacitation should be achieved also in a defined media, the composition of which approximates the environmental of the female reproductive tract (Visconti and Kopf, 1998).

Several intracellular changes are known to occur, including increases in membrane fluidity, cholesterol efflux, intracellular Ca+2 and cAMP concentrations, protein tyrosine phosphorylation and changes in swimming patterns and chemotactic motility (Breitbart and Naor, 1999). Hyperactivated motility is one of the best characterized phenomena associated to capacitated sperm. Despite this, little is known regarding the energy sources from which sperm transform their motion parameters to hyperactivation during capacitation. In fact, the source of ATP that support of sperm motility has been long debated in the field of gamete research. In mammalian sperm there are two pathways for ATP production: glycolysis that occurs along the entire length of the principal piece of the flagellum, and mitochondrial respiration, centered on mitochondria of the midpiece. Mitochondrial respiration is the most efficient source of ATP and, in this way, it has been inferred that, under normal conditions, the ATP required for sperm motility is mainly obtained through mitochondria respiration (Mukai and Okuno, 2004). Hence mitochondrial status has been related not only with sperm motility in bull (Garner and Thomas, 1999), horse (Gravance *et al*., 2000), ram (Marínez-Pastor et al. 2004), and mouse (Mukai and Okuno, 2004) but also with fertilization ability in human (Kasai *et al*., 2002). However, several works strongly indicate that mitochondria are not the unique energy source for sperm motility, and glycolisis had been also related with motility. In fact, the gene knock-out of the glycolytic enzyme GAPDH caused the appearance of no-motile sperm and a significant reduction of the ATP content (10% of the total)

despite having no deficiency in oxygen consumption (Miki *et al*., 2004). Accordingly, Marin *et al*. (2003) reported that glycolysis plays a significant role in energy source in boar sperm. This is in accordance with the presence of an active and specific glycolytic activity in mammalian sperm (Mukai and Okuno, 2004). This will be linked to the presence of a sperm specific form of glycerlaldehyde 3-phosphate dehydrogenase (GAPDH), which is tightly bound to the fibrous sheath (Kamp *et al*., 2003). In addition, several studies have documented the relationship between glycolysis and capacitation-dependent cell-signaling (Urner and Sakkas, 2003). Following all of these results, Guthrie *et al*. (2008) propose glycolysis as a specialized and sperm-specific source of ATP to maintain motility in mammalian sperm. All of these data suggest that the maintenance of sperm function would be the result of the equilibrium between the energy obtained from glycolysis and Krebs cycle.

Centering on mitochondrial function, it has been described that capacitation is related with a notorious loosening and distention of mitochondria forming the sperm mitochondrial sheath (Vorup-Jensen *et al*., 1999). We don't know the molecular processes that induce these changes, although several possibilities can be exposed. One of the most interesting would be based in changes in the actin-constituted cytoskeletal network that surrounds mitochondria together with specific actions of mitochondria-modulator proteins like mitofusin2 (MFN2). The MFN2 is especially interesting, since this mitochondria membrane protein participates in mitochondrial fusion in mammalian cells, thus contributing to the maintenance and operation of the actin-constituted mitochondrial network (Bach *et al*., 2003). The mitochondrial network controls the co-ordinate action of all of mitochondria in a cell. This allows mitochondria for controlling cellular processes such as apoptosis (Green and Reed, 1998) intracellular Ca+2 signaling (Dumollard *et al*., 2006), energy metabolism (Bach *et al*., 2003; Martins de Brito and Scorrano, 2008) and sperm capacitation (Brener *et al*., 2003).

The main aim of this work is to study the control of the changes in mitochondrial activity that are concomitant to "in vitro" capacitation (IVC) and subsequent "in vitro" progesterone-induced acrosome reaction (IVAR) in boar

spermatozoa. For this purpose, the first step was to analyze IVC- and IVAR-linked changes in mitochondrial activity through two separate techniques. The first one was the quantification of changes in the rhythm of oxygen consumption. The second one was the study of mitochondrial activity through the specific JC-1 stain procedure. The second step was to determine changes in the expression and midpiece location of both actin and MFN2 through Western blot analyses and immunocytochemistry. Our result indicate that IVC and the induction of IVAR are related to an increase in mitochondrial activity, which was also related to changes in the expression and midpiece location of both actin and MFN2. Incidentally, this is the first description of the presence of MFN2 in mammalian sperm, pointing out the importance of this protein in the control of sperm mitochondrial function.

3.3 Materials and methods

3.3.1 Boar semen collection

All procedures described within were approved by the Autonomous University of Barcelona Animal Care and Use Committee and were performed in accordance with the Animal Welfare Law issued by the Catalan Government (Generalitat de Catalunya, Spain)

Commercial artificial insemination (AI) doses from boars of proven fertility were obtained from a commercial farm (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Spain) The sperm were manually collected, maintained at 37ºC in a water bath and diluted at 2 x10^7 sperm/mL in a commercial dose extender for refrigerated semen (MR-A Extender; Kubus, S.A.; Majadahonda, Spain) and distributed in 100-mL commercial doses. Six of the 100-mL doses obtained, chosen at random, coming from different boars, were placed in portable refrigerator at 16 ºC for approximately 45 minutes, which was the time required to arrive at the laboratory as previously reported (Ramió-Lluch *et al*., 2009).

3.3.2 "In vitro" capacitation and acrosome reaction procedures.

Fifty mL of each AI-dose were washed three times by centrifugation at 600 x g for 5 min at 16 ºC and re-dilution in a 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), containing 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 4.5 mM $CaCl_2$ (NCM medium).The osmolarity was 304±5 mOsm and pH was adjusted at 7.4. After the last wash, sperm were resuspended in capacitating medium (CM medium, which is composed by NCM medium added with 5 mg/mL bovine serum albumin: BSA) at a final concentration of 50-70x10^6 sperm/mL. Incubation in CM was maintained for 4 h at 38.5ºC in a 5% CO_2 atmosphere as described by Ramió *et al.* (2008).

The induction of IVAR was carried out through incubation in the presence of progesterone, as described before (Ramió *et al*., 2008; Jimenez *et al*., 2003; Wu *et al*., 2006). For this purpose, progesterone was added to a final concentration of 10 µg/mL with boar sperm previously incubated in CM for 4 hours at 38,5ºC in a 5% CO_2 atmosphere. After thoroughly mixing, sperm were further incubated for and additional 1 hour at 38.5ºC in a 5% CO_2 atmosphere.

Sperm aliquots of 1,5 mL were taken at 0, 1, 2 , 3 and 4 hours of capacitation and 5, 15, 30 and 60 minutes after the induction of IVAR. For SDS-PAGE, sperm pellets were obtained by centrifugation (30 s at 10,000 g) and immediately frozen at -196 ºC in N_2 liquid and stored at -80ºC before use. Furthermore, other 100 µL-aliquots of sperm were fixed with 300 µL of 2% paraformaldehyde for 1 hour and stored at 4ºC before processing for indirect immunofluorescence.

3.3.3 Evaluation of the achievement of capacitation status

Percentages of viability, altered acrosomes and true acrosomal reaction were determined by using the double-staining fluorescein isothiocyanate-conjugated Arachis hypogaea (PNA) lectin and propidium iodide stain (PNA-

FITC/IP stain) as described by Busalleu *et al*. (2005). These percentages were determined after counting 200-300 spermatozoa per slide at 1000 x. Altered acrosomes were considered to be those which did not show a clear and uniform PNA lectin staining, whereas those which were considered as being subjected to a true acrosomal reaction showed, after the stimulation of IVAR, positive viability and disruption of the acrosome PNA, stain as referred to by Ramió *et al*. (2008).

3.3.4 Oxygen consumption measurement

Sperm oxygen consumption was estimated with a Clark oxygen electrode (Oxytherm Hansatech Instruments Ltd. Norfolk, UK) linked to a recorder system software (Oxygraph, Hansatech Instruments Ltd. Norfolk, UK). Water maintained at 38,5 ºC was circulated throughout the DW1 oxygen electrode chamber and constant stirring by a magnetic flea ensured homogeneous distribution of O_2. The zero was set by adding a few grains of sodium dithionite (Na_2S20_4) to the chamber, which contained 700 µl of distilled water. Measurements were made by adding 900 µL CM tempered at 38,5º C followed by 100 µL of the sperm solution. The plunger was inserted to expel air and O_2 consumption was monitored for approximately 3 minutes. The mean sperm concentration at the DW1 chamber was 80 x 10^5 sperm / mL. Data are presented as nmol O_2 consumed / viable sperm.

3.3.5 Evaluation of Mitochondrial Membrane Potential (MMP) through JC-1 stain

The lipophilic, cationic compound, 5, 5', 6, 6' -tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) has the distinct advantage of differentiating cells of high and low mitochondrial membrane potential. The maximal excitation for JC-1 is 488 nm, with an emission spectra of the monomer in the green range (530 nm) when mitochondria are inactive and the emission of JC-1 aggregates in the high orange wavelength (590 nm) when mitochondrial membrane potential is high. Huo *et al*. (2002) have described the validity of this probe for boar sperm. 1,5 mL aliquot of sperm was incubated

with 15 µL of JC1 (153 µM; T-3168. Molecular Probes, Eugene, OR) for 10 min at 37ºC in the dark. The sperm was placed on clean microscope plates with a coverslip on the bottom. Images of the live stained sperm were obtained with Leica TCS 4D confocal scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) adapted to an inverted Leitz DMIRBE microscope and a 63 x (NA 1.4 oil Leitz Plan-Apo lens (Leitz, Stuttgart, Germany) in a CO_2 atmosphere and with a thermoplate at 38.5ºC. The light source was an argon/krypton laser. Images and videos, which were further stored as TIFF-format images and AVI format videos. Images were analyzed by computerized analysis in a image processor program. A minimum of 30 spermatozoa per sample were scored. A total of 6 experiments were performed. The images obtained were processed using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA). Each spermatozoa was analyzed individually. For this purpose, the analyzed midpieces were individually delimited. After this, both green and red/orange colour intensities of these delimited midpieces were calculated by using the "Histogram" included in the program. The values obtained after the application of this procedure were further processed in order to obtain two separate intensity scales, one for each colour, with arbitrary values ranging from 1 (minimal intensity) to 256 (maximal intensity). Afterwards, the proportion of colour intensities between red/orange and green was individually calculated for each midpiece and a further statistical analysis was performed in order to yield the results described below (see the Statistics section).

3.3.6 Analysis of the expression and midpiece location of actin and mitofusin-2.

3.3.6.1 Protein extraction

Sperm pellets were homogenized by sonication in 200 µl of ice-cold 50 mM Tris-HC buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethyleneglycoltetraacetic acid (EGTA), 25 mM dithiothreitol (DTT), 1,5% (w/v) Triton X-100, 1mM phenilmethil sulfonil fluoride (PMSF), 1mM benzamidine and 10 µg/mL leupeptin. Samples were then centrifuged at

13,000 g for 15 min at 4 ºC. Supernatants were recovered for protein determination.

For actin determination a previous step of acetone precipitation was carried out. For this, 900 µL of ice-cold acetone were added to 100 µL of the recovered supernatant. After 30 minutes with briefs intervals of agitation, samples were centrifuged at 15,000 g during 15 minutes and then acetone was removed by decantation. Residues of acetone were allowed to been vaporized during 30 minutes at room temperature. Afterwards, pellet was dissolved with 100 µL of distilled water and protein determination was proceeded.

Total protein content of the samples was determined through the Bradford method (Bradford, 1976) by using a commercial kid (BioRad Laboratories; Hercules, CA, USA). Samples were stored immediately at -80 ºC until their use.

Sperm protein samples were boiled for 1 minute before been transferred to sodium dodecyl sulfate-polyacrylamide gel and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was subsequently carried out following the standard protocol (Laemmli, 1970). The total amount of protein loaded in each lane was of 20 µg, whereas 0.3 µg of commercial actin from rabbit muscle (01812, Biochemika Fluka, Buchs, Switzerland) was run in parallel as a positive control.

3.3.6.2 Western blot analysis

The Western blot analysis was carried out following the standard protocol of transferring the SDS-PAGE to nitrocellulose membranes (Burnett, 1981). Transferred samples were tested with applying an anti-actin antibody (Sigma, Saint Louis, MO USA) at a final dilution of 1:20,000 (v/v), and an anti-MFN2 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) to a final dilution of 1:200 (v/v). It is noteworthy that the utilized anti-actin antibody is useful to identify actin (G and F) in all cell types (Colás *et al*., 2009). Immunoreactive proteins were detected by using peroxidase-conjugated anti-rabbit secondary

antibody (Amersham, Buckinghamshire, UK). The reaction was developed with an ECL-Plus detection system (Amersham, Buckinghamshire, UK).

3.3.6.3 Indirect Immunofluorescence

Aliquots of 50 µL of the previously described paraformaldehyde-fixed sperm samples were spread onto poly-lysined (Poly-L-lysine solution 1 % w/v in H_2O; Sigma; Saint Louis, MO, USA) microscope slides and then were lent to air dry. After this, samples were permeabilized by incubation for 10 min at room temperature in a standard phosphate buffered solution (PBS; pH 7.4) added with 0.25% (v/v) Triton x-100. Afterwards, slides were washed three times with PBS and then they were blocked through incubation with PBS including 0.1% (v/v) Tween-20 and 1% (w/v) BSA for 30 min at room temperature. Incubation with the respective primary antibody at a final dilution of 1:100 diluted in blocking buffer was carried out at 4ºC overnight. After incubation, sperm were washed thoroughly with PBS and incubated with Alexa Fluor® 488 donkey anti-rabbit igG at a final dilution of 1:200. As negative controls, samples without primary antibody and incubated with secondary antibody were run in parallel. Slides were gently washed with PBS and then were incubated with 10 µl of a commercial solution of 4.6-diamidino-2-phenylindole hydrochloride (DAPI; 125 ng/mL. Vysis Inc., Downers Grove, USA) as both a nuclear stain and an antifade mounting solution. After covering, the slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed with colorless nail polish, and slides were stored at 4ºC in the dark until their microscope observation. Fluorescent images were obtained with Leica TCS 4D confocal scanning microscope (Leica Lasertechnik) adapted to an inverted Leitz DMIRBE microscope and a 63 x (NA 1,4 oil Leitz Plan-Apo lens (Leitz). The light source was an argon/krypton laser. Successive confocal slice of images (image thickness: from 0.5 µm) were integrated in order to perform three-dimensional spermatozoa images, which were further stored as TIFF-format images.

3.3.7 Statistics

Statistical analysis of the data obtained with JC-1 staining procedure was performed by applying the SAS statistical package (SAS, 1982). As described above, the analyzed variable was the proportion between orange/red intensity colour and green intensity colour. A previous PROC NORM PLOT procedure was performed to determine that the obtained data distribution did not follow a normal distribution. Afterwards, data were normalized by applying a Ln data transformation. A FASTCLUS procedure was then applied to distribute every individual spermatozoon into a specific sperm subpopulation. The FASTCLUS procedure performs a disjointed cluster analysis based on Euclidean distances computed from one or more quantitative parameters variables. Spermatozoa were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar fluorescence were assigned to the same cluster, whereas spermatozoa that differed were assigned to different clusters. A PROC GLM procedure was applied to evaluate significant differences ($P<0.05$) and the LSMEANS procedure was applied to list these differences. Finally, a Chi-square procedure was applied to determine the subpopulation distribution percentage of every single experiment. Once the percentage distribution per experiment was determined, new PROC GLM and LSMEANS procedures were applied to determine and list, respectively, the differences among the different points of IVC and IVAR procedures. The total number of spermatozoa analyzed following this protocol was 800. These cells were from a total of 6 separate experiments.

3.3.8 Suppliers

All of the suppliers were of analytical grade and came from Sigma, Boehringer Mannheim (Mannheim, Germany), and Merck (Darmstadt, Germany)

3.4 Results

3.4.1 Evaluation of capacitation status during "in vitro" capacitation and "in vitro" acrosome reaction

As observed in Table 1 viability of spermatozoa underwent a progressive fall during the overall incubation. The percentage of viability at 0 h of incubation was 77.3 ± 2.6% and underwent a progressive fall during the overall process of IVC arriving at 55.6 ± 4.3% after 4 h of incubation. The decline in viability further progressed after the induction of IVAR, reaching values of 42.5 ± 3.7% after 1 h of progesterone addition (Table 1). This effect on viability was accompanied by a concomitant increase in the percentage of altered acrosomes. In this way, this percentage rose from 20.8 ± 1.9% at 0 h to 38.5 ± 3.2% after 4 h of incubation. Furthermore, the percentage of spermatozoa with altered acrosomes greatly increased after IVAR induction, reaching values of 69.9 ± 6.2% after 60 min of IVAR induction (Table 1). That the increase in the percentage of altered acrosomes associated with IVAR was induced by a true acrosome reaction was indicated by the observed changes in the PNA lectin distribution. In this sense, as shown in Table 1, the percentage of sperm that showed a regular, non-capacitated PNA label at the acrosomal area at 0 h of incubation was of 50.3 ± 4.7%. This percentage decreased to 26.3 ± 3.0% after 4 h of incubation in CM. This decrease was further accentuated after progesterone addition, reaching values of 4.9 ± 0.3% after 60 min of incubation with progesterone (Table 1).

Table 1. Percentages of viability and altered acrosomes during "in vitro" capacitation and subsequent, progesterone-induced acrosome reaction.

	Incubation in capacitating medium				
Incubation time	0h	1h	2h	3h	4h
Viability (%)	77.3 ± 2.6^{a}	69.1 ± 2.0^{a}	$55.2 \pm 4.3^{b*}$	$54.7 \pm 4.4^{b*}$	$55.6 \pm 4.3^{b*}$
Altered acrosomes (%)	20.8 ± 1.9^{a}	$27.9 \pm 2.0^{b*}$	$38.7 \pm 3.0^{c*}$	$40.7 \pm 3.4^{c*}$	$38.5 \pm 3.2^{c*}$
PNA-marked acrosomal area (%)	50.3 ± 4.7^{a}	$39.0 \pm 4.2^{b*}$	$27.9 \pm 2.1^{c*}$	$28.4 \pm 4.3^{c*}$	$26.3 \pm 3.0^{c*}$
	Time after progesterone addition				
	5 min	15 min	30 min	60 min	
Viability (%)	$51.0 \pm 5.4^{b*}$	$49.4 \pm 6.1^{b*}$	$45.8 \pm 5.3^{b*}$	$42.5 \pm 3.7^{b*}$	
Altered acrosomes (%)	$48.8 \pm 4.0^{c*}$	$61.5 \pm 5.3^{d*}$	$65.3 \pm 5.0^{d*}$	$69.9 \pm 6.2^{d*}$	
PNA-marked acrosomal area (%)	$22.9 \pm 2.8^{d*}$	$21.7 \pm 2.2^{d*}$	$8.5 \pm 0.9^{e*}$	$4.9 \pm 0.3^{f*}$	

Semen quality parameters have been defined in the Material and Methods section. Results are expressed as means±S.E.M. for 16 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences among groups after an Students-Neumann-Keuls statistical analysis. Asterisks indicate significant ($P<0.05$) differences when compared with the 0 h values.

Table 2. Effect of "in vitro" capacitation and subsequent "in vitro" acrosome reaction on the proportion of each sperm subpopulation obtained after multivariate cluster analysis of the values of the proportion orange/green intensity after JC1 stain.

	Incubation in capacitating medium		Time after progesterone addition	
	0h	4h	5min	60min
Subpopulation 1	14.7 ± 0.3^{a}	47.5 ± 0.4^{b}	15.8 ± 0.4^{ac}	11.9 ± 0.4^{ad}
Subpopulation 2	6.7 ± 0.2^{a}	11.5 ± 0.3^{b}	6.9 ± 0.3^{a}	3.9 ± 0.4^{c}
Subpopulation 3	3.0 ± 0.3^{a}	5.0 ± 0.3^{b}	3.0 ± 0.3^{a}	1.6 ± 0.4^{c}
Subpopulation 4	0.8 ± 0.4^{a}	1.1 ± 0.3^{a}	1.6 ± 0.4^{b}	0.2 ± 0.4^{c}

Results are means±S.E.M. for 5 separate experiments. Different superscripts in a row indicate significant ($P<0.05$) differences among groups. The values are expressed as arbitrary units as described in Materials and Methods. Sperm subpopulations have been defined in the Results section.

3.4.2 Effects of "in vitro" capacitation and "in vitro" acrosome reaction in the rhythm of oxygen consumption

Incubation of boar sperm in the CM induced a progressive, time-dependent increase in the rhythm of oxygen consumption. This increase reached a maximum after 3-4 h of incubation (6.19 x 10^{-7} nmol O_2 / viable sperm after 3 h and 5.70 x 10^{-7} nmol O_2 / viable sperm after 4 h, see Figure 1). The induction of IVAR caused a rapid increase in oxygen consumption, which reached values of 7.76 x 10^{-7} nmol O_2 / viable sperm after 5 min of progesterone addition. Afterwards, a time-dependent decrease of oxygen consumption was observed, reaching values of 3.18 x 10^{-7} nmol O_2 / viable sperm after 60 min of the IVAR induction (Figure 1).

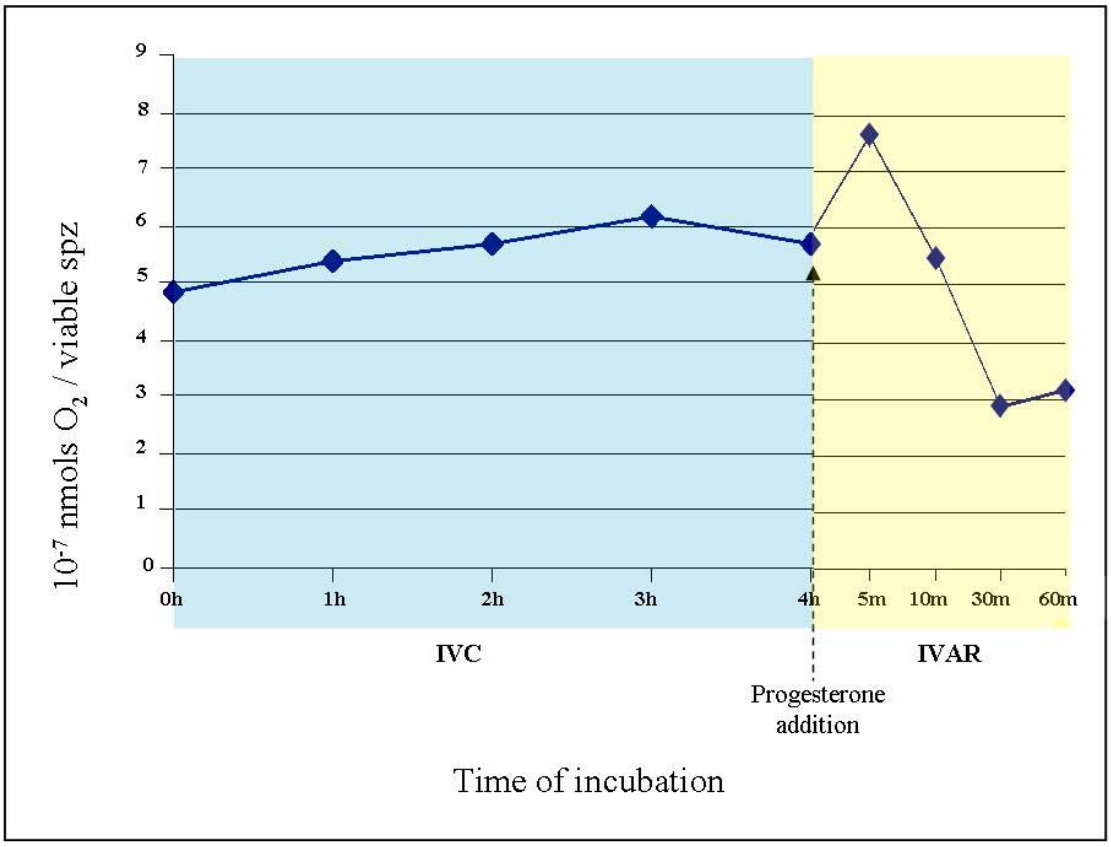

Figure 1. Oxygen consumption during IVC and IVAR . Boar sperm was subjected to "in vitro" capacitation through incubation for 4 h in the IVC medium (blue zone) and were then further subjected to progesterone-stimulated "in vitro" acrosome reaction (marked with arrows, yellow zone) as described in the Material and Methods section. Results are expressed as means for 6 separate experiments.

3.4.3 Effects of "in vitro" capacitation and "in vitro" acrosome reaction in the mitochondrial membrane potential measured through JC-1 stain

The photographical study of the images obtained after the JC-1 stain showed the presence of an especific pattern of midpiece sperm mitochondrial activation. Thus, as shown in Figure 2, if considering that green mitochondria have low MMP and red mitochondria have the most intense MMP, the appearance of red stain started in small points, mainly located at the extremes of the midpiece, from which the red JC-1 stain was extended until occupying all of the midpiece in the most activated sperm. This indicates an activation pattern based on nuclear activation points, from which the activating signal was dispersed to the other mitochondria of midpiece.

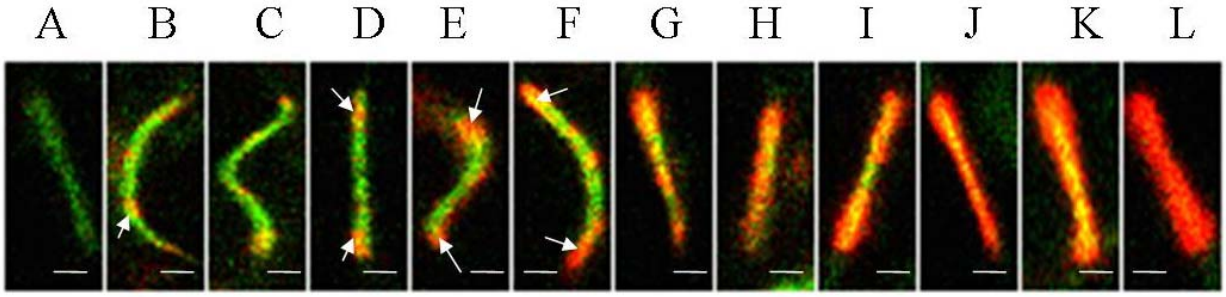

Figure 2. Location changes of mitochondria with different activities through JC-1 mitochondrial staining in boar spermatozoa. Figures show the synchronical appearance of local focuses of active mitochondria (yellow-red areas) among non-active mitochondria (green areas) in boar sperm from low (A) to maximal mitochondrial activity (L). Arrows indicate the location of initial points of mitochondrial activation in sperm with low overall mitochondrial activity. Bars indicate a real size of 2 μm.

Boar sperm showed a mean orange/green proportion of JC-1 stain of 8.2 ± 0.1 at 0 h of incubation in CM (mean ± S.E.M.). This proportion increased after incubation in the CM medium reaching values of 9.6 ± 0.1 after 4 h of incubation. The induction of IVAR induced a decrease of the mean values of the JC-1 orange/green proportion, that reached values of 4.9 ± 0.1 after 60 min of the progesterone addition. On the other hand, the JC-1 analysis rendered the presence of 4 separate sperm subpopulations in fresh semen, in accordance to

their midpiece orange/green stain proportion. These subpopulations were the following (Table 2):

Subpopulation 1: Included spermatozoa with the highest MMP activity. At 0 h 27.7% of the total spermatozoa showed a mean 14.7-fold red/orange intensity compared with the green colour.

Subpopulation 2: Spermatozoa with high MMP activity but lower than Subpopulation 1 (mean orange/red-green intensity proportion: 6.7). About 37.3% of the sperm were classified in this subpopulation at 0 h of incubation and showed a clear predominant orange fluorescence.

Subpopulation 3: Spermatozoa of this subpopulation showed lower MMP when compared with Subpopulations 1 and 2 (mean orange/red-green proportion of about 2.9). At 0 h of incubation 31.6% of the total sperm were included in this Subpopulation.

Subpopulation 4: Spermatozoa included in this Subpopulation showed the lowest MMP activity (mean orange/red-green proportion of about 0.8). At 0 h of incubation Subpopulation 1 represented a 3.66 % of the overall sperm.

The incubation of boar sperm in the CM induced clear changes in the proportion of cells included in each subpopulation. Thus, as shown in Figure 3, the percentage of sperm included in Subpopulation 1 was progressively decreasing, went from 27.7% at 0 h of incubation to 7.2% after 4 h of incubation. This decrease was concomitant with moderate increase of Subpopulations 2 (from 37.3% at 0 h of incubation to 45.2% after 4 h of incubation) and 3 (from 31.7% at 0 h of incubation to 41.6% after 4 h of incubation, see Figure 3). The induction of IVAR caused, on the contrary, a very rapid increase in the proportion of sperm included in Subpopulation 1, which reached values of 39.0% after 5 min of the addition of progesterone. Likewise, sperm in Subpopulation 4 also increased, went from 5.9% at the moment of the progesterone addition to 19.4% after 5 min of incubation (Figure 3). These increases were linked to concomitant decreases of sperm from Subpopulations

2 and 3. Afterwards, there were slight changes in the percentage of sperm included in each subpopulation, with moderate-to-slight decreases of Subpopulations 1 (24.7% after 60 min of IVAR induction) and 4 (13.6% after 60 min of progesterone addition) that were concomitant with moderate to slight increases in Subpopulations 2 (32.5% after 60 min of IVAR incubation) and 3 (29.3% after 60 min of progesterone addition, see Figure 3).

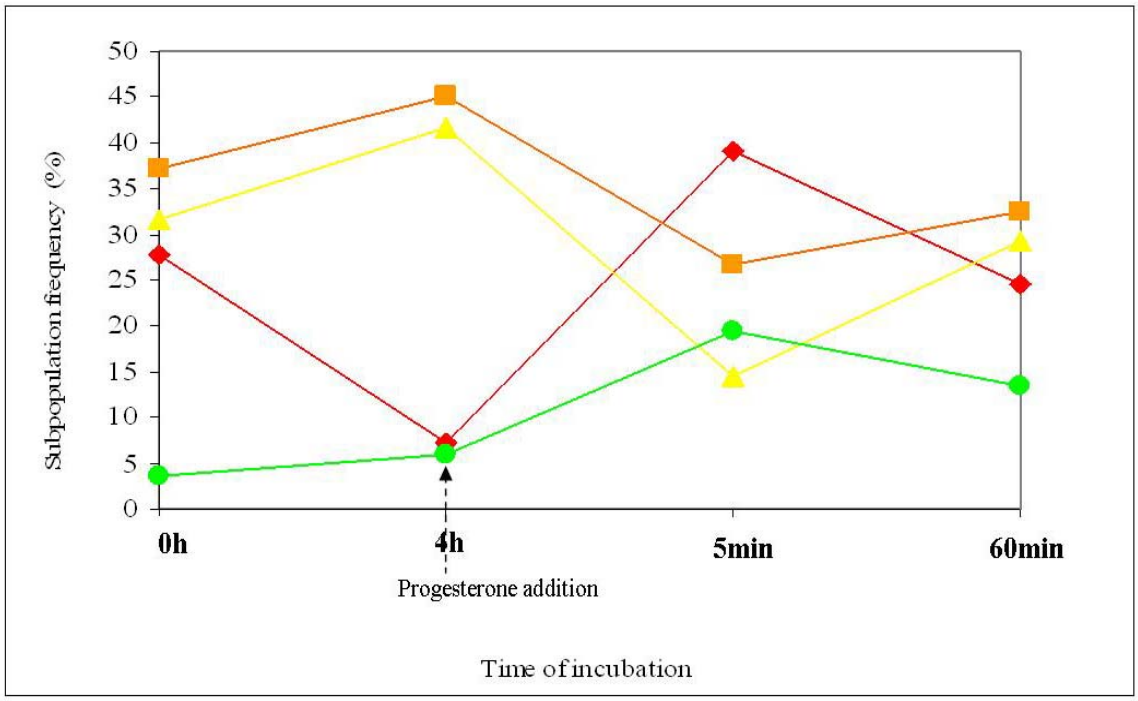

Figure 3. Dynamics of boar JC-1 sperm subpopulation during IVC and further progesterone induced IVAR. JC-1 sperm subpopulations have been defined in the Material and Methods section. ◆: Subpopulation 1. ■: Subpopulation 2. ▲: Subpopulation 3. ●: Subpopulation 4. Results shown are means± standard deviation for 5 separate experiments. Subpopulation frequency must be interpreted as the % of spermatozoa that were included in the respective subpopulation.

3.4.4. Effects of "in vitro" capacitation and "in vitro" acrosome reaction in the expression and midpiece location of actin

The Western blot analysis showed the presence of a specific band for actin of about 42 kDa. As shown in Figure 4, the incubation of sperm in the CM was concomitant with a progressive decrease in the intensity of this specific band. This decrease was subsequently increased after the induction of IVAR, reaching a minimal intensity after 60 min of the progesterone addition (Figure

4A) The immunocytochemistry showed that actin was distributed in fresh sperm along the head and the whole tail (Figure 4B). Centering in the actin midpiece distribution, the actin immunochemistry showed the specific appearance of a subequatorial actin band after 4 h of incubation with CM, together with a slight change in the intensity of midpiece actin signal, which increased at the apical area (Figure 4B). The IVAR induction induced further changes, being the most noticeable the appearance of a strong actin signal at the connecting piece, which was accompanied with a further increase in the actin signal at the apical area of the midpiece (Figure 4B).

3.4.5. Effects of "in vitro" capacitation and "in vitro" acrosome reaction in the expression and midpiece location of mitofusin-2

Our results show the presence of MFN2 in sperm both through Western blot and immunofluorescence analyses (Figure 5A). Thus, the Western blot showed the presence of an specific band of about 110 kDa in fresh sperm, accompanied with a double band of about 85 kDa. These results were in accordance with other previously published in other cellular types (Baloh *et al*., 2007; Detmer *et al*., 2007; Santel *et al*., 2003). No significant changes in the intensity of these bands were observed after IVC and subsequent IVAR whereas subsequent IVAR induced a decrease in the intense of MFN2, that was specially evident after 60 min of incubation with progesterone (Figure 5A)

Immunocytochemistry of MFN2 in fresh sperm showed a clear signal located at the midpiece in an uniform manner (Figure 5B). The incubation of sperm in the CM induced an extension of the MFN2 marking, which also appeared at the principal piece of the tail (Figure 5B). MFN2 mark was practically lost after 60 min of progesterone addition (Figure 5B).

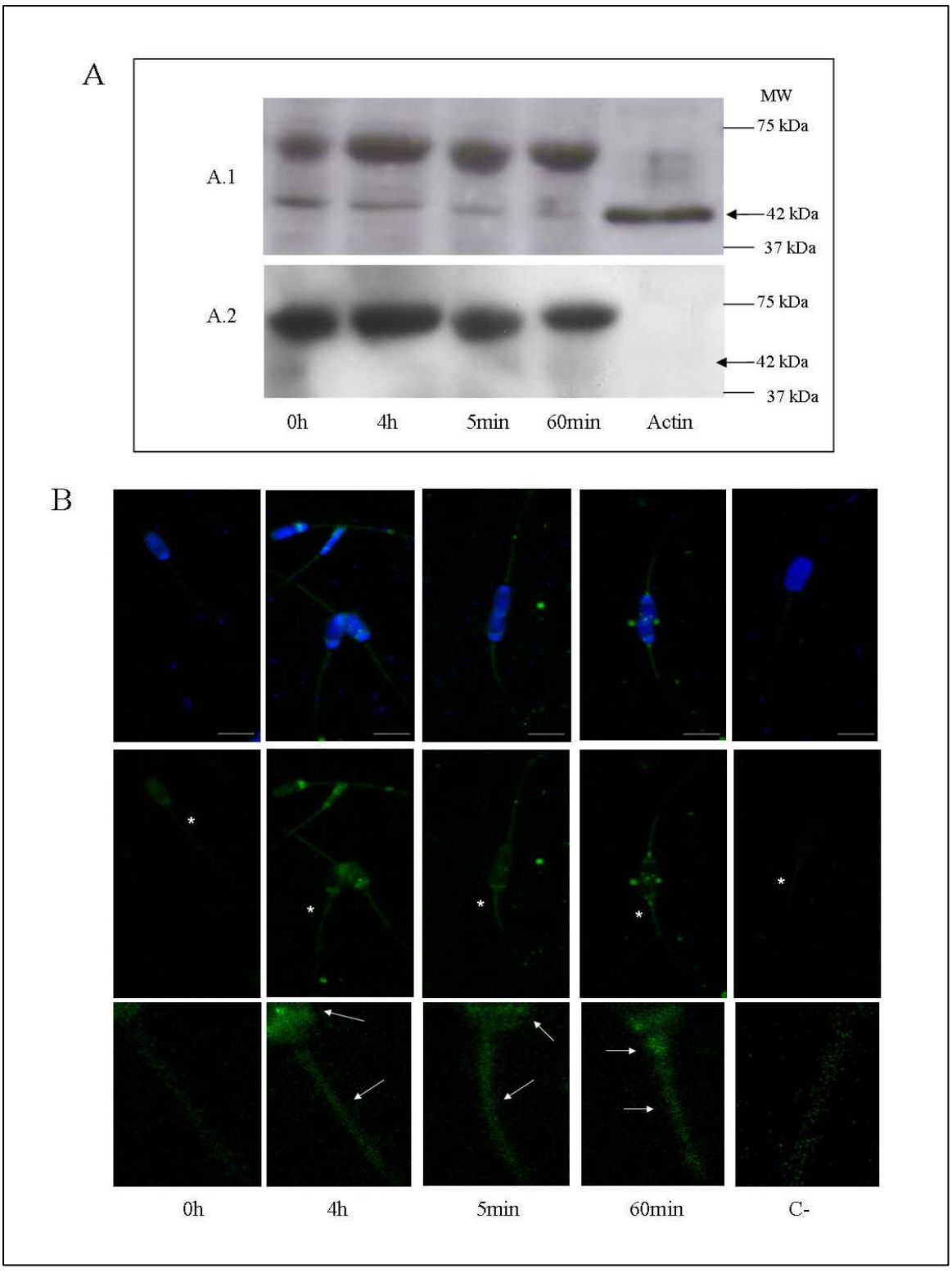

Figure 4: Western blot (A) and immunocytochemical analyses (B) of actin in boar spermatozoa. Arrowheads indicate the location of the specific signals. 0h: sperm just before incubation. 4h: sperm after 4 hours in CM at capacitation conditions as described in Materials and Methods. 5min and 60min: sperm after 5 minutes and 60 minutes of progesterone addition. A.1: Western blot performed with the specific anti-actin antibody; commercial rabbit actin (Actin) was utilized as a positive control as described in Material and Methods. A.2.: Western blot carried out without the primary antibody as negative control. In this case, the aprox 70Kda band obtained must be considered as unspecific. (B): C-: negative control. The figure shows a representative Western blot from three independent replicates. Bars indicate a real size of 7 μm

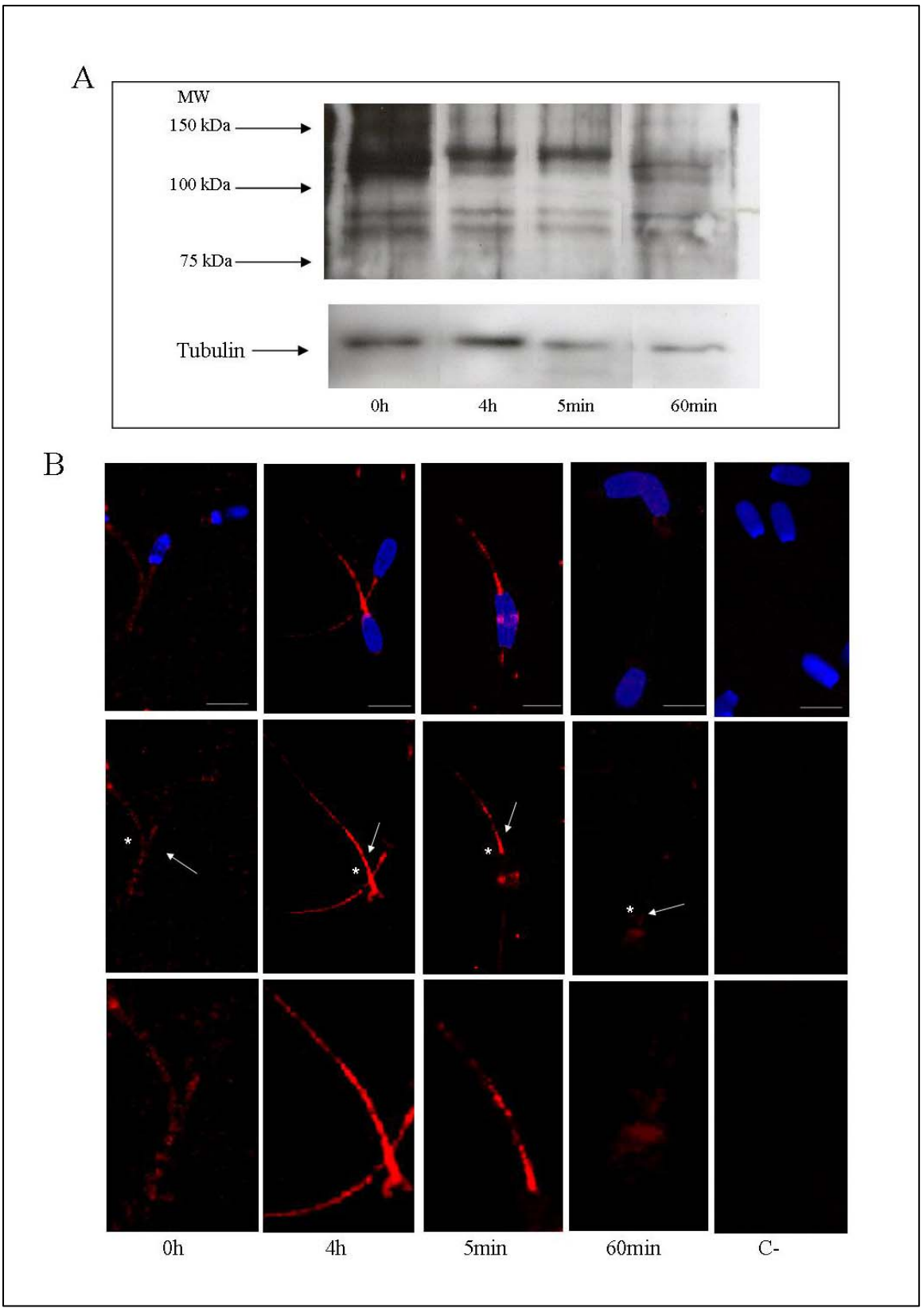

Figure 5: Western blot (A) and immunocytochemical analyses (B) of Mitofusin2 in boar spermatozoa. Arrowheads indicate the location of the specific signals. 0h: sperm just before incubation. 4h: sperm after 4 hours in CM at capacitation conditions as described in Materials and Methods. 5min and 60 min: sperm after 5 minutes and 60 minutes of progesterone addition. (A) β-Tubulin (Tubulin) was used as an internal control. The figure shows a representative Western blot from three independent replicates. Bars indicate a real size of 7 μm

3.5 Discussion

Our results clearly indicate that functional changes associated with the achievement of a feasible IVC in boar sperm are chronologically related to an increase in mitochondrial activity. Furthermore, the attainment of a feasible progesterone-induced IVAR is subsequently related to the appearance of a fast an intense peak of mitochondrial activity. Both results clearly states the importance of a very active mitochondrial function in order to attain both IVC and IVAR, as well as the importance of a precise modulation of the overall mitochondrial activity during all the sperm lifespan. In this sense, mitochondria would play a key role as a capacitation controller mechanism by not only the induction of a switch in the sperm pathways for energy production but also probably in the modulation of other inherent capacitation changes. Deeping further in this point, we must remind that mitochondria are multitasking organelles essential for normal eukaryotic cell function. They had been described to regulate crucial cellular events not only related with the oxidative energy metabolism by producing most of the cell's energy currency ATP but also in other cell processes such as Ca^{+2} signaling, generation of reactive oxygen species (ROS) and apoptosis by orchestrating signals triggered from both inside and outside the cell (Green and Reed, 1998; Martins de Brito and Scorrano, 2008; Honda and Hirose, 2003; Aihara *et al*., 2009). The molecular mechanisms underlying sperm capacitation and subsequent acrosome reaction include aspects like Ca^{+2} signaling and other apopotic-reminding changes (Visconti *et al*. 1995), as well as variations in sperm movement that would be subjected to changes in the energy sources involving concomitant changes in the mitochondrial function (Mukai and Okuno, 2004; Garner and Thomas, 1999; Gravance *et al*., 2000; Martínez-Pastor *et al*., 2004; Gaczarzewicz *et al*., 2003). All of these points stress in the importance of a finely regulated mitochondrial function during IVC and IVAR of boar sperm.

Our results indicate that progesterone-induced acrosome reaction induced an intense burst in oxygen consumption that was followed by a notable fall, suggesting a switch on energy production among IVC and IVAR. The JC-1 mitochondrial stain was in accordance with this result, as at 5 minutes after

progesterone addition there was an increase in the percentage of the most activated-mitochondria sperm subpopulation, indicating a change in mitochondrial function, specially affecting this subpopulation of spermatozoa. Our results suggest the existence of spermatozoa with heterogeneous response to capacitating stimuli in regard to their mitochondrial activity, at least. It is noteworthy that our results are the first that pointed out the existence of sperm subpopulations regarding their mitochondrial activity, which can be very useful to analyze sperm heterogeneity in an ejaculate. Additionally, these results are in accordance to other basing on separate function sperm characteristics, such as the response to “in vitro” capacitation and acrosome reaction of motility patterns (Ramió *et al*., 2008), plasmatic membrane fluidity (Harrison *et al*., 1996) and protein phosphorylation (Urner and Sakkas, 2003). In summary, our results seem to confirm that the response to capacitating stimulus of the sperm in an ejaculate are not the same for all of the cells, existing thus separate sperm subpopulations with overall different function characteristics. This leads to the question regarding a putative function specialization of the separate sperm in an ejaculate, which would lead to optimize “in vivo” fertilization. We can only speculate about this hypothesis, and several efforts are currently performed in order to separate these subpopulations to test this hypothesis.

Regarding results of JC-1 stain, it is also remarkable the putative existence of a specific pattern of mitochondrial activation along the midpiece. Thus, our observations strongly suggest that mitochondrial activation does not start in a homogeneous pattern through the entire midpiece, but from concrete points, generally located at the extremes of the midpiece, from which mitochondrial activation is radiated to the entire piece. This also suggests the existence of nucleation points, which include mitochondria especially sensitive to activation stimulus. This hypothesis would be in accordance with the results of Gaczarzewicz *et al*. (2003), which showed that in fresh sperm there was only a part of mitochondria within midpiece that were fully active, while remaining mitochondria showed a reduced ability to oxidize NADH. Thus, sensitivity to external stimuli would be different in these two mitochondrial types, reinforcing the existence of points into the midpiece with a faster and more intense response to activating factors. Remarkably, our results are in concordance with

other published indictaing that mitochondria exist in two interconverting forms. The first, as small isolated particles. The second, and more importantly, as extended filaments, networks or clusters connected with intermitochondrial junctions, named mitochondrial filaments. These mitochondrial filaments in tissues like muscle cells act as physical pathways that permit the transmission of membrane potential from the mitochondria located areas with high oxygen availability to internal mitochondria with low oxygen availability (Zorzano *et al*., 2009). Furthermore, there are experimental evidences indicating the existence of two mitochondrial populations with different respiratory activity in muscle fibre. Thus, mitochondria located in the sub-sarcolemmal region show different activity of the respiratory chain compared with the mitochondria located in the intermyofibrillar zone. Therefore, the mitochondrial filaments may permit the transmission of the mitochondrial membrane potential to those zones where the oxygen could not easily arrive due to the massive presence of contractile fibres (Zorzano *et al*., 2009; Skulachev, 2001). Moreover, it have been reported that in cell cultures from several lines, parts of the same mitochondrial filament showed different respiratory chain activities (Zorzano *et al*., 2009). This phenomenon could be due to switching off the electric conductance via intermitochondrial junctions in a filament composed of several end-to-end-joined mitochondria (Zorzano *et al*., 2009). Thus, mitochondrial filaments could act as energy transmitter cables, and protonic potential was transmitted through the mitochondrial filaments from sites that preferentially consume oxygen to those that preferentially synthesize ATP (Skulachev, 2001). Our results are in concordance with this hypothesis, reinforcing the supposition that sperm mitochondria act as mitochondrial filaments, with coordinated action and several nuclear activation points of especially sensitive mitochondria, mainly placed at the extremes of the midpiece.

Regarding the observed changes in actin expression and location, our results show that IVC and IVAR induced an increase in the actin polymerization, which makes actin much more insoluble and hence the polymerized actin was not present in the supernatants utilized for Western blot. An increase in actin polymerization linked to IVC has been previously described in boar sperm (Castellani-Ceresa *et al*., 1993). In fact, this polymerization depends on PKA

activation, protein tyrosine phosphorylation and phospholipase D activation (Breitbart *et al*., 2005). The capacitation-linked increase of actin polymerization is an important event, since it induces the migration of several plasma membrane proteins. This actin-induced protein migration, especially which of the acrosomal area, caused in turn that the affected regions became able to fuse with the egg plasma membrane as a result of change in distribution and composition of cell surface components (Breitbart *et al*., 2005; Saxena *et al*., 1986). Following with this rationale, the observed changes in the location of actin in the midpiece after IVC and IVAR, which include the appearance of a clear actin signal at the connecting piece after IVAR, also suggest that midpiece actin underwent IVC- and IVAR-changes in its polymerization status. Since actin-polymerization changes are closely related to concomitant changes in the surrounding protein environment (Breitbart *et al*., 2005; Saxena *et al*., 1986) is logical to suppose that these changes would induce variations in midpiece proteins which control mitochondria activity, modulating in this way IVC- and IVAR- induced variations of mitochondrial function.

Another remarkable point of this work is the detection for first time of sperm MNF2. The MFN2 is a dynamin family GTPase involved in the regulation of many mitochondria-modulated functions such as oxidative phosphorylation (Pich *et al*., 2005) and intracellular signaling (Chen *et al*., 2004). MFN2 and its homolog MFN1 directly participate in docking and tethering of neighboring mitochondria and outer membrane fusion (Koshiba *et al*., 2004). Thus, the existence of sperm MNF2 would be related to the modulation of sperm mitochondrial function. At this respect, we must remember that mitochondria number, shape and distribution are modified in many cell types to meet the specialized energy requirements during cellular growth and differentiation or in response to external stimuli (Honda and Hirose, 2003). Centering on spermatozoa, this is a highly differentiated and compartmentalized cell, with mitochondria tightly packed at the midpiece. It has been described that IVC induces a striking change in sperm mitochondria packaging towards a more loosely wrapped or distended morphology (Vorup-Jensen *et al*., 1999). This change would be in accordance with Cardullo and Blatz (1991), that observed a relationship between changes in tail beat frequency and concomitant variations

of mitochondrial volume This change of the mitochondrial morphology is in line with the general concept that mitochondria are dynamic organelles which can change their size and shape depending on the metabolic state of a cell (Sadava, 1993) and during spermiogenesis (Woolley, 1970; Otani *et al*., 1988). At this respect, we have observed a clear change in tail distribution of MFN2 during IVC and IVAR. Thus, the location of MFN2 was extended to the principal piece of the tail after achieving capacitation. These changes would be related with the concomitant changes in mitochondria morphology above mentioned, which, in turn, would be also related to the IVC- and IVAR-linked changes in mitochondrial function. Capacitation is a process that requires special energy requirements and Ca^{+2} signaling and, in this way it is noteworthy that mitochondrial filaments have been described to allow energy transmission between different cellular regions (Amchenkova *et al*., 1988), determining the size and dynamics of the mitochondrial Ca^{+2} pools (Legros *et al*., 2002). This implies that the capacitation-linked mitochondrial morphological changes, in which MFN2 would be related, are important in order to optimize phenomena such as the observed increase energy activity. At this respect, we must remember that boar spermatozoa lack a phosphorylcreatinine-creatinine kinase shuttle system to transport energy-rich phosphate from the mitochondria to the distal dynein ATPases (Kamp *et al*., 2003) and the extension of MFN2-linked mitochondrial filaments to the principal piece during IVC and IVAR would aid to transmit energy to the entire tail in order to activate flagellum contractibility in this area. This increase in the tail-receiving energy would be instrumental in the observed IVC- and IVAR-associated changes of sperm motility patterns, explaining thus the presence and importance of sperm MFN2.

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IV. CONCLUSIONS GENERALS / GENERAL CONCLUSIONS

CONCLUSIONS FINALS / GENERAL CONCLUSIONS

Els resultats presentats en aquesta tesi ens permeten arribar a les següents conclusions:

1.- Els resultats de l'estudi de l'estructura subpoblacional de l'ejaculat durant la capacitació I la reacció acrosòmica "in vitro" evidencien que cada una de les subpoblacions espermàtiques reaccionen de diferent manera davant als estímuls propis de la capacitació i reacció acrosòmica espermàtica. Nogensmenys, la incubació en el medi de capacitació provoca també canvis en la freqüència d'espermatozoides dins de cadascuna de les subpoblacions. Així doncs, al final de la capacitació es descobreix un augment important en el nombre d'espermatozoides de la subpoblació 4, que es caracteritza per uns elevats paràmetres de velocitat i linearitat. Contràriament, la reacció acrosòmica provoca una davallada important en aquesta subpoblació.

2.- L'estructura subpoblacional de 4 *clusters* d'espermatozoides mòtils es manté durant les diferents condicions experimentals, fet que evidencia la importància d'aquesta característica de l'ejaculat porcí.

3.- La capacitació i reacció acrosòmica "in vitro" provoca en l'espermatozoide porcí no només canvis en el patró de fosforil·lació dels residus de tirosina sinó també en els de serina i treonina. Aquests canvis inclouen l'aparició de fosforil·lació de determinades proteïnes lligada a la consecució de la reacció acrosòmica. Alhora, s'han fet notar canvis en l'estructura conformacional d'algunes proteïnes i la presentació de fosforil·lació en noves localitzacions com ara a l'acrosoma.

4.- En general s'observa un augment de la fosforil·lació en els residus tirosina, serina i treonina en l'espermatozoide porcí durant la capacitació "in vitro" seguida d'una disminució general durant la reacció acrosòmica provocada per la progesterona. Així doncs aquests resultats fan palesa la importància de la fosforil·lació en el control i regulació de processos espermàtics com la capacitació i la reacció acrosòmica en el porcí.

5.- La capacitació i la reacció acrosòmica en l'espermatozoide porcí provoquen canvis en l'activació mitocondrial. Així, la incubació en condicions capacitants i els primers minuts de la reacció acrosòmica representen un increment significant en l'activitat dels mitocondris corresponent amb un augment del consum energètic.

6.- L'activació mitocondrial durant la capacitació y reacció acrosómica "in vitro" presenta un patró concret al llarg de tota la peça intermèdia de l'espermatozoide porcí. Així, s'evidencien determinats nuclis d'activació des dels quals s'arriba a la totalitat dels mitocondris espermàtics.

7.- Tant l'actina com la mitofusina-2 tenen papers importants en el control i modulació de la funció mitocondrial espermática, ja sigui provocant canvis en l'estructura proteica de la membrana plasmàtica perimitocondrial o modificant l'estructura mitocondrial en sí. Sigui como sigui, aquests canvis modificaran alhora els contactes intermitocondrials i la transmissió d'estímuls a través de les peces intermitja i prinicpal de la cua.

Aquesta darrera pàgina us la dedico a tots els que al llarg d'aquests anys heu estat al meu costat i heu fet possible que arribés el dia en el que escrivís aquestes paraules.

MOLTES GRÀCIES A TOTS PERQUÈ SENSE VOSALTRES AQUESTA TESI NO HAURIA ESTAT POSSIBLE!

D.O.G