RAFAEL PEDROSO BETARELLI

EFEITOS DA GLUTATIONA REDUZIDA SOBRE OS PROCESSOS DE CAPACITAÇÃO E REAÇÃO ACROSSÔMICA *IN VITRO* EM ESPERMATOZOIDES SUÍNOS REFRIGERADOS

LAVRAS-MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Ciências Veterinárias, área de concentração em Reprodução Animal, para a obtenção do título de Doutor.

Prof. Dr. Márcio Gilberto Zangeronimo

Orientador

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EFFECTS OF REDUCED GLUTATHIONE OVER CAPACITATION PROCESSES AND *IN VITRO* ACROSSOME REACTION FOR REFRIGERATED SWINE SPERMATOZOON

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Ciências Veterinárias, área de concentração em Reprodução Animal, para a obtenção do título de Doutor.

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RESUMO GERAL

Um estudo foi realizado objetivando-se avaliar os efeitos da glutationa reduzida (GSH) sobre a função espermática, durante a capacitação e posterior indução da reação acrossômica *in vitro*, em sêmen suíno refrigerado. Isso foi feito por meio da avaliação de vários marcadores de capacitação *in vitro* e outros parâmetros globais de qualidade de sêmen, tais como viabilidade, motilidade total, exocitose acrossomal induzida por progesterona, fragmentação de DNA, níveis de desordens lipídicas na membrana espermática, espécies reativas de oxigênio (ERO), resíduos de cisteínas livres em extratos de cabeça e cauda espermáticas e fosforilação proteica de resíduos de tirosina. Um total de 62 ejaculados, provenientes de 35 reprodutores suínos saudáveis Pietrain, entre 2 e 3 anos de idade, foram usados nas diferentes análises deste trabalho. A adição de GSH, no meio de capacitação (CM), impediu a maioria das alterações concomitantes à capacitação. Além disso, o GSH provocou uma queda rápida e intensa da motilidade total ($P<0,05$), a qual foi mantida durante todo o período de incubação. Apesar destes resultados, o GSH não afetou o início da exocitose acrossomal *in vitro* induzida pela progesterona (IVAE) observada após 4h de incubação no CM. Os resultados mostraram que a capacitação *in vitro* de sêmen suíno está relacionada com um aumento significante tanto da quebra total das pontes dissulfeto quanto dos níveis de ERO intracellular. Esses fenômenos podem desempenhar um papel, na obtenção do estado capacitante da célula espermática, embora eles não sejam fundamentais na realização da posterior IVAE. Finalmente, a motilidade dos espermatozoides parece ser, parcialmente, controlada por mecanismos iônicos e de oxidação-redução, tal como indicou a incubação com GSH em condições separadas. Portanto este estudo demonstra a existência de vias paralelas e separadas dentro dos eventos da capacitação espermática controladas pelo GSH e, assim, contribui para uma melhor compreensão desses fenômenos e traz um novo direcionamento para trabalhos futuros dentro dessa linha de pesquisa.

Palavras-chave: Funcionalidade espermática. Espermatozoide. Fertilização. Espécies reativas de oxigênio. Pontes dissulfeto. Antioxidante.

GENERAL ABSTRACT

A study was conducted with the objective of evaluating the effects of reduced glutathione (GSH) over spermatic function during capacitation and posterior induction of acrosome reaction *in vitro*, in refrigerated pig semen. This was done by evaluating many *in vitro* capacitation markers and other global parameters of semen quality, such as feasibility, total motility, acrosome exocytosis induced by progesterone, DNA fragmentation, levels of lipid disorders in the spermatic membrane, reactive oxygen species (ROS), free cysteine residue in sperm head and tail extracts and protein phosphorylation of tyrosine residue. A total of 62 ejaculates derived from 35 healthy breeding Pietrain pigs, between 2 and 3 years of age, were used in the different analyses conducted in this work. The addition of GHS in the capacitation medium (CM) prevented most changes consequent to capacitation. In addition, the GSH caused quick fall and intense total motility ($P < 0.05$), which was maintained during the entire incubation period. Despite these results, the GH did not affect the beginning of *in vitro* acrosome exocytosis induced by progesterone (IVAE), observed after four hours of CM incubation. The results showed that *in vitro* pig semen capacitation is related to the significant increase of both total break of disulfide bonds and the levels of intracellular ROS. These phenomena can fulfill a role in obtaining the capacitating state of the spermatic cell, although they are not fundamental in performing the posterior IVAE. Finally, spermatozoid motility seems to be partially controlled by ionic and oxidation-reduction mechanisms, as was indicated by the GSH incubation in separate conditions. Therefore, this study demonstrates the existence of parallel and separated paths within the spermatic capacitation events controlled by the GSH, thus contributing for the better understanding of these phenomena, and brings a new direction for future works in this line of research.

Keywords: Spermatic functionality. Spermatozoid. Fertilization. Reactive Oxygen Species. Disulfide bonds. Antioxidant.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A indústria suinícola atua como um dos principais segmentos do mercado agropecuário mundial, sendo responsável pela produção anual de mais de 118 milhões de toneladas de carne, em 2015 (ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - OECD-FAO, 2016), além da geração de milhares de empregos. Além disso, de acordo com esse estudo, estima-se que, entre 2014 e 2023, o crescimento anual médio da produção mundial de carne suína seja de 1,2%, o que representará uma importante participação no consumo mundial de proteína animal. Esse crescimento da suinocultura deve-se à disseminação contínua de tecnologias aplicadas à produção, resultantes de pesquisas desenvolvidas, em diversas áreas como melhoramento genético, nutrição, sanidade e reprodução.

Atualmente, com a aplicação de diversas práticas e biotecnologias voltadas à reprodução de suínos, tais como a inseminação artificial, o papel do macho torna-se cada vez mais importante para a melhoria dos índices reprodutivos. Nesse sentido, muitos estudos têm sido realizados na tentativa de melhor compreender os eventos e funções envolvidos no metabolismo espermático. Entre os eventos mais importantes, destaca-se o processo de capacitação espermática que ocorre apenas no trato reprodutivo feminino, dificultando seu estudo *in situ*.

No entanto, com o surgimento de tecnologias de reprodução assistida, grande quantidade de informação tem sido recolhida sobre a forma como os espermatozoides tornam-se capacitados. Verifica-se que esse processo envolve a ativação de diversas vias de sinalização por mensageiros intracelulares, tais como Ca^{2+} e AMPc que resultam na reorganização de proteínas e lipídios da

membrana plasmática, além de mudanças nos padrões de motilidade e desestabilização da membrana acrossomal. Recentemente, com a descoberta de inúmeros agentes crioprotetores adicionados aos meios de congelamento do sêmen, novas linhas de pesquisa vêm surgindo na tentativa de se elucidar os efeitos dessas substâncias sobre o metabolismo espermático. Entre esses agentes, a glutationa reduzida (GSH) vem mostrando resultados positivos em relação à criopreservação espermática, embora seu efeito sobre a capacitação e reação acrossômica, ainda, permaneça desconhecido.

O GSH é o tiol mais abundante nas células e é considerado de vital importância, participando, principalmente, da manutenção do equilíbrio redox intracelular. Nos espermatozoides, além dessa função, o GSH, também, participa da manutenção da estabilidade da estrutura nucleoproteica. Tais propriedades funcionais estão relacionadas à sincronização de eventos essenciais no processo de capacitação espermática e consequente reação acrossômica. Nesse sentido, sugere-se que o GSH possa desempenhar um papel fundamental, na modulação da capacitação espermática, retardando os efeitos capacitantes dependendo de sua concentração. No entanto esta hipótese, ainda, não foi esclarecida. Assim, objetivou-se avaliar os efeitos do GSH sobre a função espermática, durante a capacitação e a indução da reação acrossômica pela progesterona *in vitro*, em sêmen suíno refrigerado.

2 REFERENCIAL TEÓRICO

2.1 O processo de capacitação espermática

A capacitação espermática foi descrita pela primeira vez por Austin (1951) e Chang (1951), em ratos e coelhos, respectivamente. Os autores observaram que os espermatozoides deviam permanecer no trato reprodutivo feminino, por certo período de tempo, a fim de alcançar a capacidade de fertilização. Em outras palavras, a capacitação é um processo que ocorre, naturalmente, no trato genital feminino e que confere à célula espermática a capacidade de passar pela reação acrossômica, interagir com a zona pelúcida e iniciar a fusão junto à membrana plasmática do ovócito (YANAGIMACHI, 1994). Posteriormente, a descoberta de que certos fatores no trato genital feminino eram necessários para o espermatozoide tornar-se fértil foi um passo primordial para o consequente desenvolvimento da fertilização *in vitro* (CHANG, 1955).

A capacitação espermática tem sido definida como uma combinação de eventos sequenciais e paralelos que ocorrem na cabeça (preparação para a reação acrossômica) e na cauda do espermatozoide (hiperativação). Esse processo pode ser dividido em duas fases: (1) eventos rápidos e iniciais, que compreendem a ativação de um movimento vigoroso e assimétrico do flagelo. Esses eventos ocorrem, logo que a célula espermática deixa o epidídimo, ao entrarem em contato com o fluido seminal; e (2) eventos lentos e tardios que compreendem as alterações no padrão de movimento (hiperativação), a capacidade de realizar a reação acrossômica e a fosforilação de tirosina (SALICIONI et al., 2007; VISCONTI, 2009). *In vivo*, essa última fase ocorre, durante a passagem dos espermatozoides pelo trato reprodutivo feminino, mas também, quando as células espermáticas são incubadas em meio de cultura apropriado, contendo,

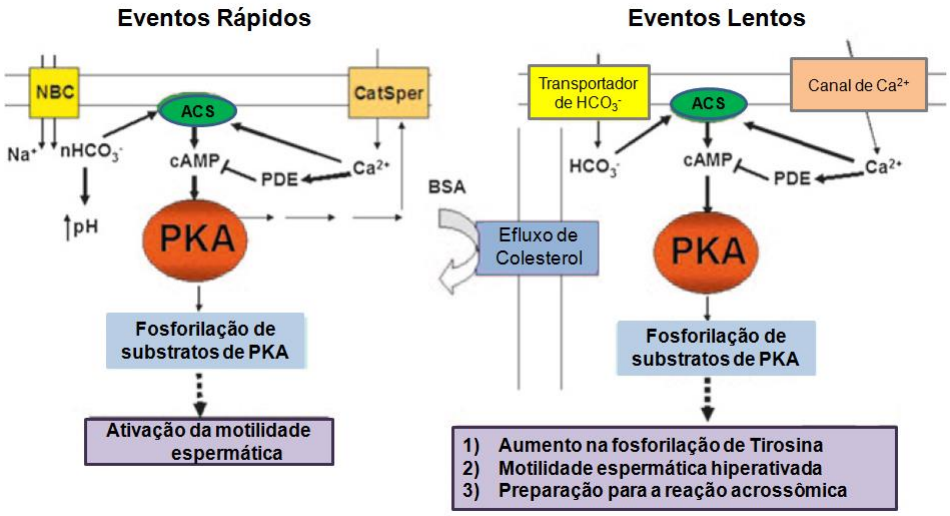
principalmente, Ca^{2+}, HCO_3^- e uma proteína removedora de colesterol, normalmente, a albumina sérica bovina (BSA).

Embora existam controvérsias sobre a possibilidade dos eventos rápidos fazerem parte da capacitação, observa-se que essa etapa é fundamental para a realização bem sucedida da fertilização. Essa etapa ocorre, alguns segundos após a ejaculação, sendo ativada pelo aumento das concentrações de Ca^{2+} e HCO_3^- presentes no fluido seminal (VISCONTI, 2009). O HCO_3^- entra na célula pelo cotransportador de Na^+/HCO_3^- (DEMARCO et al., 2003), aumentando o pH intracelular e a ativando a adenil ciclase solúvel (ACS), um tipo único de adenilato ciclase presente no espermatozoide. A principal característica dessa enzima é que ela é ativada tanto por HCO_3^- quanto pelo Ca^{2+} e não pela proteína G ou forskolina, como a maioria das adenilato ciclases transmembrana. O aumento das concentrações intracelulares de HCO_3^- produz uma assimetria da membrana plasmática, ativando as enzimas *scramblase* que translocam os fosfolpídeos de membrana, tais como a fosfatidilserina e a fosfatidiletanolamina (GADELLA; HARRISON, 2000). Esse evento facilita a remoção do colesterol da membrana espermática pelas proteínas removedoras externas, como a albumina e as lipoproteínas de alta densidade (HDL) (SALICIONI et al., 2007; VISCONTI, 2009). Ao mesmo tempo, a ACS aumenta os níveis intracelulares de AMPc e, subsequentemente, a ativação da proteína quinase A (PKA), que modula a resposta dos canais de Ca^{2+}, tais como o *CatSper*. A abertura desses canais aumenta a concentração intracelular desse íon, ajudando a manter a ACS ativa para realizar a modulação dos eventos lentos da capacitação espermática (WENNEMUTH et al., 2003) (Figura 1).

Em suínos, um evento importante que ocorre, durante a fase rápida, é a remoção de uma adesina espermática espécie-específica, AQN-1, a partir da superfície da célula espermática (EKHLASI-HUNDRIESER et al., 2005). Essa adesina desempenha um papel relevante, na formação do reservatório

espermático, em suínos, inibindo a atividade de outras proteínas associadas à superfície celular, tais como a AQN-3, AWN e P47/SED1, que estão envolvidas na interação dos espermatozoides com a zona pelúcida do ovócito. Durante a desestabilização da membrana espermática, já na fase tardia, a AQN-1 é removida da superfície do espermatozoide, liberando os espermatozoides do reservatório ovidutal e permitindo a interação espermática com a zona pelúcida (TÖPFER-PETERSEN; EKHLASI-HUNDRIESER; TSOLOVA, 2008).

Figura 1 - Bases moleculares dos eventos rápidos e lentos associados à capacitação espermática.



Legenda: Nos eventos rápidos (esquerda), o bicarbonato e o Ca^{2+} estimulam a ativação da motilidade espermática, através da ACS e PKA. Esses íons são transportados por um cotransportador Na^{+}/HCO_3^{-} (NBC) e um canal de cálcio específico espermático (CatSper). Nos eventos lentos (direita), o espermatozoide adquire a habilidade para fertilizar o ovócito, havendo o aumento na fosforilação de tirosina, presença da motilidade espermática hiperativada e preparação para a ocorrência da exocitose do acrossoma. (ACS = Adenil Ciclase Solúvel; cAMP = Adenosina Monofosfato Cíclico; PDE = Fosfodiesterases; PKA = Proteína quinase A; BSA = Albumina sérica bovina, ilustrando uma proteína removedora de colesterol). Fonte: Adaptado de Visconti (2009).

O início dos eventos lentos da capacitação, que ocorre no oviduto do trato reprodutivo feminino, é marcado pela remoção do colesterol da membrana plasmática pela HDL e pela albumina sérica, acarretando no aumento da sua fluidez (CROSS, 2004). Durante esta fase, a PKA, ativada no interior celular pelo AMPc, fosforila várias proteínas em resíduos de serina e treonina, ativando, direta ou indiretamente, várias proteínas quinases e/ou inibindo fosfatases, levando ao aumento na fosforilação de resíduos de tirosina de proteínas intracelulares, que, por sua vez, estimulam as mudanças na função espermática que ocorrem durante os eventos sincronizados da capacitação. Tais alterações são responsáveis pela hiperativação espermática; pela quimiotaxia dos espermatozoides em direção ao ovócito; pela capacidade espermática de realizar a reação acrossômica induzida por um agonista biológico presente na zona pelúcida ou pela progesterona; e, consequentemente, pela capacidade de fertilizar o ovócito (SIGNORELLI; DIAZ; MORALES, 2012). Ao final do processo, isto é, quando um espermatozoide está totalmente capacitado, a membrana plasmática apical da cabeça da célula espermática torna-se fusogênica (HARRISON, 1996, 2004), permitindo a união entre os gametas e a transferência do material genético masculino ao interior do ovócito. Os eventos tardios da capacitação espermática ocorrem sob um rigoroso controle, uma vez que esse é um processo irreversível, ou seja, se ocorrer a desestabilização da membrana espermática, na ausência do ovócito maduro, inevitavelmente, ocorrerá a morte do espermatozoide (RODRÍGUEZ-MARTÍNEZ, 2007).

2.2 Modificações pós-traducionais envolvidas com o processo de capacitação

Um dos mecanismos mais importantes que envolve a modulação das alterações estruturais e funcionais, durante a capacitação espermática, está ligado às modificações pós-traducionais pela fosforilação de serina, treonina e

resíduos de tirosina de proteínas espermáticas específicas . Esse é um mecanismo geral que está presente, em todas as células, desempenhando um papel importante, em muitos processos, incluindo a transdução de sinais, o transporte intracelular e a progressão do ciclo celular (JHA et al., 2006).

O estado de fosforilação de fosfoproteínas é controlado pela atividade de um sistema complexo de proteínas quinases específicas (PK) e fosfatases. Deve-se salientar que espermatozoides de mamíferos são transcricionalmente inativos e incapazes de sintetizar novas proteínas, portanto a dependência de mecanismos de fosforilação de proteínas, para modular funções específicas, , é maior do que em outras células do organismo (URNER; SAKKAS, 2003). Inúmeras funções estão ligadas à fosforilação de proteínas, tais como a motilidade espermática (TASH; MEANS, 1983; VIJAYARAGHAVAN et al., 1997), o reconhecimento da zona pelúcida, a afinidade entre os gametas e, por fim, à própria reação acrossômica (FLESCH; GADELLA, 2000; LEYTON; SALING, 1989; NAZ; AHMAD, 1994).

Os resíduos de tirosina, por exemplo, têm sido implicados como sendo capazes de regular a glicólise na célula espermática (ARCELAY et al., 2008). Assim, em ratos (VISCONTI et al., 1999b), humanos (LECLERC; DELAMIRANDE; GAGNON, 1996), bovinos (GALANTINO-HORNER et al., 2004), suínos (KALAB et al., 1998) e equinos (POMMER; RUTLLANT; MEYERS, 2003), a capacitação está, diretamente, relacionada aos resíduos de tirosina de proteínas espermáticas de maneira PKA-dependente (TARDIF et al., 2001).

2.3 Fatores que modulam a capacitação espermática

Como descrito anteriormente, as mudanças bioquímicas que ocorrem, durante a capacitação espermática, estão relacionadas com a composição lipídica e permeabilidade da membrana, com a concentração iônica intracelular, com o

movimento do espermatozoide e com a fosforilação de proteínas. Todos esses eventos ocorrem, quando o espermatozoide é exposto a um novo ambiente, de concentração iônica diferente, que é o trato reprodutivo feminino ou os meios de capacitação *in vitro*.

Dentre os fatores que estimulam a capacitação espermática, além dos íons cálcio e bicarbonato, existe, também, a via de regulação extracelular, que representa não só a interação de ligantes aos receptores de membrana, mas também a ativação intracelular de espécies reativas de oxigênio (ERO). A ativação dessas vias conduz à fosforilação de diferentes proteínas que estão associadas aos eventos de capacitação. Diversos fatores podem estimular ou inibir a capacitação. A seguir, serão descritas as principais substâncias capacitantes e decapacitantes envolvidas na modulação de todo o processo de capacitação espermática.

2.3.1 Fatores capacitantes

Efluxo de colesterol

O colesterol está mais concentrado, em regiões altamente organizadas da membrana plasmática do espermatozoide, mais especificamente, na região acrossomal, sob a forma de microdomínios conhecidos como balsas lipídicas, (SELVARAJ et al., 2009). O início dos eventos lentos da capacitação envolve a saída do colesterol da membrana dos espermatozoides, aumentando sua fluidez. Nos meios de capacitação *in vitro*, a presença de aceitadores de colesterol, tais como a BSA, HDL e β-ciclodextrinas promove a remoção do colesterol da membrana plasmática do espermatozoide (VISCONTI et al., 1999a). Entretanto, *in vivo*, evidências apontam a presença de albumina e HDL, no fluido folicular e ovidutal (VISCONTI et al., 1999b; YANAGIMACHI, 1994), que desempenham um papel central na iniciação e ativação das vias de transdução de sinal ligadas à capacitação (VISCONTI et al., 1999b). Nesse sentido, alguns trabalhos *in vitro*

demonstraram que a β-ciclodextrina, ao promover a remoção do colesterol da membrana, estimulou, indiretamente, a fosforilação de resíduos de tirosina mediada através da via de sinalização AMPc/PKA (BAILEY, 2010; SHADAN et al., 2004; VISCONTI et al., 1999b).

Bicarbonato

O fluido epididimal, normalmente, apresenta concentrações mais baixas de HCO_3^- em relação ao plasma seminal e ao trato reprodutor feminino. Quando o espermatozoide se encontra nessas regiões, o HCO_3^- é formado, a partir da atividade da anidrase carbônica IV presente na membrana espermática, quando em contato com maiores concentrações de CO_2, presentes no meio (WANDERNOTH et al., 2010). Da mesma forma, quando os espermatozoides são incubados em concentrações mais elevadas de CO_2 (cerca de 1%), são induzidos à capacitação e à fosforilação de resíduos de tirosina (GALANTINO-HORNER et al., 2004).

Além de aumentar o pH intracelular e ativar a ACS, o HCO_3^- também, está envolvido, na maturação funcional dos espermatozoides, ao afetar a dinâmica da membrana em termos de composição lipídica, teor de colesterol, polaridade lateral de glicolipídeos, fluidez e distribuição de fosfolípideos (GADELLA; GESTEL, 2004). Isso é decorrente da atividade de algumas proteínas translocadoras de fosfolipídeos de membrana (flipases e *scramblase*), ambas dependentes de HCO_3^-. Tais proteínas ajudam a manter a assimetria dos fosfolipídeos de membrana, na região acrossomal, durante a capacitação.

In vitro, a liberação de outro grupo de proteínas ancoradas glicosilfosfatidilinositol CD52 e CD55 induzida pelo HCO_3^- resulta na agregação de lipídios, na região apical dos espermatozoides suínos (BOERKE et al., 2014), resultando na assimetria dos fosfolipídeos. Nesse momento, a adenilato ciclase solúvel, encontrada no citosol de espermatozoides, difere da adenilato ciclase

ligada à membrana de células somáticas. No caso das células espermáticas, a ativação dessa enzima é dependente do aumento do pH intracelular propiciado pelo HCO_3-. Esse aumento no pH eleva as concentrações de AMPc no citosol. Entretanto eventos da capacitação, tais como a hiperativação e a fosforilação de tirosina, são dependentes da atividade da adenilato ciclase solúvel, enquanto a exocitose acrossomal não depende (HESS et al., 2005).

Cálcio

O cálcio (Ca^{+2}) participa em muitas funções espermáticas como a capacitação, motilidade, quimiotaxia e fertilização. Em células somáticas, o retículo endoplasmático e as mitocôndrias são as principais organelas envolvidas no armazenamento de cálcio. Entretanto, no caso da célula espermática, os locais de armazenamento de cálcio não estão, totalmente, definidos. Os prováveis locais de armazenamento são o acrossoma e a envoltura nuclear redundante (GANGWAR; ATREJA, 2015).

Na membrana espermática, existem dois tipos de canais principais envolvidos com a entrada de cálcio no espermatozoide, durante a capacitação, os canais de cálcio dependente de voltagem (CCDV) e os canais de cátion espermáticos (*CatSper*). A atividade de CCDV é, altamente, sensível a qualquer mudança no pH intracelular, durante a capacitação (NERI-VIDAURRI; TORRES-FLORES; GONZALEZ-MARTINEZ, 2006), porém o mecanismo de sensibilidade dos canais *CatSper* não é bem conhecido (XIA; REN, 2009). Esses autores relataram que glicoproteínas, presentes na zona pelúcida, podem induzir o influxo de cálcio por meio desses canais que, segundo Chung et al. (2014), estão envolvidos, na regulação da fosforilação de tirosina no flagelo, durante a capacitação. De acordo com Lishko et al. (2010), a progesterona produzida pelas células do *cumulus* está envolvida na sinalização de cálcio pela abertura dependente de voltagem do canal *CatSper*. Dessa maneira, pelo aumento do

influxo de cálcio pelos canais CatSper, a progesterona estaria envolvida na hiperativação e reação acrossômica dos espermatozoides. Por sua vez, as prostaglandinas, mas especificamente as PGE, presentes no fluido seminal (KACZMAREK et al., 2010) e no muco cervical (CHARBONNEL et al., 1982), também, estão relacionadas com processos reprodutivos por ativarem os canais *CatSper* (GANGWAR; ATREJA, 2015).

Alcalinização intracelular e potencial elétrico de membrana

O aumento do pH intracelular é vital, para a iniciação e regulação de diferentes atividades, associadas à capacitação do espermatozoide. Essa alcalinização do meio intracelular espermático é essencial tanto para a hiperativação espermática quanto para a reação acrossômica (DARSZON et al., 2006; SUAREZ, 2008).

Vários transportadores e canais estão relacionados às regulações do pH e do potencial elétrico de membrana dos espermatozoides de mamíferos, tais como bomba de Na^+/H^+, transportadores de HCO_3^-, canais *CatSper*, canais Slo3 e canais de próton dependente de voltagem 1 (HV1). Embora o mecanismo molecular e a função da hiperpolarização da membrana, durante a capacitação espermática, seja pouco esclarecida, assinala-se que ela tem uma correlação positiva com a alcalinização intracelular e é necessária para a preparação da exocitose acrossomal (DE LA VEGA-BELTRAN et al., 2012). O canal de potássio Slo3, específico de células espermáticas, está envolvido na hiperpolarização, durante a capacitação e regula a atividade do canal de Ca^{2+} *CatSper* (CHAVEZ et al., 2014). Esses dois canais são ativados pela alcalinização intracelular, sendo abertos, após uma despolarização transitória, induzida por agentes fisiológicos do oviduto, como as proteínas da zona pelúcida e a progesterona, momentos antes da reação acrossômica (OSMAN et al., 1989). Por sua vez, o HV1 é encontrado na peça principal e atua na expulsão de prótons

H^+ nessa região da célula espermática. A despolarização da membrana e a alcalinização do meio intracelular são os dois fatores principais que ativam o HV1 (LISHKO; KIRICHOK, 2010).

2.3.2 Fatores decapacitantes

Um dos aspectos mais importantes da regulação da capacitação, espermática dentro do oviduto, é que os espermatozoides têm que permanecer, por certo período não capacitados, com o acrossoma intacto até o encontro com o ovócito. Para isso, existem fatores que dificultam a capacitação espermática.

Os fatores decapacitantes são moléculas que podem ser encontrados, no plasma seminal e que recobrem e estabilizam a membrana do espermatozoide evitando sua capacitação prematura. O fator capacitante melhor caracterizado é o grupo de proteínas que compõe o plasma seminal e que formam parte da família "*Binder of Sperm*" (BSPs), identificados, primeiramente, em bovinos, mas também presentes em diferentes espécies de mamíferos, incluindo em suínos. Essas proteínas se unem aos fosfolipídeos da membrana da célula espermática e, quando são removidas junto ao epitélio do trato reprodutivo feminino, arrastam junto a elas fosfolipídeos e colesterol constituintes da membrana. Após a remoção, os espermatozoides podem se ancorar, nas zonas reservatórias ou interagir com móleculas solúveis do fluido ovidutal, desencadeando, assim, os eventos capacitantes (GORDÚN; MONDÉJAR; MIGUEL, 2013).

O zinco, presente no plasma seminal, é um inibidor muito eficiente de canais de próton dependente de voltagem 1 (HV1), prevenindo a ativação espermática prematura. Quando há a alcalinização intracelular, a inibição do HV1 por zinco, também, aumenta. A exposição dos espermatozoides aos fluidos do trato reprodutivo feminino, com menores concentrações de zinco, facilita a ativação de HV1 (LISHKO et al., 2010; LISHKO; KIRICHOK, 2010). Nessa

região, o zinco pode ser absorvido por células adjacentes ou ligado à albumina (GANGWAR; ATREJA, 2015).

Nas zonas reservatório do trato reprodutivo feminino, existem ligantes que interagem com os receptores acoplados à proteína G (GPCRs) e inibem a atividade das adenilato ciclases associadas à membrana espermática. Isso reduz a produção de AMPc em espermatozoides já capacitados e previne as reações acrossômicas espontâneas/degenerativas (FRASER, 2010). Esses ligantes foram encontrados, em espermatozoides de ratos (FRASER et al., 2006), suínos (FUNAHASHI et al., 2000) e humanos (FRASER; OSIGUWA, 2004). No entanto essa inibição da perda espontânea do acrossoma não impede que os espermatozoides desenvolvam reações acrossômicas em resposta à progesterona (GREEN et al., 1996).

Além das alterações iniciais dependentes da ACS, na arquitetura da membrana plasmática da célula espermática, existem outras alterações que envolvem a perda, desmascaramento ou rearranjo de moléculas, na superfície do espermatozoide, que dificultam a capacitação (FRASER, 2010). As adesinas espermáticas (AWN, AQN-1 e AQN-3), por exemplo, que atuam na estabilização da membrana plasmática da vesícula acrossomal, sofrem um rearranjo, a partir da superfície dos espermatozoides, durante a capacitação, em que a remoção da AQN-1 da superfície espermática permite, primeiramente, a liberação dos espermatozoides das zonas reservatório, no oviduto e, em seguida, a exposição da AWN e AQN-3 e sua consequente interação com proteínas da zona pelúcida (CALVETE et al., 1997; DOSTALOVA et al., 1994; SANZ et al., 1993). Assim, as perdas de AWN e AQN-3, durante o processo de capacitação, por exemplo, são, geralmente, referidas como fatores decapacitantes (BEDFORD; CHANG, 1962; DE LAMIRANDE; LECLERC; GAGNON, 1997). Por outro lado, a não remoção de AQN-1 dos espermatozoides pode reverter a capacitação, ou seja, ela pode reverter a célula para o estado não

fertilizante. Com o tempo, os espermatozoides podem se capacitar novamente e recuperar a fertilidade. Essa característica indica que a capacitação é "reversível", embora algumas etapas desse processo, como o acoplamento da membrana, não sejam reversíveis. Quando a capacitação é revertida por fatores decapacitantes, não há retorno de colesterol, para a membrana plasmática (FRASER, 2010).

Outro fator decapacitante, a calmodulina, é uma proteína que se une ao cálcio e está relacionada aos processos celulares de transdução de sinais que são originados por aumento nos níveis de cálcio citoplasmático. A concentração de calmodulina é reduzida, durante a capacitação e isso resulta na diminuição da atividade da Ca^{2+}-ATPase, dependente de calmodulina, encarregada de expulsar o cálcio da célula. Consequentemente, há aumento da concentração de cálcio citoplasmático. Outro efeito da redução de calmodulina é o aumento do AMPc que, como já mencionado, é o segundo mensageiro que, também, regula os fenômenos da capacitação espermática. Esse aumento da concentração de AMPc é provocado pela queda da atividade da fosfodiesterase PDE1, que é uma enzima dependente de Ca^{2+}-calmodulina (GORDÚN; MONDÉJAR; MIGUEL, 2013).

2.4 A reação acrossômica

O acrossoma é uma vesícula ácida cujo seu interior contém uma variedade de proteínas e enzimas hidrolíticas, como a hialuronidase, acrosina, fosfolipase C, colagenase, β-galactosidase, dentre outras, cuja liberação ajuda os espermatozoides a atravessarem o *cummulus ooforus* e a zona pelúcida. A reação acrossômica ocorre ao final do processo de capacitação e quimiotaxia. Nesse momento, a membrana plasmática do espermatozoide é fundida em vários pontos com a do acrossoma pelos canais de cálcio, propiciando a liberação do conteúdo acrossomal ao exterior da célula espermática e facilitando a passagem pela zona pelúcida (GORDÚN; MONDÉJAR; MIGUEL, 2013).

O processo de exocitose acrossomal é desencadeado *in vivo* por ligação de espermatozoides capacitados à zona pelúcida de ovócitos ovulados (FLESCH; GADELLA, 2000). O passo inicial na complexa via que conduz à exocitose acrossomal é o influxo de Ca^{2+} (DARSZON et al., 2006) desencadeado por proteínas da própria zona pelúcida e pela progesterona (FLESCH; GADELLA, 2000). Supõe-se que a reação acrossômica exerce, pelo menos, uma dupla função. Uma por facilitar a capacidade do espermatozoide em penetrar na zona pelúcida e outra por fundir-se com ovócitos (YANAGIMACHI, 1994). Já a indução da reação acrossômica *in vitro,* em espermatozoides suínos, pode ser alcançada pela adição de diferentes agentes ao meio de capacitação, incluindo o ionóforo de cálcio, a progesterona e proteínas da zona pelúcida (ASHWORTH et al., 1995; BERGER et al., 1989; MELENDREZ; MEIZEL, 1995; MELENDREZ; MEIZEL; BERGER, 1994; PETRUNKINA et al., 2005).

O citoesqueleto de actina, também, participa da reação acrossômica atuando na regulação da exocitose do acrossoma. Durante a capacitação, a polimerização de actina é aumentada pela ativação das vias PKA e PKC. Quando os espermatozoides capacitados são estimulados à realizarem a exocitose do acrossoma, ocorre rápida despolimerização da actina que parece estar relacionada ao aumento do cálcio citossólico. Se a despolimerização de actina é inibida, a exocitose do acrossoma induzida pela ZP é bloqueada (GORDÚN; MONDÉJAR; MIGUEL, 2013).

2.5 A participação da glutationa reduzida no processo de capacitação

O GSH (L-γ-glutamil-L-cisteinilglicina) é um tripeptídeo, ubiquamente, distribuído em células vivas. Trata-se do tiol mais importante, presente em seres vivos, sendo um composto de peso molecular de, aproximadamente, 307 contendo um grupo sulfidrila (MARTÍNEZ SARRASAGUE et al., 2006), que

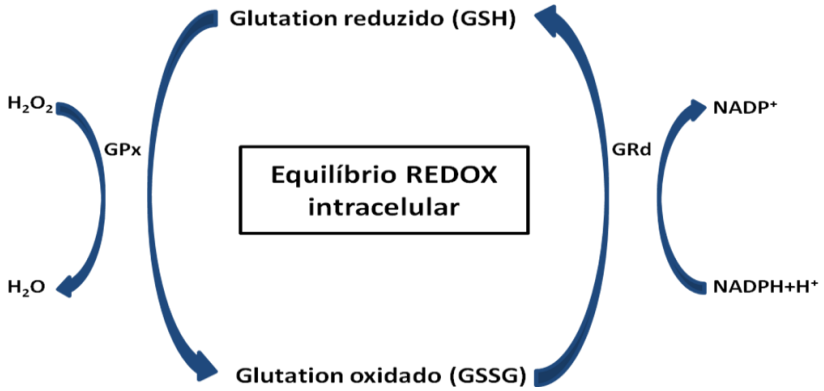
confere a esse composto ação protetora contra os danos causados pelo estresse oxidativo.

O estresse oxidativo, por sua vez, pode ser definido como qualquer desequilíbrio entre substâncias oxidantes e antioxidantes, em que as primeiras prevalecem e produzem uma cascata de radicais livres, conduzindo a um processo lipoperoxidativo (BETTERIDGE, 2000). Os efeitos da peroxidação lipídica incluem a perda irreversível de motilidade, o vazamento de enzimas intracelulares, danos ao DNA espermático (WHITE, 1993) e deficiências na penetração e fusão do espermatozoide no ovócito (AITKEN, 1995).

A glutationa apresenta-se em duas formas: a forma reduzida (GSH) e a forma oxidada (GSSG), que se formam por meio da atividade da glutationa peroxidase e a glutationa redutase. Essa inter-relação é que confere a ação protetora da glutationa contra ERO.

Como antioxidante, o GSH desempenha papel importante, no mecanismo de defesa contra o estresse oxidativo intracelular, embora, também, esteja envolvido em outras funções do organismo, tais como atuação como coenzima, participação em processos de desintoxicação, controle da permeabilidade da membrana e transporte de aminoácidos, participação na síntese proteica, entre outras (MARTÍNEZ SARRASAGUE et al., 2006). No equilíbrio redox, a glutationa peroxidase usa o GSH, para reduzir o peróxido de hidrogênio a H_2O e o GSSG resultante é reduzido a GSH pela glutationa redutase, utilizando NADPH como cofactor (Figura 2).

Figura 2 - Equilíbrio REDOX intracelular.



Legenda: Uma das principais funções atribuídas ao GSH. GPx: Glutation Peroxidase; GRd: Glutation Redutase.

A síntese do GSH ocorre em duas etapas e depende da disponibilidade de substratos, como cisteína, ácido glutâmico e glicina e dos mecanismos regulatórios. A regulação de sua síntese ocorre pela enzima gamaglutamil cisteinil sintetase (γGSC), na qual o GSH exerce o *feed back* negativo (LU, 2013). Assim, o GSH está sujeito a um constante *turnover* no organismo, sendo fígado, rins, pulmões, coração, intestinos e músculos os principais órgãos responsáveis pelo seu equilíbrio. A captação pelos demais tecidos depende da atividade da gama glutamil transpeptidase (γGT), localizada na membrana (DENEKE; FANBURG, 1989). Dentro das células, o GSH é encontrado, principalmente, nas mitocôndrias, retículo endoplasmático e núcleo e, nesse último, um aumento de sua concentração, durante a apoptose, é observado (MEISTER, 1995).

No sêmen de mamíferos, a capacidade de defesa antioxidante é constituída por diversos sistemas enzimáticos e não enzimáticos, sendo esse último representado, principalmente, pelo sistema glutation (LUBERDA, 2005). A função básica do GSH, no sêmen de mamíferos, está relacionada a suas interações com outros sistemas como um mecanismo preventivo contra os ERO

(ALVAREZ; STOREY, 1989; STRZEZEK; LAPKIEWICZ; LECEWICZ, 1999). Essa função de eliminação, exercida pelo GSH, ajuda a célula espermática a combater os efeitos do estresse oxidativo (AITKEN, 1999). Em espermatozoides humanos, o ciclo da glutationa forma um sistema de suporte básico pela remoção de H_2O_2 com a superóxido dismutase (SOD). Estudos demostraram que o H_2O_2 é o ERO mais tóxico nessas células (ALVAREZ; STOREY, 1989; GRIVEAU et al., 1995).

Existem diferenças nos níveis de glutation nos espermatozoides entre as diferentes espécies. Em suínos, essa concentração é de 0,3 nmol/10^9 espermatozoides contra 5,3 ± 2,2 nmol/10^9 espermatozoides em humanos, 5,3 ± 1,1 nmol/10^9 espermatozoides em cães (LI, 1975) e 29,3 nmol/10^9 espermatozoides em bovinos (AGRAWAL; VANHA-PERTTULA, 1988). No plasma seminal, as concentrações encontradas foram de 185,8 ± 46,7 µM em suínos (STRZEZEK; LAPKIEWICZ; LECEWICZ, 1999), 0,2 a 1,4 µM em humanos (OCHSENDORF et al., 1998) e 13 a 19 µM em touros (AGRAWAL; VANHA-PERTTULA, 1988). Em suínos, notam-se níveis, relativamente, baixos de GSH, nos espermatozoides, em comparação às demais espécies (LI, 1975), sugerindo menor atividade, desse sistema, no processo de proteção antioxidante. Assim, para diminuir os danos causados pela criopreservação, o GSH tem sido, comumente, adicionado aos meios de congelamento de sêmen (GIARETTA et al., 2015; YESTE et al., 2014).

Além de participar do processo de defesa antioxidante, o GSH, também, facilita a descondensação do pronúcleo espermático, durante a fertilização *in vitro*, em suínos (WHITAKER et al., 2008). Essa descondensação ocorre em decorrência da ativação da histona no interior do ovócito penetrado. Assim, acredita-se que o GSH, também, facilita a desagregação de protaminas que mantêm o DNA condensado, pela mudança no ambiente redox do ovócito (FUNAHASHI; SANO, 2005). Assim, o uso de GSH induz efeitos benéficos,

em muitos aspectos da produção *in vitro* de embriões suínos (ABEYDEERA, 2002), tais como crioproteção, durante o congelamento e descongelamento do sêmen e aumento da taxa de fertilização (GADEA et al., 2005).

Outos efeitos da adição de GSH, nos meios de congelamento e descongelamento de sêmen suíno, incluem o aumento da motilidade dos espermatozoides, redução dos níveis de peróxidos intracelulares e aumento da capacidade de penetração do espermatozoide no ovócito (GADEA et al., 2004, 2005; YESTE et al., 2013). A adição de GSH, a uma concentração de 2 mM, parece melhorar a estabilidade da estrutura nucleoproteica e a resistência às crioinjurias, aumentando a viabilidade espermática (YESTE et al., 2013) e a fertilidade do plantel inseminado com sêmen criopreservado (ESTRADA et al., 2014).

No entanto, embora muitos trabalhos tenham comprovado os benefícios do GSH à qualidade do sêmen, poucos estudos investigaram o mecanismo preciso pelo qual o GSH poderia mediar esse efeito protetor ou se esse poderia interferir em funções espermáticas básicas tais como a capacitação e a reação acrossômica em diferentes concentrações de GSH. Portanto estudos mais aprofundados são necessários, para elucidar o real mecanismo, pelo qual essa substância exerce seu papel e sua relação com os processos naturais do espermatozoide suíno.

3 CONSIDERAÇÕES GERAIS

O uso de substâncias antioxidantes, como o GSH, em meios de criopreservação de sêmen, está cada vez mais difundido por seus efeitos crioprotetores, melhorando as taxas de fertilização. Recentemente, com os achados a respeito do uso desses agentes crioprotetores, nos meios de congelação de sêmen suíno, novas portas, para linhas de pesquisa, abriram-se para elucidar os efeitos destas substâncias sobre o metabolismo espermático.

O processo de criopreservação induz fortes alterações na função espermática. Algumas dessas mudanças, tais como modificações nos parâmetros de movimento e nos níveis globais de fosforilação de tirosina dos espermatozoides, lembram aquelas induzidas durante o processo de capacitação. Tais semelhanças têm levado ao surgimento do termo "criocapacitação" para descrever este fenômeno. Porém os espermatozoides congelados-descongelados tornam-se desestabilizados e a criopreservação prejudica a capacidade fertilizante do sêmen quando utilizado na inseminação artificial (IA).

Desse modo, pela semelhança entre alguns eventos dos dois processos, os resultados obtidos com sêmen suíno criopreservado poderiam sugerir que o GSH e, portanto, o estado oxidativo do espermatozoide, poderia desempenhar um papel na modulação do processo fisiológico de capacitação e reação acrossômica no sêmen suíno. No entanto, ainda, faltam estudos, para determinar o papel do GSH, nos mecanismos que modulam tais processos e, assim, esclarecer esta hipótese.

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SEGUNDA PARTE – ARTIGO

ARTIGO 1 - BOAR SPERM *IN VITRO* CAPACITATION AND SUBSEQUENT PROGESTERONE-INDUCED ACROSOME EXOCYTOSIS ARE CONCOMITANT WITH INCREASES IN INTRACELLULAR LEVELES OF BOTH FREE CYSTEINE RADICALS AND OVERALL REACTIVE OXYGENE SPECIES: EFFECTS OF REDUCED GLUTATHIONE

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TITLE: Boar sperm *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis are concomitant with increases in intracellular leveles of both free cysteine radicals and overall reactive oxygene species: effects of reduced glutathione

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ABSTRACT

Incubation for 4h of boar sperm in a medium (CM) specifically designed to achieve *in vitro* capacitation (IVC) induced a significant ($P<0.05$) increase in the intracellular levels of free cysteine radicals and both peroxides and superoxides. These increases were concomitant with capacitation-like changes of parameters like sperm motility, membrane fluidity, mitochondrial membrane potential tyrosine phosphorylation levels of protein P32 and overall location of protein tyrosine phosphorylation. The addition of reduced glutation at the CM prevented the majority of the capacitation-concomitant changes, excepting that of P32. Furthermore, GSH causes a rapid and intense drop of total motility, which was maintained during all of the incubation period. Despithe these results, GSH did not affect the launching of progesterone-induced *in vitro* acrosome exocytosis (IVAE) observed after 4h of incubation in the CM. On the other hand, the addition of GSH together with progesterone after 4h of incubation in CM did not affect again the launching of IVAE, while there were few and slight effects in the majority of the other analyzed parameters. Despite this, the addition of GSH after 4h of incubation together with progesterone induced again a drop in total motility, although in a much less intensity than that observed when GSH was added at 0h of incubation. Our results seem to indicate that boar sperm IVC is related with a significant increase of both overall broken disulfide bonds and intracellular ROS levels. These phenomena could play a role in the achievement of the capacitation status, although they are not instrumental in the achievement of subsequent progesterone-induced IVAE. Finally, sperm motility seems to be partially controlled by ionic and redox mechanisms, as the incubation with GSH in separate conditions would indicate.

Keywords: Spermatozoa. Sperm function. Antioxidant. Nucleoprotein struture. phosphorylation. Free-cysteine residues. Fertilisation.

RESUMO

A incubação por 4 horas de sêmen suíno em um meio (CM) especificamente preparado para ativar a capacitação espermática *in vitro* (IVC) induziu um aumento significativo ($P<0,05$) nos níveis intracelulares de resíduos de cisteínas livres e de peróxidos e superóxidos. Esses aumentos foram concomitantes com alterações em parâmetros relacionados à capacitação, tais como a motilidade espermática, fluidez de membrana, potencial de membrana mitocondrial, níveis de fosforilação de proteína P32 em tirosina e localização geral da fosforilação de proteínas em resíduos de tirosina. A adição de glutationa reduzida (GSH) ao CM impediu a maioria dessas alterações concomitantes à capacitação, exceto a da P32. Além disso, a GSH causa uma rápida e intensa queda da motilidade total, que foi mantida durante todo o período de incubação. Apesar desses resultados, a GSH não afetou a ativação da exocitose acrossomal *in vitro* induzida por progesterona (IVAE) observada após 4 horas de incubação no CM. Por outro lado, a adição de GSH junto com a progesterona após 4 horas de incubação no CM não afetou novamente a ativação da IVAE, embora houveram poucos e leves efeitos na maioria dos outros parâmetros analizados. Apesar disso, a adição de GSH após 4 horas de incubação junto com a progesterona induziu novamente uma queda na motilidade total, embora em uma intensidade muito menor do que a observada quando a GSH foi adicionada no início da incubação. Nossos resultados parecem indicar que a IVC em sêmen suíno é relacionada com um aumento significativo tanto de pontes dissulfeto rompidas totais quanto de níveis intracelulares de espécies reativas de oxigênio (ROS). Esse fenômeno pode desempenhar um papel no alcance do estado capacitante, embora ele não seja essencial na realização da subsequente IVAE induzida pela progesterone. Finalmente, a motilidade espermática parece ser parcialmente controlada por mecanismos iônicos e de oxidação-redução, como a incubação com GSH em condições específicas indicariam.

Palavras-chave: Espermatozoide. Função espermática. Antioxidante. Estrutura nucleoprotéica. Fosforilação. Resíduos de cisteínas livres. Fertilização.

1 INTRODUCTION

One of the most important processes that undergo mammalian sperm after ejaculation is capacitation. This process, which leads to the achievement of fully sperm fertilizing ability, is achieved inside the female reproductive tract (CHANG, 1984; FRASER, 1998). This characteristic difficults the study of capacitation in *in situ* conditions. However, with the advent of assisted reproductive technologies through of *in vitro* studies, a large amount of information has been gathered on how the sperm achieved capacitation through a sequence of biochemical modifications that lead to the establishment of fully fertilizing ability. These modifications involve the activation of several signaling pathways through variations in the phosphorylation status of a wide array of proteins that is linked to the increase of intracellular messengers such as cAMP and Ca^{2+}, the reorganization of plasma proteins and membrane lipids and changes in motility patterns (APARICIO et al., 2007; BALDI et al., 2000; SIGNORELLI; DIAZ; MORALES, 2012; VISCONTI, 2009; VISCONTI et al., 1998). Finally, the destabilization of acrosomal membrane of sperm increases the ability to bind to the oocyte zona pellucida (BALDI et al., 2000; FRASER et al., 1998).

Recently, with the findings regarding the use of cryoprotectants agents in the freeze extenders of boar semen, new doors for research lines opened to elucidate the effects of these substances on the sperm metabolism. The cryopreservation process induces strong changes in sperm function. Several of these changes, such as modifications in motion parameters and in the overall tyrosine phosphorylation levels of sperm proteins remind those induced during the capacitation process (BAILEY; BLODEAU; CORMIER, 2000; NARESH; ATREJA, 2015; TALUKDAR; AHMED; TALUKDAR, 2015; WYSOCKI; KONCICKA; STRZEZEK, 2009). The similarity of these effects has lead to several authors to describe this phenomenon as “cryocapacitation”, since these

thawed spermatozoa have fertilizing ability, albeit reduced, without needing of a previous capacitation process. As indicated, the cryocapacitation process impairs the fertilizing ability of semen when applied in a standard artificial insemination (AI) procedure (BAILEY; BLODEAU; CORMIER, 2000; TALUKDAR; AHMED; TALUKDAR, 2015). Thus, any system that allows users of frozen semen to minimize the cryocapacitation-linked alterations would be very useful to optimize the feasibility of frozen boar semen for AI. In this way, the addition of reduced glutathione (GSH) to the freezing medium has shown excellent "in vivo" fertility results when applied to boar semen (ESTRADA et al., 2014). Several of the improving effects that the utilization of GSH has on boar semen freezing are linked to the reversion of some of the cryocapacitation –related alterations, as changes in sperm motility (ESTRADA et al., 2014; YESTE et al., 2013). It is noteworthy that GSH is the most abundant thiol in cells and is considered of vital importance, among other functions, for the maintenance of the intracellular redox balance (JACOB et al., 2003). Taking this into account, results obtained in boar frozen-thawed sperm could suggest that GSH and hence the precise oxidative status of boar sperm could play a role in the modulation of boar sperm capacitation process. However, this hypothesis remains to be elucidated.

Regarding the effect of GSH on boar semen cryopreservation, it has been described that the addition of GSH in semen freezing-thawing media counteracts the freezing-thawing linked alterations in the overall integrity of sperm disulfide bounds among proteins (CHATTERJEE; DE LAMIRANDE; GAGNON, 2001), as well as increases sperm motility, reduces ROS levels and increases the penetration ability of the spermatozoa into oocytes (GADEA et al., 2004, 2005). The GSH also seems to maintain a stable nucleoprotein structure (YESTE et al., 2013), and thereby, depending on their concentration in the

extender, this set of cell properties could interfere the sperm capacitation, wich is a process when occur a range of synchronized events.

Taking into account all of the above described results, the main aim of this study was to evaluate the effects of reduced glutathione on the achievement of boar sperm *in vitro* capacitation and subsequent *in vitro* progesterone-induced acrosome exocytosis. This was done through the evaluation of several *in vitro* capacitation markers and other overall semen quality parameters such as percentages of viability, total motility, true progesterone-induced acrosome exocytosis and DNA fragmentation and levels of sperm membrane lipid disorder, reactive oxygen species (ROS), free-cysteine residues in both head and tail sperm extracts, and phosphorylation of protein tyrosine residues.

2 MATERIALS AND METHODS

2.1 Seminal samples

A total of 62 ejaculates collected from 35 healthy Pietrain boars of proven fertility aged 2 to 3 years, obtained on different days were used in this study. These animals were housed in climate-controlled buildings (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), fed with an adjusted diet (2.3 kg/day) and provided with water *ad libitum*. The sperm-rich fractions were collected manually using the hand-gloved method, diluted at a final sperm concentration of 2×10^7 sperm⁄ml in a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain), and cooled down to 16–17 °C. Diluted semen was then distributed in 90 ml comercial AI doses that were placed in a thermal packaging bags at 16ºC for approximately 45 min, which was the time required to arrive at the our laboratory.

2.2 Experimental design and *in vitro* capacitation and acrosome exocytosis procedures

In all cases, each ejaculate was divided in five samples in accordance with the experimental treatments and it was used to evaluate the sperm parameters (motility, free-cysteine residues, DNA fragmentation, sperm viability, acrosome integrity, sperm membrane lipid disorder, ROS levels, mitochondrial membrane potential and phosphorylation of protein tyrosine residues). Each measured parameter used a number of ejaculates (n) within the total number. There were two separate experimental designs. In the first one, we tested the effects of the addition of GSH from the start of the incubation in a capacitating medium, in order to determine the effects of GSH in the achievement of a feasible IVC. The second experimental design was conducted by addition of GSH after the achievement of the IVC status and sinultaneoulsy to the induction of progesterone-modulated IVAE, in order to determine the

putative effects of GSH in the achievement of IVAE in fully *in vitro* capacitated boar sperm. Thus, in both experimental designs there were 5 separate experimental points: a positive control (C+) in which cells were incubated in a capacitation medium (CM) containing $NAHCO_3$ and bovine serum albumin (BSA), three different GSH treatments in which cells were resuspended in CM that was supplemented either at the 0h or after 4h of incubation time with increasing concentrations of GSH ($C_{10}H_{17}N_3O_6S$; GSH, Sigma-Aldrich®, St Louis, MO, USA), and a negative control (C-), which the sperm were re-diluted in a non-capacitating medium (NCM) without GSH, $NaHCO_3$ and BSA.

For the experimental design conducted to determine the effects of GSH from the 0h time of incubation onwards, 50 mL of each semen sample was utilized through division between all five treatments. For this purpose, the 50-mL aliquot was firstly centrifuged at 600*g* for 10 min at 16ºC and initially resuspended at a final concentration of $20\text{-}30\times10^6$ sperm ⁄mL in NCM. The NCM was composed by 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 $NaHPO_4$, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (osmolarity= 289 mOsm/Kg±3.8 mOsm/Kg). After this resuspension, 40 mL were separated and added with 5 mg ⁄mL BSA and 37.6 mM $NaHCO_3$ in order to modify the NCM to the CM (pH=7.4; osmolarity=304mOsm/Kg±5mOsm/Kg). The remaining 10 mL-aliquot was lent to be incubated in NCM to obtain the C-experimental point. Subsequently, the 40-mL CM suspension cell was further divided in four 10-mL aliquots. One of these 10-mL aliquots was lent withput further addition of any substance, whereas the other three 10-mL CM-aliquots were added with GSH to final concentrations of 1mM, 2mM and 5mM, respectively. All five 10-mL aliquots were incubated for 4 h at 38.5ºC in a 5% CO_2 atmosphere, as described in Ramió et al. (2008). Sperm aliquots were taken

at 0, and 4 hours of incubation to perform the appropriate analysis. After 4h of incubation, cells were subjected to the induction of progesterone-activated *in vitro* acrosome exocytosis as described before (JIMÉNEZ et al., 2003; WU; CHIANG; CHENG, 2006). For this purpose, 10 mg/mL progesterone (code number P0130; Sigma; Saint Louis, USA) was added to boar sperm previously incubated in the capacitation medium for 4 h at 38.5 ºC in a 5% CO_2 atmosphere. After thorough mixing, the sperm was further incubated for an additional 1h at 38.5 ºC in a 5% CO_2 atmosphere. During this subsequent incubation time, aliquots were taken at 1 min, 5 min and 60 min after the addition of progesterone to perform the required analyses.

Samples utilized for the second experimental design conducted to determine the effect of GSH on IVAE of previosly capacitated samples were treated in a similar way to that described above. The main difference was that in the GSH experimental points, GSH was added only after 4h of incubation in the CM and simultaneosly to progesterone. The utilized GSH concentrations were also of 1mM, 2mM and 5mM. Finally, in this second experimental design, aliquots were also taken after 0h and 4h of incubation in CM and after 1min, 5min and 60 min of the simultaneous addition of progesterone and GSH.

2.3 Analysis of parameters indicative of the achievement of both *in vitro* capacitation and *in vitro* acrosome exocytosis

As previously indicated, parameters determined in order to analyse the achievement of both IVC and IVAE were the computer-assisted (CASA) motility analysis, the spectrophotometric determination of the free-cysteine residues in both head and tail sperm extracts, microscopic counting of the percentage of DNA fragmentation, cytometyric analysis of sperm viability, acrosome integrity, membrane lipid disorder, ROS levels, mitochondrial membrane potential (MMP) and the immunological detection of

phosphorylation levels of protein tyrosine residues, with an especial emphasis in the P32 protein as an specific capacitation marker of boar sperm (BRAVO et al., 2005).

2.4 Sperm motility analysis

Sperm motility analysis was performed by utilizing a comercial CASA system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based upon the analysis of 25 consecutive digitalized photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10 x 0.30 PLAN objective lens, Olympus-Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image capturing of one photograph every 40 ms. Five to six separate fields were taken for each replicate, and 5 replicates were run per sample and treatment. The obtained sperm motility descriptors were described following Ramió et al. (2008). Settings taken into account for all of the utilized motility parameters were as following:

Range of particles area: 10–80 μm^2.

Connectivity: a minimum of 11 images for all parameters, but a minimal of 10 images for only the mean amplitude of lateral head displacement (ALH).

Parameter ranges:

Curvilinear velocity (VCL): 1–500 μm⁄s.

Linear velocity (VSL): 1–500 μm⁄s.

Mean velocity (VAP): 1–500 μm⁄s.

Straightness coefficient (STR): 10–98%.

Linear coefficient (LIN): 10–98%.

Wobble coefficient (WOB): 10–98%.

Mean amplitude of lateral head displacement (ALH): 0–100 μm.

Frequency of head displacement (BCF): 0–100 Hz.

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 onto a warmed (37ºC) slide and covered with a 22×22 mm coverslip. Finally, total motility was defined as the percentage of spermatozoa that showed a VAP>10 µm⁄s.

2.5 Evaluation of free-cysteine residues in sperm nucleoproteins

The determination of free cysteine radicals in sperm head and tail proteins as an indirect measure of disrupted disulfide bridges within nucleoproteins was carried out following the protocol adapted to boar spermatozoa and described by Flores et al. (2011). Briefly, samples were centrifuged at 600*g* and 16ºC for 10 min and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150mM NaCl, 1% (v:v) Nonidet, 0.5% (w:v) sodium deoxycholate, 1mM benzamidine, 10 µg/mL leupeptin, 0.5mM phenylmethylsulfonyl fluoride, and 1mM Na_2VO_4. Spermatozoa were subsequently homogenized through sonication (Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany). Obtained homogenates were centrifuged at 850*g* for 20 min at 4ºC. The resultant supernatants were reserved to measure the free-cysteine in sperm tail proteins and the pellets were resuspended in 300 µL of Tris buffer to measure the free-cysteine in sperm head proteins.

The levels of free cysteine radicals in sperm nucleoproteins were determined in the samples obtained by using the 2,2'-dithiodipyridine technique (2,2'-dipyridyl disulfide; Sigma-Aldrich) as described by Brocklehurst, Stuchbury and Malthouse (1979). With this purpose, 10-µL aliquots of resuspended, isolated sperm heads obtained as described previously and other 10-µL aliquots of resultant supernatants were added to 990 mL of an aqueous solution of 0.4mM 2,2'-dithiodipyridine. Aliquots of 10 µL of standard cysteine solutions with concentrations ranging from 0.1mM to 5mM (Sigma-Aldrich)

were also added to 990 µL of 0.4mM 2,2'-dithiodipyridine for evaluation. In all cases, mixtures were incubated at 37ºC for 60 min, and levels of free cysteine radicals were finally determined through spectrophotometric analysis at a wavelength of 340 nm. The results obtained were normalized through a parallel determination of the total protein content of samples by the Bradford method (1976), using a commercial kit (Quick Start Bradford Protein Assay; BioRad, Hercules, CA, USA). Five replicates per sample and treatment were evaluated, and the corresponding mean ± SEM was calculated.

2.6 Evaluation of DNA fragmentation

DNA fragmentation was assessed using a commercial sperm chromatin dispersion test (SCDt) kit specifically designed for boar spermatozoa (Sperm-Halomax®-Sus for fluorescence microscopy; ChromaCell S.L., Madrid, Spain) and following the manufacturer's instructions. This test is based on the different response that intact and fragmented DNA show after a deproteinization treatment, and previous reports have shown that the results obtained with this technique strongly correlated with those obtained with other tests, like the neutral comet assay (ENCISO et al., 2006).

Briefly, the lysing buffer included in the commercial kit was tempered to 22°C and vials containing low-melting agarose were incubated at 100°C for 5 min in a water bath. Vials were then left in another water bath at 37°C for 5 min to equilíbrate the agarose temperature. Twenty-five µL of each sperm sample (at a final concentration of 10^7 spermatozoa/mL) were added to a vial prior to mixing it thoroughly. One drop of 25 µL containing the spermatozoa in agarose was placed onto the treated face of the slides provided with the kit and covered with a glass coverslip to avoid air-bubble formation.

Slides were placed on a cooled plate within a fridge and left at 4°C for 5 min. The coverslip was then removed and 50 µL of lysis solution per slide were

added. An incubation step at 22°C for 5 min was performed, prior to washing for 5 min with miliQ® water. The slides were subsequently dehydrated by three steps of 2 min each with aqueous dilutions of 70% (v:v) ethanol, 90% (v:v) ethanol and 100% (v:v) ethanol. Finally, sperm samples were stained with propidium iodide (PI, 2.5 µg/mL; Molecular Probes®, Eugene, OR, USA) and mounted in DABCO antifading medium (DABCOTM anti-fading medium; Sigma-Aldrich®). Samples were observed under an epifluorescence microscope (Zeiss AxioImager Z1; Karl Zeiss) at 1000× magnification. Three counts of 250 spermatozoa each using three different slides were carried out per sample and treatment, prior to calculating the corresponding mean±SEM. Spermatozoa with fragmented DNA exhibited a large and spotty halo of chromatin dispersion, whereas spermatozoa with non-fragmented DNA exhibited only either an small halo or not halo at all.

2.7 Flow cytometric analysis

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC) (LEE et al., 2008). These analyses were conducted to evaluate some sperm functional parameters, such as sperm viability (membrane integrity), sperm membrane lipid disorder, acrosome integrity, and intracellular peroxide and superoxide levels. In each case, the sperm concentration in each treatment was adjusted to 1×10^6 spermatozoa/mL in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below (i.e. SYBR-14/PI, M540/YO-PRO®-1, PNA-FITC/PI, H2DFCDA/PI, HE/YO-PRO®-1, JC-1 or PI after hypotonic treatment to correct raw data).

Samples were evaluated through a Cell Laboratory QuantaSCTM cytometer (Beckman Coulter, Fullerton, CA, USA; Serial Number AL300087,

Technical specification at https://www.beckmancoulter.com/wsrportal/ajax/downloadDocument/721742AD.pdf?autonomyId=TP_DOC_32032&documentName=721742AD.pdf). This instrument, which had not been altered in the original configuration provided by the manufacturer, was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW. In our case, only the singleline visible light (488 nm) from the argon laser was used to perform the analyses. Cell diameter/volume was directly measured with the Cell Lab QuantaTM SC cytometer employing the Coulter principle for volume assessment, which is based on measuring changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system, thus, has forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10 µm Flow- Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Optical filters were also original and they were FL1, FL2 and FL3. In this system, the optical characteristics for these filters were as follows: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, band pass filter: 525 nm, detection width 505– 545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm); and FL3 (red fluorescence): long pass filter: 670/30 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence (SYBR14, PNA-FITC, YO-PRO®-1 and H2DFCDA), whereas FL3 was used to detect red fluorescence (M-540, HE and PI).

Sheath flow rate was set at 4.17 µL/min in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE vs. SS dot plots) for a minimum of 10000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 µm) and cell

aggregates (particle diameter>12 µm). Therefore, the sperm-specific events, which usually appeared in a typically L-shaped scatter profile, were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described below, compensation was used to minimize spill-over of the fluorescence into the a different channel.

Information on the events was collected in List-mode Data files (LMD), and these generated files were then analysed using Cell Lab Quanta_SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL1 vs. FL3) and to analyse the cytometric histograms. In PNA-FITC/PI, H2DFCDA/PI and HE/YO-PRO®-1 assessments, data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina et al. (2010). Each assessment for each sample and parameter was repeated three times in independent tubes prior to calculating the corresponding mean ± SEM.

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes® (Invitrogen, Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma).

Sperm viability (SYBR-14/PI)

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by Garner and Johnson (1995). Briefly, sperm samples were incubated at 38 °C for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 10 µM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, whereas PI fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: (i) viable green-stained spermatozoa ($SYBR\text{-}14^{+}$/ PI^{-}); (ii) non-viable red-stained spermatozoa ($SYBR\text{-}14^{-}/PI^{+}$); and (iii) non-viable spermatozoa that were stained both Green

and red (SYBR-14$^+$/PI$^+$). Non sperm particles (debris) were found in the SYBR-14$^-$/PI$^-$ quadrant.

Single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI/FL-3 channel (2.45%).

Sperm membrane lipid disorder compatible with capacitation (M540/YO-PRO®-1)

Lipid disorder of boar sperm membrane was evaluated by Merocyanine-540 (M540) and YO-PRO-1 co-staining, following a modified procedure from Rathi et al. (2001). Briefly, spermatozoa were stained with M540 (final concentration: 400 µM) and YO-PRO®1 (final concentration: 40 µM) and incubated for 10 min at 38 °C in the dark. Red fluorescence from M540 was collected through FL-3 and green fluorescence from YO-PRO®1 was collected through FL-1. A total of four sperm populations were observed in flow cytometry dot plots: (i) viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO®-1$^-$); (ii) viable spermatozoa with high membrane lipid disorder (M540$^+$/YOPRO®-1$^-$); (iii) non-viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO®-1$^+$) and (iv) non-viable spermatozoa with high membrane lipid disorder (M540$^+$/YO-PRO®-1$^+$). In this test, data were not compensated.

Acrosome integrity (PNA-FITC/PI)

Acrosome integrity was assessed by costaining the spermatozoa with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocianate (FITC) and PI, according to the procedure described by Nagy et al. (2003). Briefly, spermatozoa were stained with PNA-FITC (final concentration: 2.5 µg/mL) and PI (final concentration: 10 µM) and incubated at

38 °C for 10 min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3. Spermatozoa were identified and placed in one of the four following populations: (i) viable spermatozoa with intact acrosomes (PNA-FITC$^-$/PI$^-$); (ii) viable spermatozoa with exocytosed acrosomes (PNA-FITC$^+$/PI$^-$); (iii) non viable cells with intact acrosomes (PNA-FITC$^-$/PI$^+$); and (iv) non-viable cells with damaged acrosomes (PNA-FITC$^+$/PI$^+$). Following this classification, the percentage of true acrosome exocytosis (TAE) was based in the percentage of viable cells that simultaneously showed acrosome alteration.

Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC spill over into the PI/FL-3 channel (2.45%).

Assessment of intracellular Reactive Oxygen Species levels (H2DCFDA/PI and HE/YOPRO®-1)

Intracellular peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) levels were determined using two different oxidation-sensitive fluorescent probes: 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) and hydroethidine (HE). Following a procedure modified from Guthrie and Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed by costaining the spermatozoa either with PI or with YO-PRO®-1.

In the case of peroxides, spermatozoa were stained with H2DCFDA at a final concentration of 200 µM and PI at a final concentration of 10 µM, and incubated at 25 °C for 60 min in the dark. H2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2′,7′-dichlorofluorescein (DCF) upon oxidation (GUTHRIE; WELCH, 2006). This DCF fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3.

Unstained and single-stained samples were used for setting the EV gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

In the case of superoxides, samples were stained with HE (final concentration: 4 µM) and YO PRO®-1 (final concentration: 40 µM) and incubated at 25 °C for 40 min in the dark (GUTHRIE; WELCH, 2006). Hydroethidine is freely permeable to cells and is oxidized by $O_2^{-\bullet}$ to ethidium (E) and other products. Fluorescence of ethidium (E^+) was detected through FL-3, and that of YO-PRO®-1 was collected through FL-1. Data were not compensated.

Data are expressed as means ± SEM of percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF^+ fluorescence) and of viable spermatozoa with high $O_2^{-\bullet}$ levels (high ethidium fluorescence; E^+).

Mitochondrial membrane potential

The MMP was analyzed by applying the methode described in Gillan, Evans and Maxwell (2005). For this purpose, samples were incubated with the flurochrome 5,5'-6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) to a final concentration of 0.3µM at 38ºC for 10 min in the dark. Afterwards, samples were processed through the flux cytometer. There emision filters FL1 and FL2 were utilized to differentiate two subpopulations. The first subpopulation were sperm with high MMP, which presented JC-1 aggregates with and orange staining. The second subpopulaiton were cells with low MMP, which presented JC-1 monomers with a green staining. Data were not compensated.

2.8 Western blot assay

Ten ejaculates, five belonging to capacitation test and five to acrosome reaction test, were selected for the Wetern blot assay. For this purpose, 1 mL-

aliquot belonging to each experimental point was centrifuged at 1000*g* for 30 seconds and pellets were stored at -80°C until the beginning of the assay. When stated, pellets were then resuspended and sonicated in 300µL of ice-cold lysis buffer (pH 7.4) containing 50mM Tris-HCl, 1mM EDTA, 10mM EGTA, 25mM dithiothreitol, 1,5 % (v:v) Triton® X-100, 1mM phenylmethanesulfonyl fluoride, 10 µg/mL leupeptin, 1mM ortovanadate and 1mM benzamidine. After 30 min in ice, the homogenized suspensions were centrifuged at 4°C at 10,000 rpm for 20 min and total protein amount in supernatants was calculated through the Bradford method (1976) using a commercial kit (Bio-Rad Laboratories; Fremont, CA, USA). Afterwards, samples were added to a loading buffer (1:5; v:v) containing 250mM Tris-HCl (pH 6.8), 50mM dithiothreitol, 10% (w:v) SDS, 0.5% (v:v) bromophenol blue and 50% (v:v) glicerol and stored at -20°C until their subsequent assay.

To perform Western blot analyses, samples were loaded into 0.75 mm gels containing 10% acrylamide (w:v) to perform SDS-PAGE (LAEMMLI, 1970). After running the gels at constant voltage (180V), the activated proteins of gels were transferred to an immun-blot low-fluorescence polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in 7 min using the Trans-Blot® Turbo Transfer System (Bio-Rad) with Trans-Blot® Turbo Midi Transfer Packs. Membranes were subsequently submerged for 60 min in blocking solution consisted in a Tris-buffered saline solution added with 5% (w:v) BSA and 0.1% (v:v) Tween-20. After this time, membranes were then submerged in blocing solution containing the approppriate concentration of the utilized primary antibody. Membranes were incubated with the antibody at 4°C for a minimum of 8h. The utilized primary antibody were a mouse monoclonal clone PY20 anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich; St. Louis, Missouri, USA) and a mouse monoclonal anti-beta tubulin (ref. T5201; Sigma-Aldrich; St. Louis, Missouri, USA). In both cases, the utilized dilution was of

1:1000 (v:v). Beta-tubulin was used as an internal standard to normalize the volume of protein bands, stripping and reprobing membranes when necessary.

After three washes, the immunoreaction was tested for 60 min using a horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse secondary antibody (Dako; Glostrup, Denmark) at a dilution of 1:5000 (v:v) in blocking solution.

After 6 washes, the reaction was developed for 2 min with a chemiluminescent HRP substrate (ImmunoCruz Western Blotting Luminol Reagent; Santa Cruz Biotechnology®, Dallas, Texas, USA) according to the manufacter's instructions and the membranes were revealed. Prestained protein standards with a molecular mass range of approximately 250–10 kDa were used. Finally, the image analysis system ImageJ 1.49 (National Institute of Health, USA) was used to quantify the changes in intensity of various bands.

2.9 Detection of phosphotyrosine residues location in boar sperm through inmunocytochemistry

The treatments tested in this analysis were both C+ and C- and just a concentration of reduced glutathione, namely, 2mM GSH 2 mM. Eight ejaculates, four belonging to capacitation test and four to acrosome reaction test, were selected for the inmunocytochemistry assay.

Immunocytochemistry of phosphotyrosine in boar sperm was carried out as following: Aliquots of 200 µL were centrifuged at 1000*g* for 30 s and pellets were fixed through of the resuspension in 400 µL of 4% (w:v) paraformaldehyde in PBS. Sperm samples were spread onto poly-lysined (Poly-L-lysine solution 1 % w⁄v in H2O; Sigma, St 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 with 0.3% (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 5% (w⁄ v) BSA for 60 min at 20ºC. Incubation with the specific mouse monoclonal clone PY20 anti-phosphotyrosine antibody (ref. P4110; Sigma-Aldrich; St. Louis, Missouri, USA) as in Medrano et al. (2006) at a final dilution of 1:200 (v:v) in PBS was carried out a minimum of 8h at 4ºC. After incubation, the sperm was washed thoroughly three time with PBS at 4ºC and incubated with the corresponding Alexa Fluor® 488 donkey anti-mouse secondary antibody to a final dilution of 1:500 (v:v). Slides were gently washed three time with PBS at 4ºC and were then incubated with 10µL of a commercial solution of 4.6-diamidino-2-phenylindole hydrochloride (DAPI, 125 ng ⁄ ml; Vysis Inc., Downers Grove, IL, USA) as both a nuclear stain and an anti-fade mounting solution. After being covered, the slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed with colourless nail polish, and slides were stored at 4ºC in the dark until their microscopic observation. Fluorescent images were obtained with a Leica TCS 4D confocal scanning microscope (Leica Lasertechnik, Heidelberg, Germany) adapted to an inverted LeitzDMIRBE microscope and a 63 × (NA 1.4 oil) Leitz Plan-Apo lens (Leitz, Wetzlar, Germany). The light source was an argon ⁄ krypton laser. Successive confocal slices of images (image thickness: 0.5 µm) were integrated to perform three-dimensional spermatozoa images, which were further stored as TIFF-format images.

2.10 Statistical evaluation of results

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 2.0; SPSS Inc.; Chicago, Illinois, USA), and data from all assessments are presented as the mean ± standard error of the mean (SEM). Data were first tested for normality and variance homogeneity through Shapiro-Wilk and Levene tests, respectively. When required, data (x) were

transformed using the arcsine square root (arcsin $\sqrt{x}$) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (i.e. composition of capacitation media) and the intrasubject factor was the incubation time (i.e. 0h, 4h, 4h 1 min, 4h 5 min, 4h 60 min). In all cases, each sperm functional parameter was the dependent variable, and multiple post-hoc comparisons were calculated using Sidak's test.

When no transformation remedied the normality, non-parametric procedures were used with raw data. Friedman's test and the Wilcoxon matched-pairs test were performed as non-parametric alternatives to repeated measures ANOVA. In all statistical analyses, the minimal level of significance was set at $P<0.05$.

3 RESULTS

3.1 In vitro capacitation and subsequent progesterone-induced acrosome exocytosis

Incubation of sperm cells in both CM and NCM showed a progressive drop on viability, although this dynamics was more accentuated in the case of cells incubated in the NCM (Figs. 1A,B). The addition of GSH in the CM at 0h of incubation had no remarkable effect on viability (Fig. 1A). The addition of GSH with progesterone together after 4h of incubation in CM did not affect the percentage of viability under any circumstances (Fig. 1B).

Values of TAE in sperm incubated in CM were maintained very low after 4h of incubation. Nevertheless, the addition of progesterone induced a progressive and continuous increase in TAE levels until 60 min after the addition of the hormone (64.7%±2.8, see Fig. 1C). This phenomenon was not observed in cells incubated in the NCM (Figs. 1C,D). The addition of GSH both at 0h of incubation and after 4h of incubation and with progesterone together did not significantly modify the dynamics observed in the control, CM spermatozoa (Figs. 1C,D).

3.2 Effects of GSH on the sperm motility during the capacitation and acrosome reaction

Incubation of boar sperm in the control CM did not induce any significant modification in the percentage of total motility during the first 4h of incubation (Fig. 1E). The percentage of total motility was subsequently maintained after the addition of progesterone, although there was a significant decrease 60 min after the addition of the hormone (Fig. 1E) On the contrary, sperm incubated in the control NCM showed much lower initial total motility values, and these lower values were maintained, and even worsened, during all of the incubation time (Figs. 1E,F). The addition of GSH at time 0 caused an

immediate, concentration-dependent drop of total motility. The addition of progesterone after 4h of incubation in CM temporarily stopped the GSH-linked decrease of total motility, although this decrease was again observed after 60 min of the progesterone addition Fig. 1E). Likewise, the addition of GSH with progesterone together was linked with a remarkable decrease of total motility when compared to control cells after 60 min of incubation (Fig. 1F).

The results of motility parameters for all treatments are presented in Tables 1-3. The incubation in the control CM induced a progressive, time-dependent increase in VSL, LIN, STR and WOB values. This effect was concomitant with a decrease of mean VCL and mean ALH. These changes are similar to those previously published in the same conditions (COVARRUBIAS et al., 2015; YESTE et al., 2015), and were assumed to be concomitant with the achievement of a feasible IVC status. The addition of progesterone after 4h of incubation in CM induced a rapid increase of VCL, VAP and mean ALH that was concomitant with a decrease of LIN, STR and WOB values. These changes were transitory, with a clear reversion after 5 min of the addition of progesterone. The observed progesterone-induced changes of motility parameters were practically abolished after 60 min of progesterone addition (Tables 1-3). The addition of GSH at the time 0h of incubation had, in an overall view, slight effects of boar sperm motility parameters dynamics (Tables 1-3). Meanwhile, the addition of GSH after 4h of incubation in CM with progesterone together increased VCL, VSL and BCF values whereas counteracted the progesterone-induced effects observed on LIN, STR and WOB, although only at the higher concentrations of GSH of 2mM and specially 5mM (Tables 1-3).

3.3 Effects of GSH on the free-cysteine residues in both head sperm and tail sperm extracts and in the DNA fragmentation percentage during the achievement of both *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis

The incubation of sperm in the CM caused a progressive increase in the levels of free cysteine in the sperm head (from 3.0 nmol/µg protein±0.3 nmol/µg protein at 0h of incubation to 17.4 nmol/µg protein±1.7 nmol/µg protein after 4h of incubation), appearing a gradual decline after the induction of the acrosome exocytosis (Fig. 2A). The addition of GSH at the start of the incubation inhibited this increase, whereas the addition of GSH and progesterone together after 4h of incubation in CM accelerated the observed decrease in sperm incubated in the CM alone (Figs. 2A,B).

Likewise to that observed in sperm head extracts, levels of free cysteine residues in tail sperm extracts were progressively increased when cells were incubated in CM for 4h (from 3.3 nmol/µg protein±0.4 nmol/µg protein at 0h of incubation to 8.4 nmol/µg protein±1.0 nmol/µg protein after 4h of incubation, see Fig. 2C). Afterwards, free cysteine levels were roughly maintained after the progesterone addition, reaching values of 9.2 nmol/µg protein±1.1 nmol/µg protein after 60 min of progesterone incubation (Fig. 2C) The addition of GSH at the CM at 0h of incubation prevented the capacitatiom-related increase at all of tested concentrations (Fig. 2C). Meanwhile, the addition of GSH after 4h of incubation together with progesterone did not have any clear effect of sperm taill free cysteine residues levels (Fig. 2D).

Finally, the percentage of boar sperm that showed signs of DNA fragmentation was very low in all of the tested experimental points. Only a very slight increase was observed in cells incubated in NCM at the end of the experimental procedure (Figs. 2E,F). The addition of GSH both at 0h of

incubation and together with progesterone after 4h of incubation in the CM did not cause any significant effect on this parameter (Figs. 2E,F).

3.4 Effects of GSH on the sperm membrane lipid disorder compatible with capacitation during the capacitation and acrosome reaction

Incubation of boar sperm in the control CM was concomitant with a noticeable and significant ($P<0.05$) increase in the percentage of viable cells with capacitation-like changes in membrane fluidity (from 8.0%±1.3% at 0h of incubation to 52.1±4.8% after 4h of incubation, see Fig. 3A). The subsequent addition of progesterone cursed with a progressive decrease of the percentage, reaching values of 37.8%±3.6% after 60 min of further incubation (Fig. 3A). The addition of GSH at 0h of incubation significantly ($P<0.05$) diminished the observed increase in the percentage of viable cells with capacitation-like membrane fluidity changes, reaching the maximal counteracting effects at GSH concentrations of 2 mM and 5 mM (Fig. 3A). On the contrary, the addition of GSH after 4h of incubation together with progesterone did not significantly modify the dynamics of the percentage of viable spermatozoa with capacitation-like membrane fluidity changes after the IAVE induction (Fig. 3B).

3.5 Effects of GSH on the mitochondrial membrane potential during the capacitation and acrosome reaction

Incubation of sperm in CM for 4h induced a steady increase in the percentage of cells with high MMP, which went from 23.1%±3.7% at 0h of incubation to 54.6%±4.3% after 4h of incubation (Fig. 3C). The addition of progesterone induced a further increase in this percentage, reaching peak values of 70.9%±5.3% after 1 min of the IVAE induction. Afterwards, the percentage of high MMP sperm decreased, reaching values of 57.7%±4.2% after 60 min of the progesterone addition (Fig. 3C). The addition of GSH at all of the tested concentrations at 0h of incubation in the CM counteracted the increase in the

percentage of high-MMP cells observed after 4h of incubation in CM as well as the further, progesterone-linked increase of this parameter (Fig. 3C). Likewise, the addition of GSH after 4h of incubation in CM together with progesterone prevented the progesterone-induced peak of the high MMP cells percentage observed 1 min after the progesterone addition, but only at GSH concentrations of 2mM and 5mM (Fig. 3D). When the mean signal intensity of high MMP cells was determined, an increase was observed in control cells after 4h of incubation in CM (Fig. 3E,F). The MMP levels reached after 4h of incubation were maintained after the addition of progesterone (Fig. 3E,F). The addition of GSH at 0h of incubation prevented this increase at all of the tested GSH concentrations, although the maximal effects were observed at GSH concentrations of 5mM (Fig. 3E). On the contrary, the addition of GSH after 4h of incubation in the CM together with progesterone did not significantly modify the dynamics observed in control cells at any of the tested experimental points (Fig. 3F).

3.6 Effects of GSH on the intracellular superoxide levels during the capacitation and acrosome reaction

The percentage of viable sperm with high intracellular superoxide levels significantly ($P<0.05$) increased after 4h of incubation in CM (from 11.6%±1.7% at 0h of incubation to 17.5%±1.9% after 4h of incubation, see Fig. 4A). The subsequent addition of progesterone induced a temporary decrease in this percentage which, again raised after 5 min of the addition of the hormona, reaching higher values after 60 min of incubation (Figs. 4A,B). Concomitantly, the intensity of the mark seen in viable sperm with high superoxide levels also increased during the 4h incubation in CM (Figs. 4C,D). However, there was no decrease of this intensity after the progesterone-induced IVAE (Figs. 4C,D). The addition of GSH at 0h of incubation counteracted the observed increase in the

percentage of sperm with high superoxide levels at all of the tested concentrations, as well as partially counteracted the observed increase in the mean intensity signal (Fis. 4A,C). This counteracting effect of GSH was progressively diminishing after the addition of progesterone, observing no effects after 60 min of incubation with the hormone (Figs. 4A,C). The addition of GSH after 4h of incubation simultaneously with progesterone did not induce any significant effect either in the percentage of viable cells with high superoxide levels or the mean intensity signal of these cells (Figs. 4B,D).

3.7 Effects of GSH on the intracellular peroxide levels during the capacitation and acrosome reaction

The percentage of viable sperm with high intracellular peroxide levels significantly ($P<0.05$) increased after 4h of incubation in CM from 2.4%±0.7% at 0h of incubation to 18.6%±2.0% after 4h of incubation (Fig. 5A). This percentage was roughly maintained after the addition of progesterone for all of the incubation period (Figs. 5A,B). This dynamics was accompanied with a time-dependent increase of the mean intensity of signal in sperm with high intracellular peroxide levels (Figs. 5C,D). The addition of GSH at 0h of incubation diminished the observed increases in both the percentage of cells with high peroxide levels and the mean intensity of signal in cells with high peroxide concentration (Figs. 5A,C). Furthermore, the addition of GSH after 4h of incubation in CM together with progesterone induced a rapid and significant ($P<0.05$) decrease of both the percentage of viable sperm with high peroxide leveles and the mean intensity of signal in high-peroxide cells, with more marked effect at GSH concentrations of 5mM (Figs. 5B,D).

3.8 Effects of GSH on boar sperm tyrosine phosphorylation levels of P32 protein after the achievement of IVC and subsequent progesterone induced IVAE during the achievement of *in vitro* capacitation and subsequent *in vitro* acrosome exocytosis

The images of the P32 expression are presented in Figs. 6 and 7. The incubation of boar sperm in CM for 4h induced an increase the in phosphotyrosine intensity signal of the P32 protein. This increase was maintained, and even increased, after the addition of progesterone , although the phosphotyrosein P32 signal was much reduced after 60 min of the hormone addition. The addition of GSH at the time 0h of incubation in the CM had no noticeable effects on the P32 phosphotyrosine signal during the first 4h of incubation, although there was a decrease of this signal after the progesterone treatment, which was more intense at GSH concentrations of 5mM (Fig. 6A). The addition of GSH after 4h of incubation in CM together with progesterone did not show any remarkable effect on the P32 phosphotyrosine signal. (Fig. 7).

3.9 Effects of GSH on boar sperm tyrosine phosphorylation location patterns during the achievement of *in vitro* capacitation and subsequent *in vitro* acrosome reaction

Boar sperm incubated 0h in the CM showed an overall slight phosphotyrosine signal form all of the cell, while the maximal intensity of this signal was located at the post-acrosomal area of the head (Figs. 8 and 9). The incubation during 4h in the CM increased the intensity of the post-acrosomal signal. The addition of progesterone after 4h of incubation in CM induced a very noticeable increase of phosphotyrosine signal intensity located at the post-acrosomal area, which was accompanied with a noticeable signal in the entire sperm tail (Figs. 8 and 9). This increase of both post-acrosomal and tail intensity phosphotyrosine signal was maintained after 5 min of progesterone addition,

although both signals were clearly diminished after 60 min of incubation (Figs. 8 and 9). The addition of 2mM GSH at time 0h of incubation in CM counteracted the observed increase in post-acrosomal signal, as well as the appearance of a clear tail signal after the progesterone induction (Fig. 8). On the contrary, the addition of 2mM GSH after 4h of incubation in CM together with progesterone did not modify the IVAE-related phsohpotyrosine signal changes (Fig. 9). The only remarkable data was the maintenance of the tail signal after 60 min of the addition of both 2mM GSH and progesterone together after 4h of previous incubation in the CM (Fig. 9).

4 DISCUSSION

The results showed here indicate that GSH-sensitive mechanisms are important, although not totally instrumental, in the achievement of a feasible boar sperm IVC and subsequent, progesterone-induced IVAE. This is concluded by the fact that although GSH was unable to inhibit the achievement of the progesterone-induced IVAE, several important markers of the achievement of boar sperm IVC like motion parameters and changes of sperm membrane fluidity and the overal tyrosine phosphorylation status are altered by GSH. These results suggest the existence of parallel and separate pathways controlling the achievement of both IVC and IVAE in a manner in which the alteration of some of these separate pathways will not yield a complete paralysis of final progesterone-induced IVAE.

The existence of separate and parallel pathways controlling boar sperm IVC/IVAE has been in fact previously suggested. Thus, a previous work suggested the existence of separate pathways with different sensitivity to extracellular calcium levels that showed specific effects on specific capacitation markers like motility and sperm membrane fluidity. However, at the contrary to that observed by GSH, extracellular calcium was instrumental in the achievement of progesterone-induced IVAE (YESTE et al., 2015). This is not surprising, since the strong relationship between a proper calcium influxs and the achievement of IVAE has been widely demonstrated (AITKEN; NIXON, 2013; COSTELLO et al., 2009; PUBLICOVER; HARPER; BARRATT, 2007). In fact, our rsults seem to suggest that the achievement of progesterone-induced IVAE was not entirely dependent of a proper functioning of Ca^{2+}-independent pathways linked to the appearance of important capacitation characteristics like the hyperactivated movement or the increase in membrane fluidity.

Taking the above comments into account, the main question would be centered on what Ca^{2+}-independent pathway/s are affected by GSH during the

achievement of boar sperm IVC. We don't exactly know what would be these pathways. However, the obtained results can yield some suggestions on this point. Thus, it is noteworthy that the achievement of IVC was accompannied with an increase in the free cysteine radicals levels in both sperm head and tail. This increase was concomitant with parallel, if slight, increases of intracellular ROS levels and overall mitochondrial activity as indicated the JC-1 analysis. All of these phenomena were counteracted by GSH. This is not surprising, since GSH has a known anti-ROS action that was concomitant with a role of protective agent of disulfide bonds (YESTE et al., 2012). Furthermore, the anti-ROS action of GSH would induce a concomitant lowering effect on mitochondrial activity, since mitochondria are the main location in which ROS are produced in sperm (MURPHY, 2009). Meanwhile, the increase of both free cysteine radical levels and the mitochondrial activity leading to a subsequent increase of intracellular ROS concentration can be a role in the achievement of IVC by themselves.

Centering on free cysteine radicals, this parameter would be a direct indicator of the integrity of disulfide bonds among proteins and also in nucleoprotein structures. In this manner, the maintenance of a tight nucleoprotein structure in mammalian sperm could be partially controlled by the number of disulfide bonds established between nucleoproteins and DNA (FLORES et al., 2011). This relationship is especially evident in frozen-thawed boar sperm, in which the freezing-thawing related alterations of the nucleoprotein structure are concomitant with a significant increase in the number of broken head sperm disulfide bonds in a way that is counteracted by the addition of GSH (FLORES et al., 2011; YESTE et al., 2012).

Concomitantly, disulfide bonds are instrumental in the maintenance of the sperm tail structure by involvement of bonds among the separate proteins of flagellum, fibrous sheath and longitudinal columns (SUTOVSKY et al., 1997).

In this way, the exact union strenght among the separate components that regulate motility at the sperm tail will have a strong influence in the exact type of motion pattern that sperm achieve at a determined moment. Thus, the increase in the number of broken tail disulfide bonds would results in specific changes in the motion pattern that sperm present, being thus one of the mechanisms that could be related with the appearance of the boar sperm capacitation-specific motion pattern. Taking this hypothesis into account, it would be possible that the protecting effect of GSH on tail disulfide bonds could be one of the mechanisms whereby GSH alters sperm motility in the CM medium, avoiding thus the establishment of capacitation-specific motion patterns.

However, disulfide bonds are not the only point on GSH is acting. As previously indicated above, IVC was realted with a slight increase in ROS levels that can be a consequence of an increased activity of boar sperm mitochondria during the achievement of IVC. Furthermore, this putative increase in mitochondrial activity could be also linked with the assumption of the capacitation-specific motility pattern that can be observed in boar sperm after 4h of incubation in the CM. In fact, a close relationship between mitochondrial activity and boar sperm motility has been already published, although this relationship is not directly related with the overall ATP intracellular levels but with the specific chemiosmosis activity (RAMIÓ-LLUCH et al., 2013). In any case, ROS increase is very modest, which would imply that no deletereous effects would be caused by it. On the contrary, these changes in ROS levels could play a role in launching several of the molecular phenomena linked with the achievement of capacitation. In this sense, several reports indicate the ability of ROS to initiate capacitation or to modulate its progression (DE LAMIRANDE et al., 1997; FORD, 2004; O'FLAHERTY; DE LAMIRANDE; GAGNON, 2006). At this respect, it is noteworthy that ROS can modulate the activity of a myriad of separate protein kinases and phosphatases, like protein

kinase C, microtubule-associated proteins kinases and several protein phosphatases (COSENTINO-GOMES; ROCCO-MACHADO; MEYER-FERNANDES, 2012; SON et al., 2013). Remarkably, our results show that the lowering of ROS leveles induced by GSH was concomitant with impairment in the dynamics of the overall sperm protein tyrosine phosphorylation observed during the achievement of IVC. This could be important taking into account that the above mentiones protein kinases and phosphatases are involved in the regulation of sperm capacitation through acting on several of the molecular changes affected by the GSH addition such as motility and changes in membrane fluidity (ICKOWICZ; FINKELSTEIN; BREITBART, 2012; NAOR; BREITBAR, 1997; SIGNORELLI et al., 2013). Nevertheless, the putative regulatory action of ROS on boar sperm IVC would be modest, taking into account the low intracellular ROS levels that boar sperm cells have, specially when compared with other species such as bovine or human (BILODEAU et al., 2000; MAZZILI et al., 1995). In any case, this putative ROS role would not control launching of progesterone-linked IVAE, since the GSH-induced ROS lowering will not affect this process. In any case, more work is needed in order to elucidate the putative role of ROS, and also mitochondria activity, in the launching of boar sperm capacitation.

The addition of GSH had little effect on boar sperm that previously reached the capacitation status and were then led to the progesterone-induced IVAE launching. This is obvious in the majority of determined parameters, with exception of motility, and much more sligthly, head sperm free cysteine residues, MMP and intracellular peroxide levels. In any case, the overall effects of GSH are less dramatic when added after the achievement of IVC. These results could be the consequence of an overall less sensitive function status of boar capacitated sperm in front of GSH. In fact, after 4h of incubation in CM boar sperm have underwent the majority of changes that have been shown to be

sensitive to the GSH action, such as modifications of ROS levels, membrane fluidity and even strenght of nucleoprotein structure. These changes showed to be not reversible, since the addition of GSH did not revert them. Under a mechanistical point of view, results suggest that the action mechanisms by which GSH acts on disulfide bonds and even ROS production/mitochondrial activity are inactive or in a no-sensitive status in front GSH during and after the achievement of IVC and subsequent IVAE. This function change could be related with the already described function changes at IVC/IVAE. Thus, a sudden peak of O_2 consumption and ATP synthesis has been described immediately after the launching of progesterone-induced IVAE. This peak seems to be related with the assumption of a fully coupled status of boar sperm mitochondria, which seem to be uncoupled or with low activity before and after IVAE (RAMIÓ-LLUCH et al., 2013). On the other hand, GSH inhibits total motility both when added at 0h of incubation in CM and after the IVAE launching. This results suggest that the mechanism of action of GSH on boar sperm motility were not entirely dependent on variations of both tail sperm free cysteine radicals and intracellular ROS levels. At this moment, we don't know what would be this/these mechanism/s, although one hypothesis would be a mechanism related with the control of potential, and hence redox-sensitive enzymes regulating tail contractibility. In this way, proteins like the Na^+/K^+-dependent ATPase is a well known regulator of sperm motility (KOÇAK-TOKER; AKTAN; AYKAÇ-TOKER, 2002). One of the mechanisms by which the activity of this ATPase is controlled is through changes in redox status (LIU et al., 2012). This could be key in our case, since the anti-oxidative action of GSH would act on the overall boar sperm redox status, modulating thus activities of proteins like the Na^+/K^+-dependent ATPase. This action, in turn, would modify the overall boar sperm motility. Thus hypothesis would also explain the fact that the addition of GSH to frozen-thawed boar sperm increases

motility of thawed sperm (YESTE et al., 2012), in a very different manner to that observed in IVC/IVAE conditions. This striking difference, in fact would be a consequence of the existence of very separate overall redox status in fresh samples, frozen-thawed cells and sperm subjected to incubation in the CM medium. However, more work is needed in order to elucidate this point.

In summary, boar sperm IVC is related with a significant increase of both overall broken disulfide bonds and intracellular ROS levels. These phenomena could play a role in the achievement of the capacitation status, although they are not instrumental in the achievement of subsequent progesterone-induced IVAE. Finally, sperm motility seems to be partially controlled by ionic and redox mechanisms, as the incubation with GSH in separate conditions would indicate.

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Table 1- Effects of reduced glutation on mean velocity parameters of boar spermatozoa subjected to *in vitro* capacitation (IVC) and subsequent progesterone-induced acrosome exocytosis (IVAE). (Continue)

		0h	4h	1 min post-P4	5 min post-P4	60 min post-P4
VCL (µm/sec)	Negative Control	52.4±1.1^{a*}	61.8±1.7^{b*}	101.6±3.5^{c*}	94.0±2.9^{c*}	112.2±4.4^{d*}
	Positive Control	62.6±1.8^{a}	54.7±2.9^{b}	70.5±2.8^{c}	67.5±3.1^{ac}	70.8±5.3^{c}
	1mM GSH IVC	61.7±1.9^{a}	60.1±5.0^{ab}	60.2±4.3^{ab}	56.3±2,8^{b*}	78.0±6.2^{c}
	2mM GSH IVC	63.4±2.1^{a}	37.1±1,5^{b*}	58.0±3.6^{a*}	60.6±4.2^{a}	64.5±4.0^{a}
	5mM GSH IVC	58.2±1.7^{a}	41.7±3.0^{b*}	58.0±5.2^{a*}	47.2±5.6^{b*}	39.0±3.4^{b*}
	1mM GSH IVAE	62.6±1.8^{a}	54.7±2.9^{b}	75.1±4.7b*	68.0±4.8^{b}	91.8±6.9^{c*}
	2mM GSH IVAE	62.6±1.8^{a}	54.7±2.9^{b}	83.8±5.6^{a}	77.4±5.2^{a}	84.4±6.0^{a}
	5mM GSH IVAE	62.6±1.8^{a}	54.7±2.9^{b}	78.2±5.2^{a}	70.7±5.6b*	86.9±6.4b*
VSL (µm/sec)	Negative Control	13.9±0.7^{a*}	50.4±5.3^{b*}	50.7±6.8^{b*}	57.6±7.1^{b*}	81.7±7.8^{c*}
	Positive Control	20.8±0.9^{a}	25.2±1.5^{b}	26.5±2.6^{b}	27.5±3.4^{b}	32.3±2.0^{b}
	1mM GSH IVC	21.3±1.1^{a}	26.8±3.3^{b}	28.8±4.3^{b}	31.1±4.1^{b}	26.5±4.0^{b}
	2mM GSH IVC	22.0±1.4^{a}	17.4±1.7^{a*}	27.6±4.1^{b}	28.8±1.8^{b}	30.7±2.8^{b}
	5mM GSH IVC	21.4±1.6^{a}	16.5±2.3^{b*}	31.1±6.3^{b}	20.2±3.9^{a*}	14.9±1.7^{a*}
	1mM GSH IVAE	20.8±0.9^{a}	25.2±1.5^{b}	30.7±6.6^{b}	39.8±6.8b^{c*}	42.5±7.1^{c*}
	2mM GSH IVAE	20.8±0.9^{a}	25.2±1.5^{b}	23.4±1.4^{b}	31.0±5.4^{bc}	34.1±5.6^{c}
	5mM GSH IVAE	20.8±0.9^{a}	25.2±1.5^{b}	31.7±5.5^{b}	39.1±6.0^{b}	55.4±7.9^{c*}

Table 1 - Effects of reduced glutation on mean velocity parameters of boar spermatozoa subjected to *in vitro* capacitation (IVC) and subsequent progesterone-induced acrosome exocytosis (IVAE). (Conclusion)

		0h	4h	1 min post-P4	5 min post-P4	60 min post-P4
VAP (μm/sec)	Negative Control	$23.8\pm0.9^{a*}$	$47.8\pm4.9^{b*}$	$47.4\pm5.0^{b*}$	$55.3\pm5.9^{b*}$	$78.6\pm7.0^{c*}$
	Positive Control	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	38.8 ± 2.2^{b}	39.1 ± 2.3^{b}	44.3 ± 2.7^{b}
	1mM GSH IVC	32.1 ± 1.4^{a}	33.5 ± 3.0^{a}	45.3 ± 5.0^{b}	37.1 ± 2.5^{ab}	40.6 ± 5.0^{ab}
	2mM GSH IVC	31.9 ± 1.1^{a}	$22.2\pm1.4^{b*}$	37.5 ± 4.2^{a}	36.9 ± 4.6^{a}	42.7 ± 4.1^{b}
	5mM GSH IVC	32.6 ± 1.6^{a}	$23.3\pm1.8^{b*}$	36.9 ± 6.5^{a}	$31.4\pm3.1^{a*}$	$19.2\pm2.1^{b*}$
	1mM GSH IVAE	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	30.1 ± 1.0^{a}	36.4 ± 2.1^{b}	46.2 ± 3.2^{c}
	2mM GSH IVAE	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	37.9 ± 2.0^{b}	44.9 ± 2.9^{b}	44.5 ± 3.1^{b}
	5mM GSH IVAE	31.4 ± 1.0^{a}	32.1 ± 1.1^{a}	$45.4\pm2.8^{b*}$	$50.7\pm3.3b^{c*}$	$59.8\pm3.9^{c*}$

Motility parameters and the achievement of both IVC and IVAE have been already described in the appropriate Material and Methods section. Negative Control: cells subjected to incubation in the non-capacitating medium (NCM). Positive Control: sperm subjected to incubation in a standard capacitating medium (CM). 1mM GSH IVC, 2mM GSH IVC, 5 mM GSH IVC. Cells subjected to incubation in the CM in the presence of the indicated concentrations of reduced glutathione from the time 0h of incubation. 1mM GSH IVAE, 2mM GSH IVAE, 5mM GSH IVAE: boar sperm subjected to incubation in standard CM for 4h and then added with progesterone (P4) and reduced glutathione at the indicated concentrations together. Results are expressed as means±S.E.M. for 6 separate experiments. Different letters in a row indicate significant ($P<0.05$) differences among separate incubation times of the same treatment line. Asterisks indicate significant ($P<0.05$) differences when compared with the results of the Positive Control line at the same incubation time. Statistical analyses were carried out as described in the Material and Methods section.

Table 2 - Effects of reduced glutation on mean velocity coefficients of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (Continue)

		0h	4h	1 min post-P4	5 min post-P4	60 min post-P4
LIN (%)	Negative Control	24.0±1.1^{a*}	74.1±6.3^{b*}	76.6±6.9^{b*}	75.7±6.5^{b*}	81.6±7.2^{b*}
	Positive Control	34.2±1.4^{a}	50.1±2.9^{b}	33.9±2.5^{a}	41.8±2.1^{c}	50.5±3.0^{b}
	1mM GSH IVC	35.8±2.0^{a}	37.8±4.8^{a*}	58.9±5.5^{b*}	59.3±5.2^{b*}	37.1±4.0^{a*}
	2mM GSH IVC	34.0±1.6^{a}	38.6±2.0^{a*}	45.6±4.9^{ab*}	45.8±4.6^{ab}	49.4±2.2^{b}
	5mM GSH IVC	32.7±1.7^{a}	40.1±4.2^{b*}	63.3±6.2^{c*}	37.2±5.6^{b}	43.5±1.4^{b*}
	1mM GSH IVAE	34.2±1.4^{a}	50.1±2.9^{b}	40.5±4.4^{b}	39.1±4.6^{b}	58.2±6.0^{c}
	2mM GSH IVAE	34.2±1.4^{a}	50.1±2.9^{b}	39.1±4.6^{b}	42.0±4.9^{b}	60.6±7.8^{c}
	5mM GSH IVAE	34.2±1.4^{a}	50.1±2.9^{b}	38.3±5.2^{b}	40.4±4.9^{b}	70.8±7.3^{c*}
STR (%)	Negative Control	59.6±2.2^{a*}	83.0±5.4^{b*}	82.8±5.5^{b*}	84.4±5.9^{b*}	83.9±5.1^{b*}
	Positive Control	64.9±1.2^{a}	74.2±3.5^{b}	59.9±2.2^{c}	63.3±3.9^{a}	71.6±3.7^{b}
	1mM GSH	65.4±1.5^{a}	45.5±5.6^{b*}	79.1±4.0^{c*}	79.8±4.1^{c*}	56.9±5.5^{b*}
	2mM GSH	64.4±1.9^{a}	61.4±2.3^{a*}	64.4±4.8^{a}	76.0±2.3^{b*}	68.6±4.6^{a}
	5mM GSH	63.6±1.9^{a}	58.7±5.5^{a*}	85.2±3.0^{b*}	65.6±6.2^{a}	75.9±2.5^{a}
	1mM GSH IVAE	64.9±1.2^{a}	74.2±3.5^{b}	58.1±2.7^{c}	64.1±4.2^{c}	71.1±4.2^{c}
	2mM GSH IVAE	64.9±1.2^{a}	74.2±3.5^{b}	76.5±4.9^{b*}	79.1±5.4^{b*}	74.1±4.8^{b}
	5mM GSH IVAE	64.9±1.2^{a}	74.2±3.5^{b}	87.0±6.1^{b*}	87.6±6.7^{b*}	88.3±7.1^{b*}

Table 2 - Effects of reduced glutation on mean velocity coefficients of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. (Conslusion)

		0h	4h	1 min post-P4	5 min post-P4	60 min post-P4
WOB (%)	Negative Control	46.1 ± 1.1^{a}	$87\text{-}0\pm5.2^{b*}$	$79.3\pm5.5^{b*}$	$78.5\pm5.0^{b*}$	$88.3\pm7.1^{b*}$
	Positive Control	50.8 ± 1.3^{a}	62.1 ± 2.5^{b}	52.6 ± 2.2^{a}	58.9 ± 1.0^{b}	65.6 ± 4.3^{b}
	1mM GSH	52.1 ± 1.7^{a}	65.0 ± 5.8^{b}	$70.6\pm4.0^{b*}$	$69.8\pm4.8^{b*}$	$53.8\pm5.0^{a*}$
	2mM GSH	51.7 ± 1.5^{a}	58.6 ± 1.3^{ab}	$63.4\pm2.8^{b*}$	57.8 ± 4.6^{b}	$53.9\pm4.6^{ab*}$
	5mM GSH	$49,3\pm1.8^{a}$	56.3 ± 4.6^{a}	$74.7\pm6.1^{b*}$	53.9 ± 3.1^{a}	$54.7\pm1.8^{a*}$
	1mM GSH IVAE	50.8 ± 1.3^{a}	62.1 ± 2.5^{b}	51.3 ± 3.1^{a}	58.7 ± 2.3^{b}	65.3 ± 5.1^{b}
	2mM GSH IVAE	50.8 ± 1.3^{a}	62.1 ± 2.5^{b}	57.5 ± 4.9^{b}	56.4 ± 3.1^{b}	63.7 ± 4.8^{b}
	5mM GSH IVAE	50.8 ± 1.3^{a}	62.1 ± 2.5^{b}	$71.9\pm5.3^{c*}$	$74.7\pm5.5^{c*}$	$82.5\pm6.2^{c*}$

Motility coefficients and the achievement of both IVC and IVAe have been already described in the appropriate Material and Methods section. Negative Control: cells subjected to incubation in the non-capacitating medium (NCM). Positive Control: sperm subjected to incubation in a standard capacitating medium (CM). 1mM GSH IVC, 2mM GSH IVC, 5 mM GSH IVC. Cells subjected to incubation in the CM in the presence of the indicated concentrations of reduced glutathione from the time 0h of incubaiton. 1mM GSH IVAE, 2mM GSH IVAE, 5mM GSH IVAE: boar sperm subjected to incubation in standard CM for 4h and then added with progesterone (P4) and reduced glutathione at the indicated concentrations together. Results are expressed as means±S.E.M. for 6 separate experiments. Different letters in a row indicate significant ($P<0.05$) differences among separate incubation times of the same treatment line. Asterisks indicate significant ($P<0.05$) differences when compared with the results of the Positive Control line at the same incubation time. Statistical analyses were carried out as described in the Material and Methods section.

Table 3 - Effects of reduced glutation on mean head sperm movement values of boar spermatozoa subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.

		0h	4h	1 min post-P4	5 min post-P4	60 min post-P4
Mean ALH (μm)	Negative Control	3.01±0.09^{a}	2.41±0.15^{b}	2.24±0.16^{b*}	3.02±0.20^{c*}	4.17±0.35^{d*}
	Positive Control	3.07±0.08^{a}	2.29±0.12^{b}	2.93±0.13^{ac}	2.64±0.16^{c}	2.71±0.14^{ac}
	1mM GSH IVC	3.10±0.10^{a}	2.14±0.13^{b}	2.00±0.19^{b*}	2.52±4.8^{b}	2.95±0.22^{a}
	2mM GSH IVC	3.06±0.09^{a}	1.96±0.15^{b}	2.18±0.14^{c*}	2.77±0.16^{ad}	2.47±0.06^{d*}
	5mM GSH IVC	3.08±0.11^{a}	1.89±0.10^{b}	1.71±0.19^{b*}	2.56±0.14^{c}	1.81±0.10^{b*}
	1mM GSH IVAE	3.07±0.08^{a}	2.29±0.12^{b}	3.12±0.21^{ac}	2.61±0.21^{abc}	2.57±0.29^{abc}
	2mM GSH IVAE	3.07±0.08^{a}	2.29±0.12^{b}	2.74±0.19^{ac}	3.36±0.31^{c}	3.01±0.22^{c}
	5mM GSH IVAE	3.07±0.08^{a}	2.29±0.12^{b}	3.49±0.26^{c*}	3.28±0.27^{c*}	3.09±0.25^{c}
BCF (Hz)	Negative Control	6.63±0.10^{a*}	10.88±0.41^{b*}	11.24±0.45^{b*}	11.98±0.63^{b*}	14.18±0.74^{c*}
	Positive Control	7.76±0.13^{a}	8.17±0.23^{a}	7.88±0.24^{a}	8.00±0.22^{a}	7.78±0.24^{a}
	1mM GSH IVC	7.84±0.14^{a}	7.91±0.22^{a}	7.28±0.10^{b*}	10.17±0.37^{c*}	9.25±0.36^{c*}
	2mM GSH IVC	7.71±0.15^{a}	6.49±0.22^{b*}	8.13±0.25^{a}	8.87±0.24^{b*}	7.60±0.24^{a}
	5mM GSH IVC	7.69±0.17^{a}	8.05±0.24^{a}	7.58±0.24^{a}	8.47±0.32^{b}	6.70±0.18^{c*}
	1mM GSH IVAE	7.76±0.13^{a}	8.17±0.23^{a}	8.88±0.37^{b*}	10.29±0.49^{b*}	10.86±0.55^{b*}
	2mM GSH IVAE	7.76±0.13^{a}	8.17±0.23^{a}	8.63±0.29^{b*}	10.59±0.58^{b*}	10.34±0.47^{b*}
	5mM GSH IVAE	7.76±0.13^{a}	8.17±0.23^{a}	11.42±0.38^{b*}	11.68±0.47^{b*}	12.29±0.53^{b*}

Sperm head movement values and the achievement of both IVC and IVAe have been already described in the appropriate Material and Methods section. Negative Control: cells subjected to incubation in the non-capacitating medium (NCM). Positive Control: sperm subjected to incubation in a standard capacitating medium (CM). 1mM GSH IVC, 2mM GSH IVC, 5 mM GSH IVC. Cells subjected to incubation in the CM in the presence of the indicated concentrations of reduced glutathione from the time 0h of incubaiton. 1mM GSH IVAE, 2mM GSH IVAE, 5mM GSH IVAE: boar sperm subjected to incubation in standard CM for 4h and then added with progesterone and reduced glutathione at the indicated concentrations together. Results are expressed as means±S.E.M. for 6 separate experiments. Different letters in a row indicate significant ($P<0.05$) differences among separate incubation times of the same treatment line. Asterisks indicate significant ($P<0.05$) differences when compared with the results of the Positive Control line at the same incubation time. Statistical analyses were carried out as described in the Material and Methods section.

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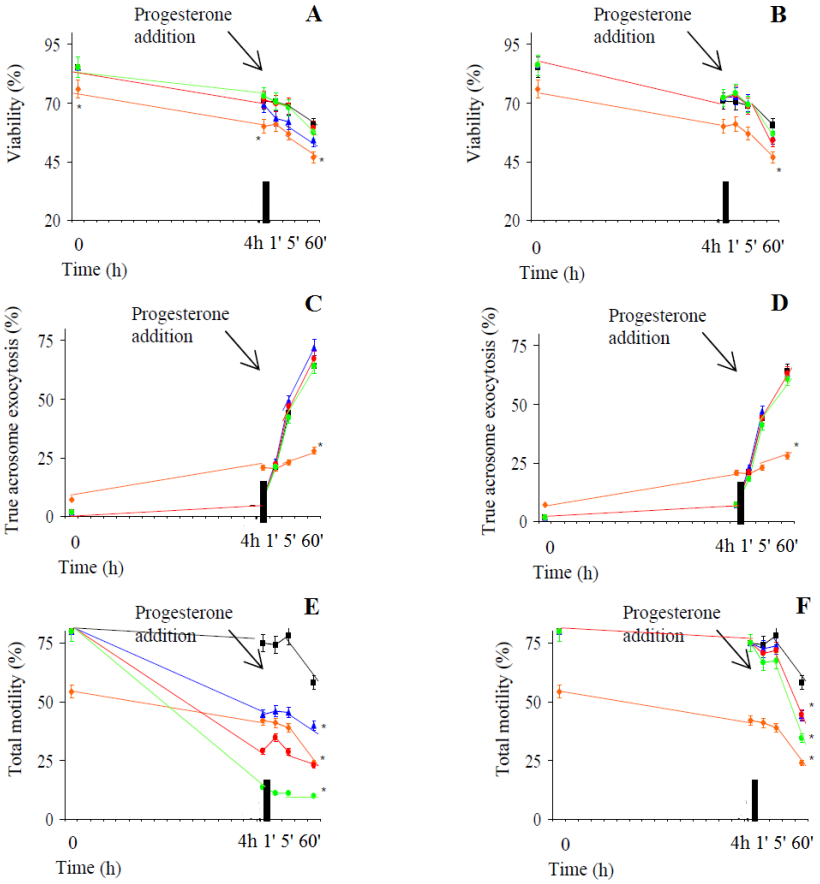
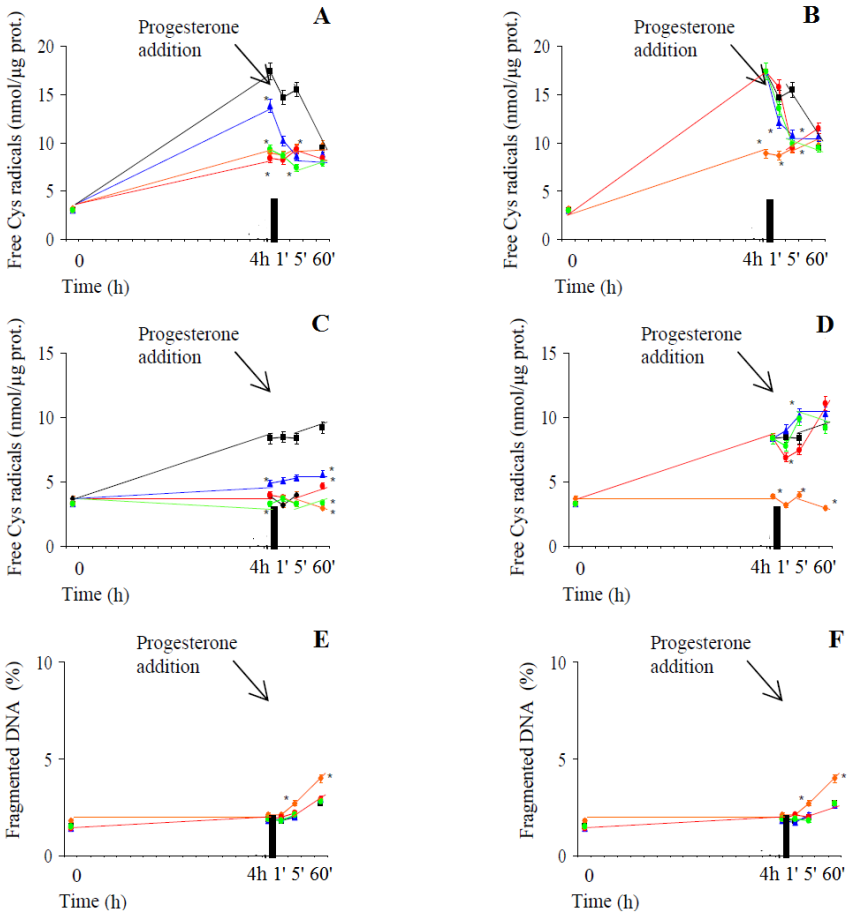
ANEXO A - FIGURAS

Figure 1 - Effects of reduced glutathione on percentages of viability, true axrosome exocytosis and total motility of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis. Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section.



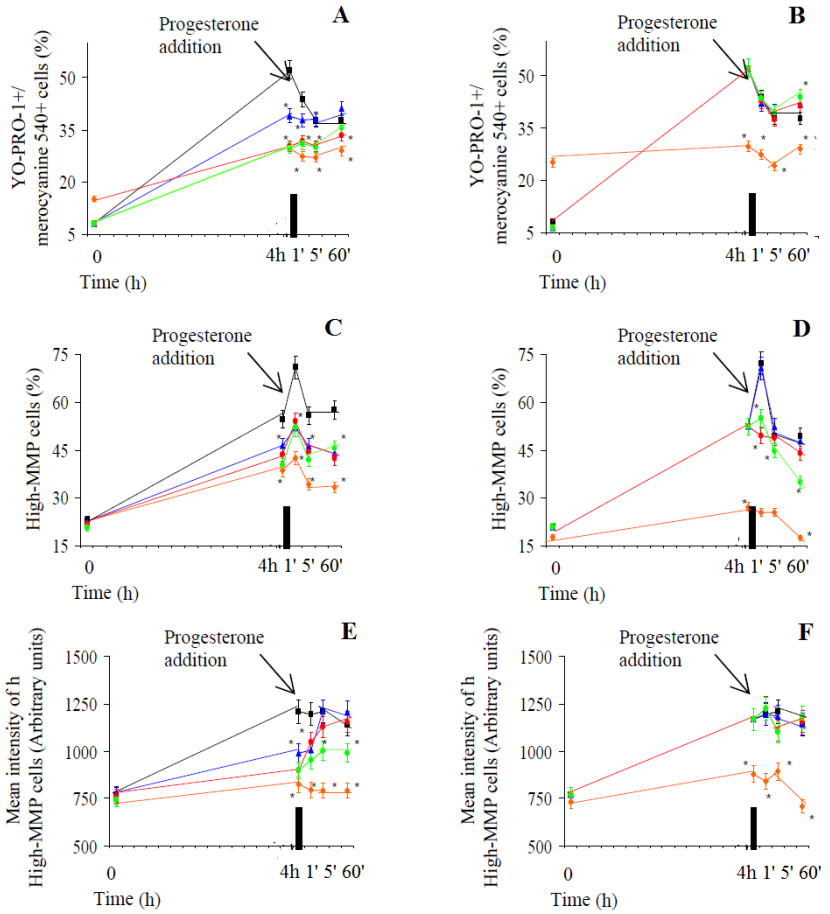
Note: A,B): percentages of viability. C,D): percentages of true acrosome exocytosis. E,F): percentages of total motility. A,C,E): results obtained after incubation of sperm in the presence of GSH from the time 0h of incubation. B,D,F): results obtained through the addition of GSH after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means±S.E.M. for 6 separate experiments.

Figure 2 - Effects of reduced glutathione on both head and tail sperm free cysteine radicals levels and the percentage of cells with fragmented DNA of boar sperm subjected to i*n vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.



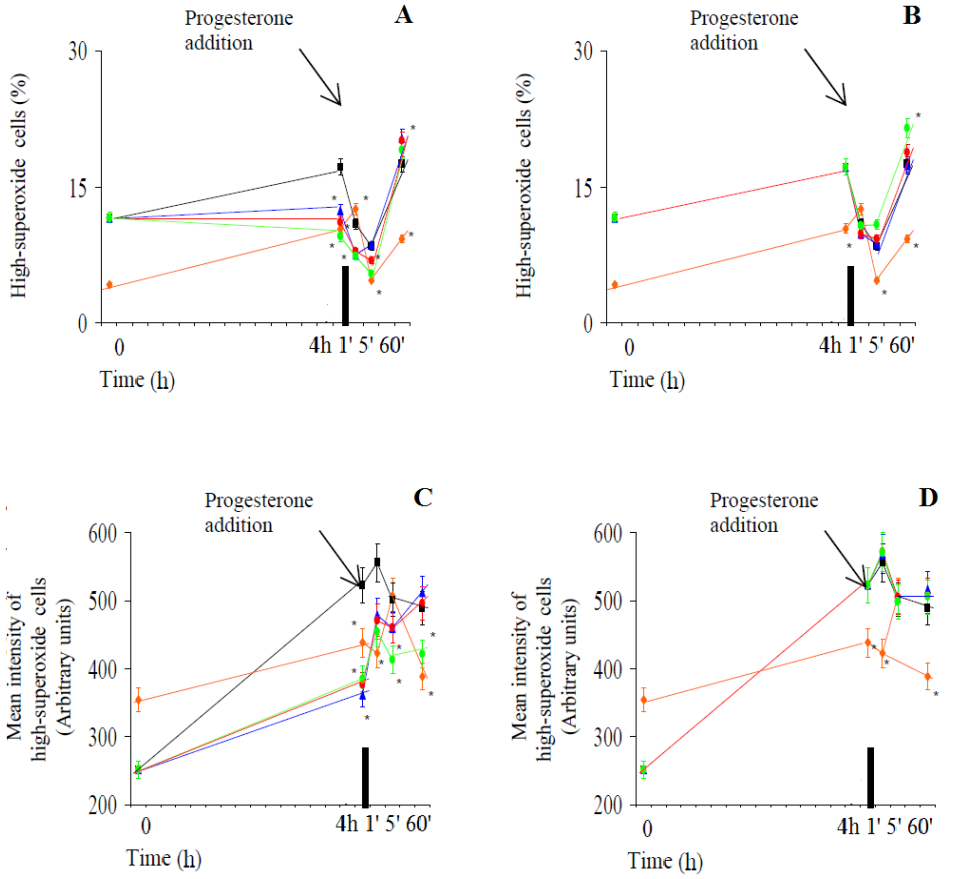
Note: Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): head sperm free cysteine radicals. C,D): tail sperm free cysteine radicals. E,F): percentages of sperm with fragmented DNA. A,C,E): results obtained after incubation of sperm in the presence of GSH from the time 0h of incubation. B,D,F): results obtained through the addition of GSH after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means±S.E.M. for 6 separate experiments.

Figure 3 - Effects of reduced glutathione on sperm membrane fluidity changes and mitochondrial activity of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.



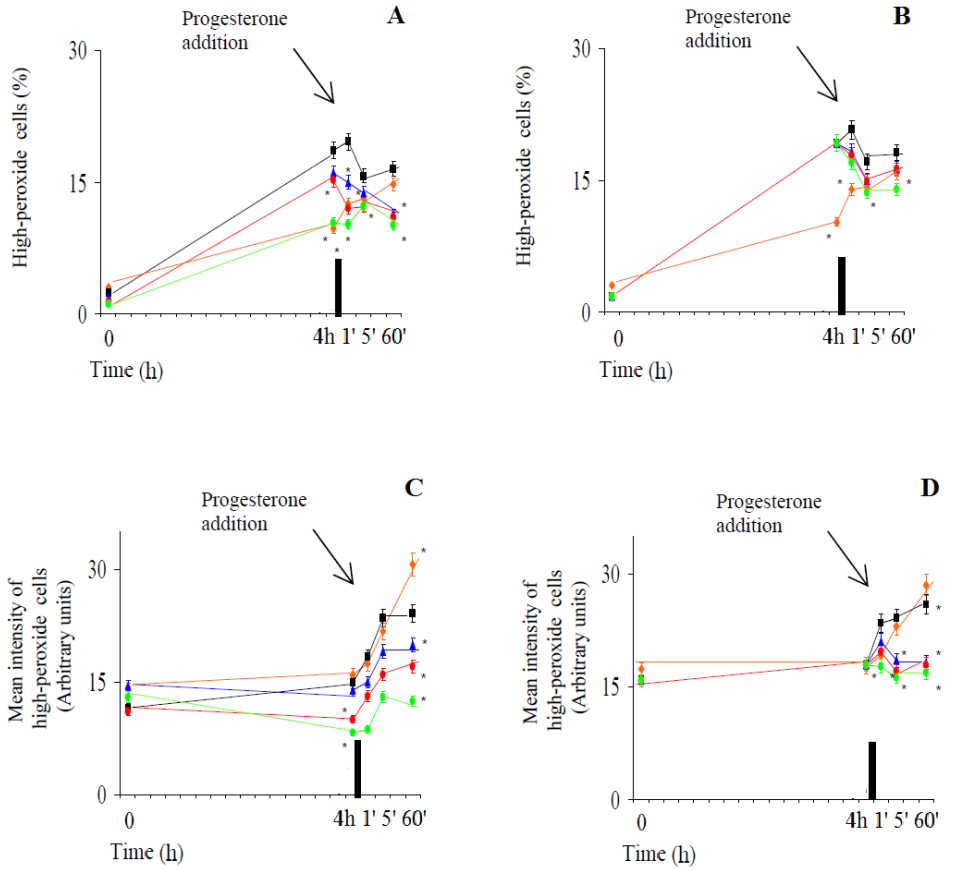
Note: Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of sperm with capacitation-like changes in membrane fluidity (YO-PRO-1/merocynaine 540+ cells). C,D): percentages of sperm with high mitochondrial membrane potential. E,F): mean staining intensity of cells with high mitochondrial membrane potential. A,C,E): results obtained after incubation of sperm in the presence of GSH from the time 0h of incubation. B,D,F): results obtained through the addition of GSH after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means±S.E.M. for 6 separate experiments.

Figure 4 - Effects of reduced glutathione on intracellular superoxide leveles of boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.



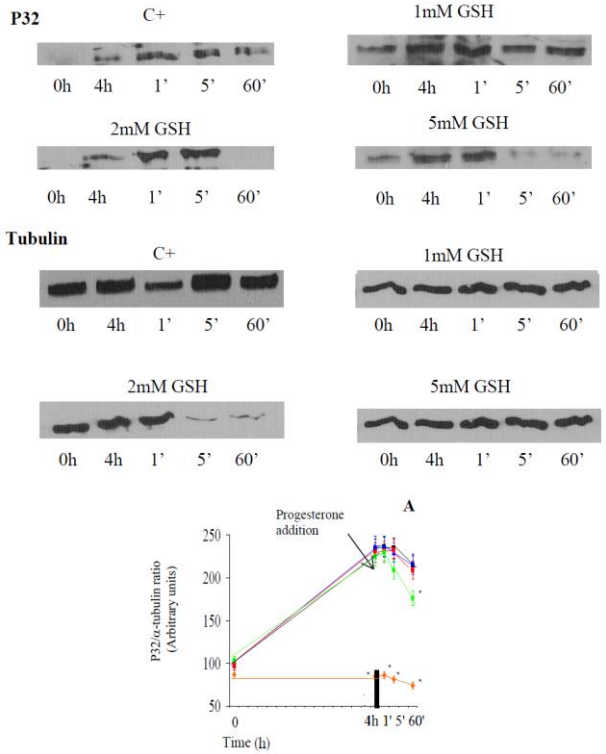
Note: Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of sperm with high intracellular superoxide levels. C,D): mean staining intensity of cells with high intracellular superoxide levels. A,C,E): results obtained after incubation of sperm in the presence of GSH from the time 0h of incubation. B,D,F): results obtained through the addition of GSH after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means±S.E.M. for 6 separate experiments.

Figure 5 - Effects of reduced glutathione on intracellular peroxide leveles of boar sperm subjected to i*n vitro* capacitation and subsequent progesterone-induced acrosome exocytosis



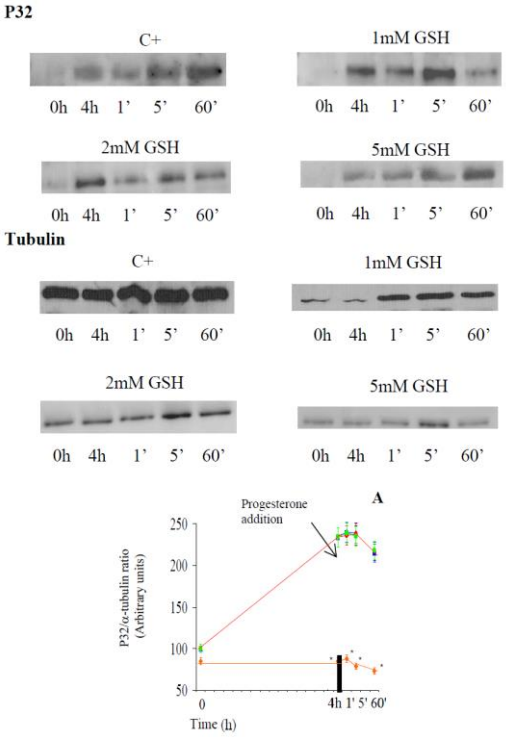
Note: Parameters and the achievement of both IVC and IVAE have been described in the Material and Methods section. A,B): percentages of sperm with high intracellular peroxide levels. C,D): mean staining intensity of cells with high intracellular peroxide levels. A,C,E): results obtained after incubation of sperm in the presence of GSH from the time 0h of incubation. B,D,F): results obtained through the addition of GSH after 4h of incubation in the appropriate medium, either CM or NCM, together with progesterone. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone. Figure shows means±S.E.M. for 6 separate experiments.

Figure 6 - Effects of reduced glutathione added at the 0h time of incubation on the tyrosine phosphorylation intensity levels of the P32 protein in boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.



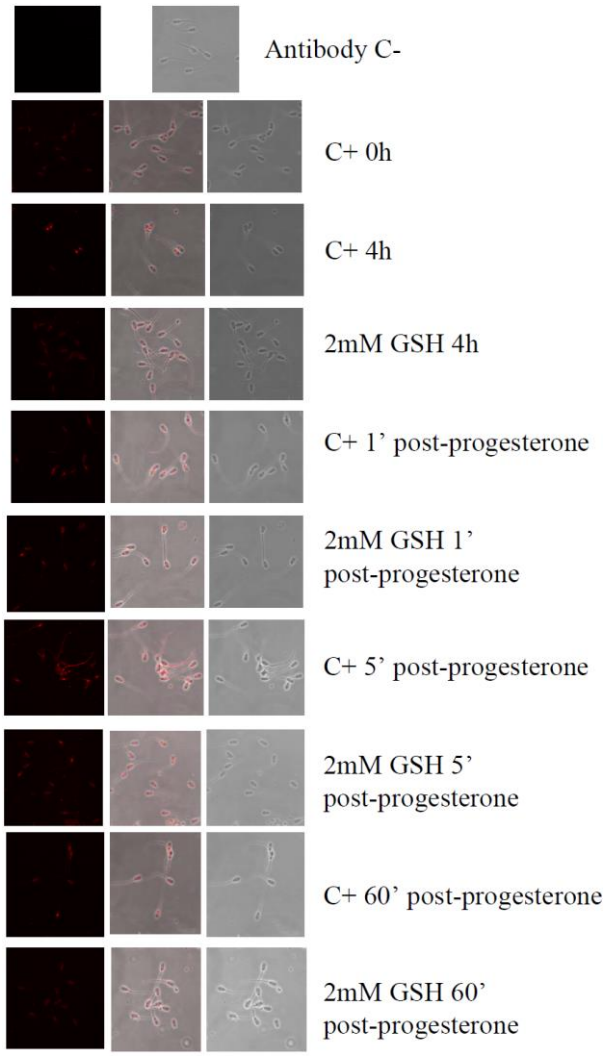
Note: Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. P32: P32 bands obtained through the Western blot analysis. Tubulin: α-tubulin bands obtained in the corresponding Western blot in which P32 phosphorylation has been previously analyzed. In this case, both P32 and α-tubulin bands are from the same samples. C+: sperm incubated in the CM medium. 1mM GSH: cells incubated in the CM medium and added with 1mM GSH from the 0h time of incubation. 2mM GSH: cells incubated in the CM medium and added with 2mM GSH from the 0h time of incubation. 5mM GSH: cells incubated in the CM medium and added with 5mM GSH from the 0h time of incubation. 0h,4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Bands shown here are representative for 5 separate experiments. A: Figure showing the index P32 intensity band/α-tubulin intensity band for each experimental point. Figure was obtained after analyzing 5 separate experiments. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone.

Figure 7 - Effects of reduced glutathione added after 4h of incubation together with progesterone on the tyrosine phosphorylation intensity levels of the P32 protein in boar sperm subjected to *in vitro* capacitation and subsequent progesterone-induced acrosome exocytosis.



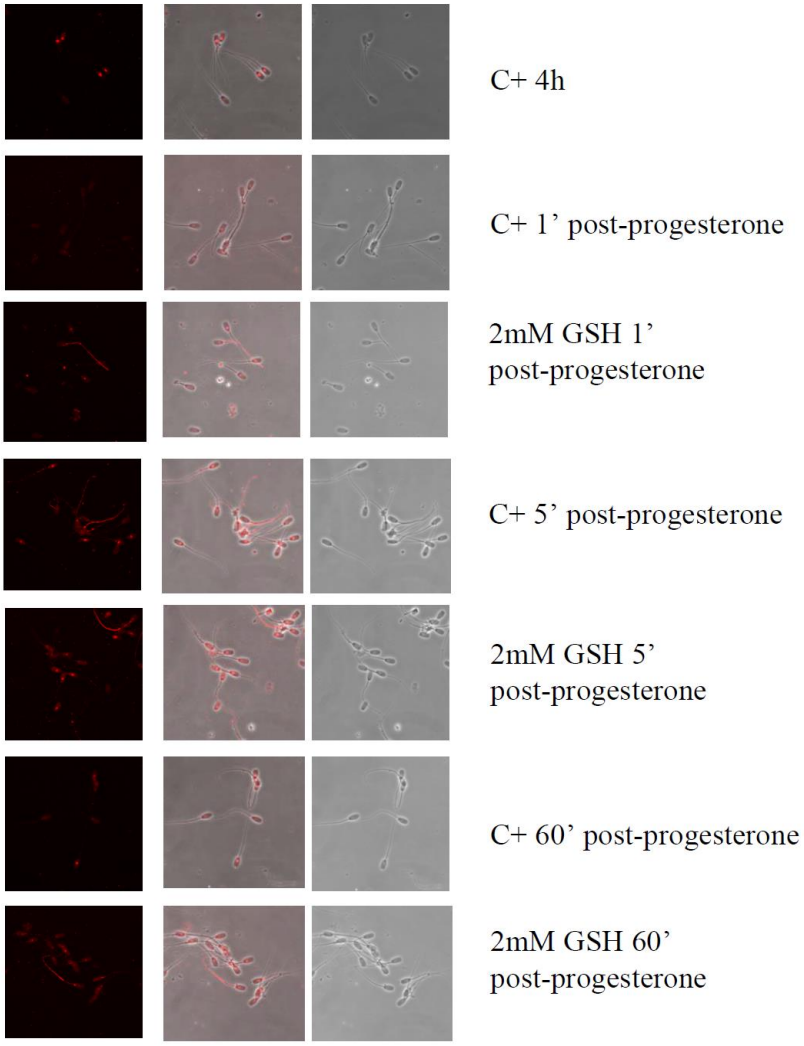
Note: Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. P32: P32 bands obtained through the Western blot analysis. Tubulin: α-tubulin bands obtained in the corresponding Western blot in which P32 phosphorylation has been previously analyzed. In this case, both P32 and α-tubulin bands are from the same samples. C+: sperm incubated in the CM medium. 1mM GSH: cells incubated in the CM medium and added with 1mM GSH from the 0h time of incubation. 2mM GSH: cells incubated in the CM medium and added with 2mM GSH from the 0h time of incubation. 5mM GSH: cells incubated in the CM medium and added with 5mM GSH from the 0h time of incubation. 0h,4h: incubation times. 1',5',60': incubation times after the addition of progesterone. Bands shown here are representative for 5 separate experiments. A: Figure showing the index P32 intensity band/α-tubulin intensity band for each experimental point. Figure was obtained after analyzing 5 separate experiments. ♦: sperm incubated in the NCM medium. ■: cells incubated in the CM medium. ▲: spermatozoa incubated in CM in the presence of 1mM GSH. ●: spermatozoa incubated in CM in the presence of 2mM GSH. ●: spermatozoa incubated in CM in the presence of 5mM GSH. Asterisks indicate significant ($P<0.05$) differences when comparing with results obtained in cells incubated in the CM alone.

Figure 8 - Changes in location of overall protein tyrosine phosphorylation of boar sperm subjected to *in vitro* capacitation and subsequent progesterone acrosome exocytosis in the presence of 2mM reduced glutathione added at the time 0h of incubation.



Note: Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. C+: sperm incubated in the CM. 2mM GSH: sperm incubated in the CM added with 2mM GSH. Antibody C-: cells incubated in the presence of secondary antibody but without previous contact with the anti-phosphotyrosine primary antibody. 0h,4h: incubation times in the initial medium.1',5',60': incubation times after the addition of progesterone following a previous 4h incubation time. Photographs shown here are representative for 5 separate experiments.

Figure 9 - Changes in location of overall protein tyrosine phosphorylation of boar sperm subjected to *in vitro* capacitation and subsequent progesterone acrosome exocytosis in the presence of 2mM reduced glutathione added at the same time that progesterone after a previous 4h incubation period.



Note: Techniques and the achievement of both IVC and IVAE have been described in the Material and Methods section. C+: sperm incubated in the CM. 2mM GSH: sperm incubated in the CM added with 2mM GSH. 0h,4h: incubation times in the initial medium.1',5',60': incubation times after the addition of progesterone following a previous 4h incubation time. Photographs shown here are representative for 5 separate experiments.