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Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm

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Abstract

Repeatable methods for IVF have not been established in the horse, reflecting the failure of standard capacitating media to induce changes required for fertilization capacity in equine sperm. One important step in capacitation is membrane cholesterol efflux, which in other species is triggered by cholesterol oxidation and is typically enhanced using albumin as a sterol acceptor. We incubated equine sperm in the presence of calcium, BSA, and bicarbonate, alone or in combination. Bicarbonate induced an increase in reactive oxygen species (ROS) that was abolished by the addition of calcium or BSA. Bicarbonate induced protein tyrosine phosphorylation (PY), even in the presence of calcium or BSA. Incubation at high pH enhanced PY but did not increase ROS production. Notably, no combination of these factors was associated with significant cholesterol efflux, as assessed by fluorescent quantitative cholesterol assay and confirmed by filipin staining. By contrast, sperm treated with methyl-β-cyclodextrin showed a significant reduction in cholesterol levels, but no significant increase in PY or ROS. Presence of BSA increased sperm binding to bovine zonae pellucidae in all three stallions. These results show that presence of serum albumin is not associated with a reduction in membrane cholesterol levels in equine sperm, highlighting the failure of equine sperm to exhibit core capacitation-related changes in a standard capacitating medium. These data indicate an atypical relationship among cholesterol efflux, ROS production, and PY in equine sperm. Our findings may help to elucidate factors affecting failure of equine IVF under standard conditions.

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Introduction

Mammalian spermatozoa remain incapable of fertilization until they undergo a maturational process in the female reproductive tract known as capacitation (Austin 1951, Chang 1951). Capacitation encompasses a number of intricately related and poorly understood events, including an increase in intracellular bicarbonate and calcium levels, in association with plasma membrane reorganization as a result of cholesterol depletion (Flesch & Gadella 2000, Gadella & Van Gestel 2004). These initial events are associated with a complex intracellular cascade modulated via the adenylyl cyclase–cAMP–protein kinase A (PKA) pathway, whose final hallmark is an overall increase in phosphorylation of tyrosine residues (PY) of a large number of proteins (Visconti *et al*. 1999*a*). Loss of membrane cholesterol is a vital part of this pathway; Visconti *et al*. (1999*b*) demonstrated that suppression of cholesterol efflux inhibited the increase in PY in mouse sperm, although this could be bypassed by direct activation of the cAMP pathway (Visconti *et al*. 1999*b*, Wertheimer *et al*. 2008). Once capacitated, sperm become capable of undergoing subsequent fertilization-related processes when exposed to the appropriate stimuli, including zona pellucida (ZP) binding, the acrosome reaction, oocyte penetration, and, finally, fusion with the female gamete (Visconti *et al*. 1998, 2011).

Capacitation can be achieved *in vitro* in most species by incubation of sperm in a medium that imitates the environment of the female reproductive tract (Yanagimachi 1994, Freeman & England 2013), typically the one containing BSA, calcium, and bicarbonate (standard capacitating medium). Addition of BSA, a cholesterol acceptor, is associated with cholesterol efflux from the sperm plasma membrane in boar, human, and

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mice (Boerke *et al*. 2008, Tulsiani & Abou-Haila 2011). Cholesterol efflux not only supports PY but also allows phospholipid scrambling that will in turn yield plasma membrane lipid microdomain reorganization promoting sperm–ZP binding and the acrosome reaction (Boerke *et al*. 2008). Cholesterol efflux in sperm has been shown to be dependent upon active externalization of the sterol to make it available to sterol acceptors in the medium; this takes place through members of the ATP-binding cassette (ABC) transporter superfamily in mice (Morales *et al*. 2012). ABCA1, ABCA7, ABCA17, and ABCG1 have been detected in mouse sperm (Morales *et al*. 2008) and ABCA1 has also been identified in dog sperm (Palme *et al*. 2014). Inhibition of ABCA1, ABCA7, and ABCG1 in mouse sperm in turn inhibits IVF, confirming that cholesterol efflux plays a core role in the physiology of capacitation and gamete interaction.

During capacitation, a mild increase in reactive oxygen species (ROS) production occurs in mammalian spermatozoa (O'Flaherty *et al*. 2006). Different oxygen radicals such as superoxide anion, nitric oxide, and hydrogen peroxide have been related to sperm capacitation (Griveau *et al*. 1994, de Lamirande *et al*. 2009, Rodriguez *et al*. 2011) and the addition of ROS yields an increase in PY in a variety of species (Baumber *et al*. 2003, Roy & Atreja 2008, Kota *et al*. 2010, Dona *et al*. 2011). It has recently been shown that ROS generation during capacitation may be necessary for cholesterol efflux from the sperm plasma membrane. ROS oxidizes cholesterol to form oxysterols; this appears to be associated with depletion of both oxysterols and unoxidized cholesterol from the plasma membrane (Brouwers *et al*. 2011, Boerke *et al*. 2013). It is postulated that formation of oxysterols may activate the sterol transporter proteins, which make sterols available to cholesterol acceptors such as albumin (Boerke *et al*. 2013).

Non-physiological cholesterol acceptors such as cyclodextrins (cyclic oligosaccharides consisting of 7α (1–4)-glucopyranose units) have been shown to also alter the cholesterol content of sperm cell membranes (Ohtani *et al*. 1989, Choi & Toyoda 1998, Flesch *et al*. 2001) and induce an increase in PY (Osheroff *et al*. 1999, Visconti *et al*. 1999*c*). Interestingly, the cholesterol complexing agent, methyl-β-cyclodextrin (MβCD), does not require cholesterol oxidation in order to effectively remove cholesterol from the sperm plasma membrane (Boerke *et al*. 2013).

No repeatable method for efficient equine IVF has yet been established, indicating a failure to effectively capacitate equine spermatozoa. In the absence of fertilization, induction of PY has been used as a marker for capacitation-related changes in equine sperm. McPartlin *et al*. (2008) reported that incubation of equine sperm at a concentration of 10×10^6/ml in air in a modified Whitten's (MW) medium, supplemented with bicarbonate, calcium, and BSA, was associated with increased PY. We have recently demonstrated that it is the increase in environmental pH associated with incubation of this bicarbonate-containing medium in air, in the presence of a low sperm concentration, that is the core mediator of PY in this treatment, as high environmental pH (∼7.9, the pH found in the mare's uterus post-inseminaton; Gonzalez-Fernandez *et al*. 2012) was associated with increased PY even in the absence of added bicarbonate or BSA. Notably, in equine sperm incubated at standard pH (≤7.4), both calcium and BSA exerted an inhibitory effect on PY (Gonzalez-Fernandez *et al*. 2012, 2013). These findings are in contrast to those described in murine sperm in which BSA or other cholesterol acceptors promote PY in the standard medium (Visconti *et al*. 1999*b*).

BSA is typically added to media during attempts at equine sperm capacitation, with the assumption that it serves to remove cholesterol from the equine sperm plasma membrane (Baumber *et al*. 2003, Pommer *et al*. 2003, Thomas *et al*. 2006, McPartlin *et al*. 2008, 2009). However, little work has been done to examine the relationship of serum albumin or other cholesterol acceptors with membrane cholesterol efflux, ROS production, or induction of PY in equine sperm. An increase in PY associated with treatment with MβCD was reported in equine sperm, but this was examined only in media containing activators of the cAMP–PKA pathway (Pommer *et al*. 2003); such activators essentially bypass the dependence of PY on cholesterol efflux (Visconti *et al*. 1995). Similarly, Bromfield *et al*. (2014) have recently evaluated factors affecting cholesterol reorganization patterns and PY in equine sperm, but essentially only in the presence of pentoxifylline and dibutyryl cAMP. Incubation of equine sperm with MβCD in the basal medium (not containing stimulators of the cAMP–PKA pathway) promoted changes in measures of membrane fluidity and cholesterol organization, but did not significantly increase PY; remarkably, the effects of BSA on these parameters in the basal medium were not reported (Bromfield *et al*. 2014).

The inhibitory effect of BSA on PY in equine sperm under standard incubation conditions (Gonzalez-Fernandez *et al*. 2012) suggests that albumin is not acting as a cholesterol acceptor under these conditions. The aim of this study was to test the influence of factors utilized in the standard capacitating medium (BSA, calcium, and bicarbonate), and the effects of environmental pH, on the capacitation-related events of ROS production, cholesterol efflux, PY, and binding of capacitated equine sperm to the ZP. The latter was tested in a heterologous (bovine) system, as validated previously (Coutinho da Silva *et al*. 2012), due to scarcity of equine oocytes. These findings may help to determine, in equine sperm, factors influencing the physiological changes canonically associated with capacitation, thus helping to illuminate mechanisms underlying the failure of equine IVF and adding to our understanding of comparative sperm biology.

Materials and methods

Chemicals and reagents

All reagents were purchased from Sigma–Aldrich, Inc. unless otherwise stated.

Media

The basal medium used was that utilized by McPartlin *et al.* (2008), i.e., MW medium, consisting of 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose (anhydrous), 22 mM HEPES, 2.4 mM sodium lactate, and 1.0 mM pyruvic acid. The medium with bicarbonate was prepared by adding 25 mM $NaHCO_3$ (MW+Bic). For some media, 2.4 mM $CaCl_2$ was added (MW+Bic+Ca). Media containing calcium, bicarbonate, and BSA (7 mg/ml) were designated MW+++. All media were adjusted by varying (NaCl) to achieve an osmolality of 290–300 mOsm/kg; initial pH was adjusted to 7.25, 7.9, or 8.5, depending upon treatment, using 1 M NaOH. When used, bicarbonate was added 1 h before the experiment and all media were maintained at 37 °C in air until the beginning of the experiment, at which time the pH was determined again and adjusted to the desired value, if necessary.

Semen collection and processing

Semen was collected from seven mature light-breed stallions, using an artificial vagina (Missouri Model AV, Nasco, Ft. Atkinson, WI, USA). A nylon in-line filter (Animal Reproduction Systems, Chino, CA, USA) was used to eliminate the gel fraction. All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training. Procedures performed in Texas were approved by the Institutional Animal Care and Use Committee at Texas A&M University. The stallions used at the University of Porto were maintained according to institutional and European regulations; the ejaculates used were aliquots of commercial doses used in the reproductive clinic of the Center of Animal Reproduction of Vairão (University of Porto) with the owner's consent. Each experiment utilized a minimum of one ejaculate from each of three different stallions.

Sperm were processed as described previously (Gonzalez-Fernandez *et al.* 2013). Briefly, the sperm-rich fraction was diluted 1:5 (v:v) in the MW medium in a 15-ml conical tube. The sperm suspension was centrifuged at 400 ***g*** for 10 min at room temperature (22–24 °C; RT), then the supernatant was discarded, and the pellet was resuspended in 10 ml of MW and centrifuged again at 400 ***g*** for 10 min at RT. The sperm concentration in the resulting pellet was then determined using a NucleoCounter SP-100 (Chemometec, Allerød, Denmark) and the suspension adjusted to a final concentration of 10×10^6 sperm/ml. If addition of MβCD was performed, the medium was supplemented with the designated treatment before final sperm dilution. Aliquots of the final sperm suspension (500 µl) were placed in 5-ml round-bottom plastic tubes (BD Falcon; San Jose, CA, USA). The tubes were incubated at 37 °C in air for 4 h.

Cholesterol quantification

Sperm were incubated in basal MW (initial pH 7.25) containing different concentrations of MβCD (0.1, 1, and 3 mM) or in MW supplemented with 25 mM bicarbonate, 7 mg/ml BSA (Sigma A7030), and 2.4 mM $CaCl_2$ (MW+++). Incubation was performed at a concentration of 10×10^6 sperm/ml, at 37 °C in air for 4 h in a final volume of 500 µl. For this experiment, sperm from four different stallions were used ($n=4$ ejaculates total).

Sperm cholesterol levels were quantified using Amplex Red cholesterol assay kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). All samples were incubated in duplicate. Sperm cholesterol extraction was performed using a modification of a protocol described previously (Nicholson & Ferreira 2009). Briefly, after incubation, the samples were transferred to 1.5 ml Eppendorf tubes and centrifuged at 5000 ***g*** for 4 min at room temperature (RT). After centrifugation, the supernatant was removed and the samples were washed with 1 ml of PBS. The samples were centrifuged again (5000 ***g***, 4 min at RT), the supernatant was removed, and 25 µl of the Amplex Red reaction buffer 1× (RB 1×) were added to the samples. The samples were thoroughly pipetted to disaggregate the pellet and were placed at 4 °C for 10 min to allow for protein and cholesterol extraction. After this procedure, the samples were centrifuged for 10 min at 16 000 ***g*** at 4 °C. The supernatants containing the sperm extracts were transferred to 100-µl Eppendorf tubes and immediately used for protein quantification, using the Bio-Rad DC Protein Assay according to the manufacturer's instructions.

For cholesterol assay, 15 µl of the sperm extracts of each sample were placed in a 96-well plate and 35 µl of RB 1× were added to each well to reach a final sample volume of 50 µl. After this, an equivalent volume of Amplex Red working solution (300 µM Amplex Red, 2 U/ml cholesterol oxidase, 2 U/ml cholesterol esterase, and 2 U/ml HRP) was added. The samples were incubated at 37 °C in the dark for 30 min and fluorescence (565 nm excitation; 585 nm emission) was measured using a 96-well plate in a Synergy MX microplate reader (Bio-Tek, Winooski, VT, USA). Cholesterol values were calculated using the cholesterol standard provided in the kit and normalized to the protein content.

Filipin assay

Equine sperm were prepared and incubated as described previously, at an initial pH of 7.25. The experiment was carried out using one ejaculate from each of three different stallions. One hundred sperm were evaluated per ejaculate.

Plasma membrane distribution of cholesterol was determined using filipin, a fluorescent polyene macrolide antibiotic that binds specifically to cholesterol (Flesch *et al.* 2001). The protocol used is a modification of the one described by Shadan *et al.* (2004). After incubation, samples were transferred to 1.5-ml Eppendorf tubes and were centrifuged at 5000 ***g*** for 4 min at room temperature. The supernatant was removed, 490 µl of PBS were added to each tube, and the pellet was carefully resuspended. A stock solution of 10 mg/ml of

filipin was prepared in 100% ethanol; 10 µl of this solution were added to 490 µl of the sperm solution (10×10^6 sperm/ml) yielding a final concentration of 200 µg/ml of filipin and 2% ethanol. The samples were incubated in the dark at RT for 30 min, washed twice by centrifugation (5000 ***g*** for 4 min at RT) in 1 ml of PBS, and the pellet was resuspended in 40 µl of PBS. Twenty microliters of this suspension were smeared on a slide and were mounted using ProLong Antifade (Life Technologies Corporation) according to the manufacturer's instructions. The samples were stored in the dark overnight, sealed using nail polish, and visualized the following day under a fluorescence microscope (excitation 365 nm, emission >420 nm). Samples were evaluated using an Olympus BX60 fluorescence microscope (New Hyde Park, NY, USA) equipped with a 20× objective. At least 100 sperm per sample were evaluated visually.

Effect of medium components on sperm cholesterol content

Sperm were incubated in basal MW (initial pH 7.25) or in MW supplemented with 25 mM bicarbonate (MW+Bic), 2.4 mM $CaCl_2$ (MW+Ca), or 7 mg/ml BSA A7030 (MW+BSA), alone or in combination. In addition, MW+++ was tested at initial pH 7.9 and pH 8.5. The incubation was performed at a concentration of 10×10^6 sperm/ml, at 37 °C in air for 4 h in a final volume of 500 µl (one ejaculate from each of four stallions; $n=4$). Cholesterol quantification was performed as described earlier.

Effect of albumin type and concentration on sperm cholesterol content

To evaluate whether the unexpected lack of cholesterol efflux after incubation with BSA in the previous experiment was due to albumin type or concentration, sperm cholesterol content was evaluated after processing sperm as above, but incubating in MW containing 0, 7, or 20 mg/ml of five types of albumin: BSA 7030 (Sigma; heat-shock fractionated, fatty-acid free), BSA 9418 (Sigma; ethanol fractionated, fatty-acid free), equine serum albumin (eSA, Rocky Mountain Biologicals, Missoula, MT, USA), equine SA treated to remove lipid (LD-eSA) (via methanol and chloroform treatment, as described by Wessel & Flugge (1984)) and human serum albumin (hSA; Sigma A1887; heat-fractionated, fatty-acid free). Based on the results of the previous experiment, 3 mM MβCD was used as a positive cholesterol efflux control. One ejaculate from each of three different stallions was evaluated.

Owing to the large amount of albumin added in this study, the final sperm number, rather than protein concentration, was used to determine the cholesterol content. To do this, after removal of the final supernatant for cholesterol assay, the pellet was resuspended in 500 µl PBS. The concentration of sperm in this suspension was determined using NucleoCounter as described earlier. The results of the cholesterol assay were then expressed in ng of cholesterol/million sperm.

To determine the cholesterol content of the albumin preparations themselves, MW was prepared containing 0, 7, or 20 mg/ml of the respective albumin preparations; then, the cholesterol assay was performed as outlined above but using 5 µl of the medium, rather than sperm suspension, added to 25 µl RX1 in the initial step.

Evaluation of ROS production

Sperm were incubated in basal MW (initial pH 7.25) or in MW supplemented with 25 mM bicarbonate (MW+Bic), 2.4 mM $CaCl_2$ (MW+Ca), or 7 mg/ml BSA A7030 (MW+BSA), alone or in combination. In addition, MW+++ was tested at initial pH 7.9 and pH 8.5, and MW was tested with 0.1, 1, and 3 mM MβCD. The incubation was performed at a concentration of 10×10^6 sperm/ml, at 37 °C in air for 4 h in a final volume of 500 µl (three ejaculates from three stallions; $n=3$).

The protocol used was a modification of that designed by Minervini *et al.* (2010). Briefly, after the incubation, 100 µl of each sample were resuspended in 900 µl of prewarmed PBS and 2′,7′-dichlorofluorescein diacetate (DCFDA) was added to reach a final concentration of 5 µM. This suspension was incubated at 37 °C for 30 min in the dark; in the last 5 min, 1 µl of propidium iodide (PI) was added (final concentration, 1 µM). DCFDA is a cell-permeable non-fluorescent probe that becomes de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation by ROS, emitting at a wavelength of 530 nm (detected by FL1); cell viability was simultaneously assessed by detection of fluorescence of the non-permeant probe PI, emitting at a wavelength of 617 nm (detected by FL3). After the dye–sperm co-incubation, 10 000 sperm were evaluated using a Beckman Coulter EPICS XL flow cytometer (Coulter Corporation, Inc., Miami, FL, USA) equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, CA, USA) performing 15 mW at a wavelength of 488 nm and the EXPO 2000 software. For each experiment, the non-stained cells were delimited in the quartile denominated as B3 as shown in Supplementary Figure 2, see section on supplementary data given at the end of this article; this region was delimited using a non-stained control. Cells were considered positively stained when PI or DCFDA fluorescence was detected above this threshold. In our study, only live sperm producing ROS were considered (B4 quadrant of Supplementary Figure 2). Representative scatterplots are shown in Supplementary Figure 2.

Immunocytochemistry for PY

Sperm were incubated under the same conditions as for the previous experiment. Two ejaculates from each of three stallions were evaluated. Following incubation, sperm were centrifuged for 3 min at 5000 ***g*** and washed with 1 ml of PBS. After centrifugation, the pellet was resuspended in 1 ml of PBS and the cell concentration was adjusted to 5×10^6 spermatozoa/ml. Ten microliters of the sample were placed on a slide pretreated with poly-L-lysine. After 10 min, spermatozoa were fixed with 4% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.1% Triton X-100 (v:v) in PBS for 10 min. After three washes with PBS, sperm were blocked with 3% BSA in PBS for 60 min. Primary incubation with antiphosphotyrosine MAB (4G10) (diluted 1:500) was

performed in 3% BSA in PBS at 4 °C overnight. The sample was then washed with PBS and incubated with an anti-mouse IgG (FITC)-conjugated secondary antibody in 3% BSA in PBS for 1 h at RT. After three washings with PBS, the samples were mounted on a slide with Slowfade gold antifade solution (Molecular Probes) according to the manufacturer's instructions. Samples were evaluated using a CyScope Plus HP-Partec fluorescence microscope (Swedesboro, NY, USA) equipped with a 100× objective. One hundred sperm per sample were examined, and the number of stained tails was counted.

In vitro *maturation and preparation of bovine oocytes*

Bovine ovaries were collected at a nearby abattoir and were maintained at 30 °C in saline solution (0.9% NaCl) during transport (2 h total). After arrival at the laboratory, the ovaries were thoroughly rinsed with PBS at 37 °C and the oocytes were aspirated from 2–8-mm follicles using a 10 ml plastic syringe attached to a 20-ga hypodermic needle. Oocytes with three or more layers of compact cumulus cells were cultured in tissue culture medium 199 (TCM-199) with Earle's salts and 25 mM HEPES supplemented with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT, USA), 10 mU/ml of follicle-stimulating hormone (FSH; Life Technologies Corporation), 10 mU/ml of luteinizing hormone (LH; Life Technologies Corporation), and penicillin–streptomycin (10 U/ml of penicillin and 10 µg/ml of streptomycin). Five hundred microliters of this media were placed in four well Nunc chambers, covered with cell culture mineral oil (500 µl) and allowed to equilibrate for at least 3 h before placement of oocytes in a 5% CO_2/95% air incubator with 100% humidity set at 39 °C. Up to 50 oocytes were placed in each well and incubated for 24 h.

After maturation, cumulus cells surrounding the oocytes were removed by vortexing the complexes in a 15-ml conical tube for 3 min in 4 ml of PBS supplemented with 0.4% hyaluronidase and 0.2% polyvinylalcohol (PVA). After vortexing, the oocytes were transferred to TCM-199 supplemented with 10% FBS. The oocytes were divided randomly into groups of five to seven oocytes and were washed three times in their respective medium before placement in the binding droplets, as described below.

Heterologous binding assay

The protocol used was a modification of that reported previously (Choi *et al.* 2003). Briefly, sperm were incubated at initial pH 7.25 in MW, MW+Bic+Ca, MW+++, or MW+++ with 0.1 mM or 1 mM MβCD. To examine whether the effect of BSA might be modulated by pH, sperm were also incubated in MW+++ at initial pH 7.9. Treatment with 3 mM MβCD and media devoid of calcium, as used in previous studies, were not evaluated (with the exception of basal MW as a control), because subjective sperm motility decreased dramatically in these treatments (data not shown). This experiment was performed using a minimum of four ejaculates from each of three different stallions: stallion IP, stallion JC, and stallion XA. Spermatozoa were incubated at 37 °C in air for 4 h at a concentration of 10×10^6 sperm/ml in 500 µl medium, as described earlier.

For sperm–oocyte co-incubation, the cumulus-denuded bovine oocytes were placed in 90-µl droplets of the medium under oil before addition of sperm. The droplets were prepared with MW, MW+Bic+Ca, or MW+++ at pH 7.25 or with MW+++ at pH 7.9, for sperm capacitated in the homologous treatments. Sperm capacitated with MβCD were co-incubated with oocytes in droplets of MW+++ pH 7.25. To directly compare binding at equivalent pH, an additional treatment using sperm incubated in MW+++ at pH 7.9 and co-incubated with oocytes in MW+++ at pH 7.25 was conducted.

To each oocyte-containing droplet, 10 µl of the corresponding sperm suspension (10×10^6 sperm/ml) were added, to yield a final concentration of 1×10^6 sperm/ml. Once sperm had been added, the plates containing the droplets were placed in a 5% CO_2/95% air incubator at 39 °C and 100% humidity for 2 h. After gamete co-incubation, the sperm–oocyte complexes were pipetted three times each in PBS supplemented with 0.2% of PVA, through a wide-bore glass pipette, to remove loosely attached spermatozoa. The sperm–oocyte complexes were then fixed in 4% formaldehyde in PBS supplemented with 0.2% PVA for 12 h at 4 °C. Then, the complexes were washed with PBS supplemented with 0.2% PVA and stained with Hoechst 33342 at 5 µg/ml at 37 °C for 10 min in the dark. The complexes were mounted on slides using glycerol, covered with a cover slip, fixed using nail polish, and allowed to dry. The number of sperm bound to each oocyte was assessed using a CyScope Plus HP-Partec fluorescence microscope (Swedesboro, NY, USA) equipped with a 100× objective.

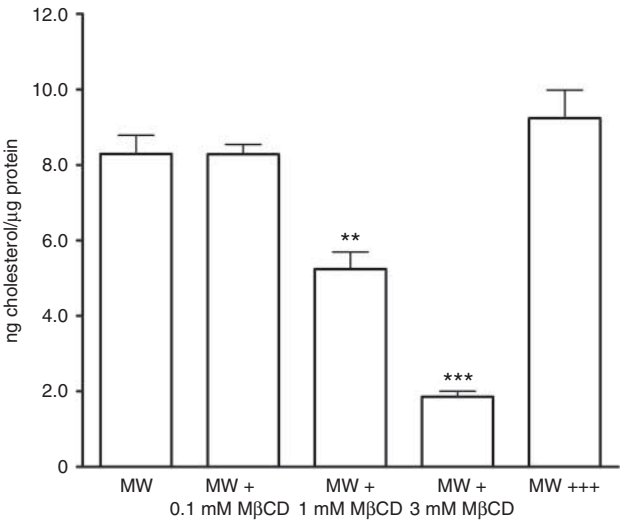

Figure 1 Effect of MβCD on sperm cholesterol as assessed by fluorescent Amplex Red assay. Equine sperm were incubated for 4 h in MW+++ or in MW supplemented with 0.1, 1, or 3 mM MβCD. The amount of cholesterol in the sperm lysate was measured and normalized to its protein content (ng cholesterol/µg protein). The experiment was carried out using five ejaculates from four different stallions ($n=4$). Values bearing **($P<0.01$) or ***($P<0.001$) differ significantly from the MW treatment and the error bars represent the S.E.M.

Statistical analysis

All data were first examined using the Shapiro–Wilk test to determine their distribution. All data resulting from the ROS production experiment, viability assays, PY immunofluorescence, and cholesterol determinations (with exceptions presented later), showed a normal distribution and differences between treatments were established using a one-way ANOVA. When differences were found, the Holm–Sidak method was used to perform multiple comparisons (treatment vs control group) for the cholesterol determinations, a Tukey *post-hoc* test was used to compare pairs of values in the ROS and a Student–Newman–Keuls *post-hoc* test was used for the PY experiments. For the heterologous binding experiments, in view of their non-Gaussian distribution, an ANOVA on ranks test was used. As the group sizes were not equal in heterologous binding experiments, a Dunn's test *post hoc* was used; a Student–Newman–Keuls *post-hoc* test was used to compare pairs of values in the cholesterol determination. Statistical significance was set at $P<0.05$. Analyses were performed using SigmaPlot ver. 12.0 for Windows (Systat Software, Chicago, IL, USA).

Results

Cholesterol content and distribution in equine sperm

Results of the cholesterol quantification assay, evaluated on sperm incubated in basal MW (devoid of BSA, calcium and bicarbonate) treated with different concentrations of MβCD to induce cholesterol efflux, and in sperm incubated in standard capacitating media (containing BSA, calcium, and bicarbonate; MW+++), are presented in Fig. 1. Sperm cholesterol contents (ng cholesterol/μg of protein; mean±S.E.M.) were 8.64±0.51, 8.69±0.46, 5.24±0.45, 2.08±0.26, and 10.17±1.1 for sperm incubated in MW supplemented with MβCD at 0, 0.1, 1 or 3 mM, or in MW+++ respectively. Values for treatment groups containing 1 or 3 mM MβCD were significantly lower than values for treatment groups containing 0 or 0.1 mM MβCD ($P<0.01$). No cholesterol efflux was detected in sperm exposed to BSA (MW+++) in comparison to the basal MW used as a control ($P>0.05$).

These results show a lack of effect of BSA on cholesterol content in equine sperm; however, it is possible that the distribution of cholesterol within the plasma membrane may be affected, as reported previously in porcine sperm after the addition of BSA (Flesch *et al.* 2001). Thus, we assessed sperm incubated in the above treatments by filipin staining (Fig. 2). No differences were detected in the distribution pattern of filipin fluorescence for sperm incubated in MW (0 mM MβCD), 0.1 mM MβCD, or MW+++ treatments; however, a substantial decrease in overall filipin fluorescence was detected in 1 and 3 mM MβCD

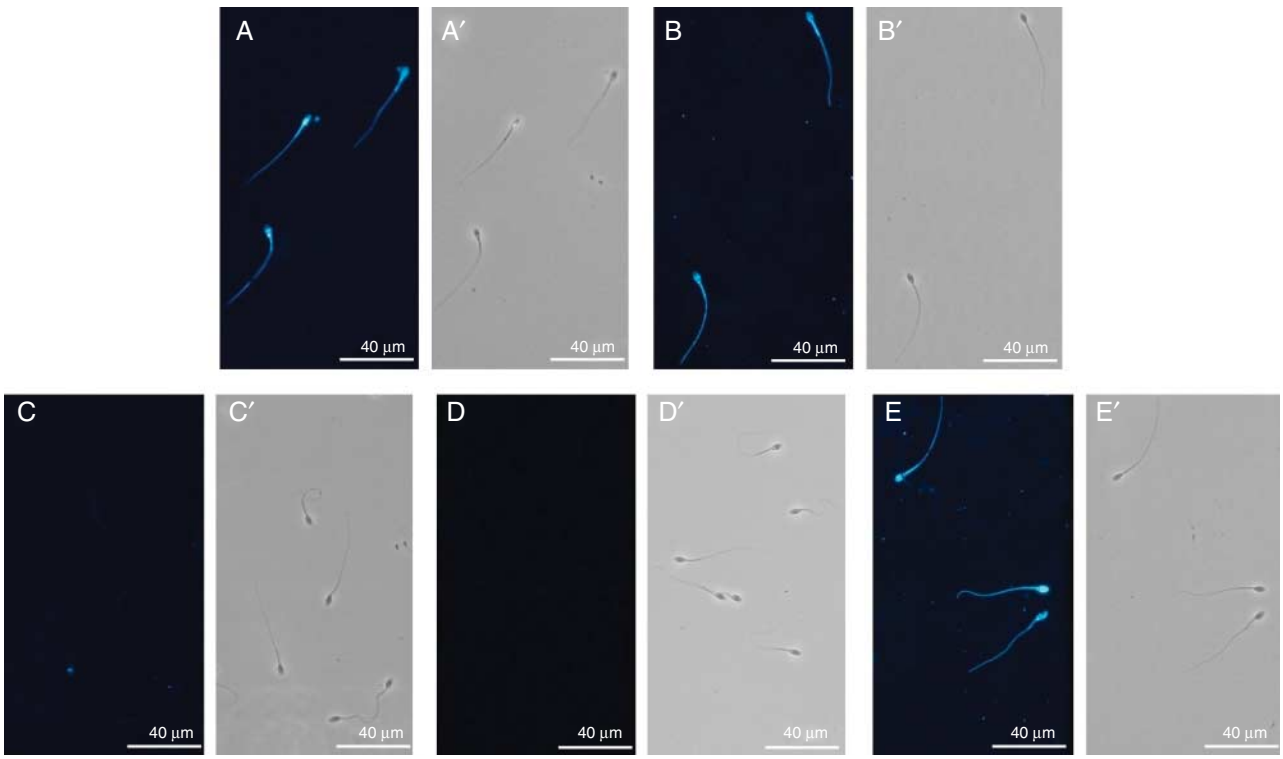

Figure 2 Distribution of equine sperm cholesterol assessed by filipin staining. Fluorescent microscopy (A, B, C, D, and E) and light microscopy (A′, B′, C′, D′, and E′) of equine sperm stained using filipin and observed at 20× magnification. The letters represent the different media: (A) MW; (B) MW +0.1 mM MβCD; (C) MW +1 mM MβCD; (D) MW +3 mM MβCD; and (E) MW+++, pH 7.25. The images shown are representative of three independent experiments (three stallions, one ejaculate per stallion; 100 sperm per sample).

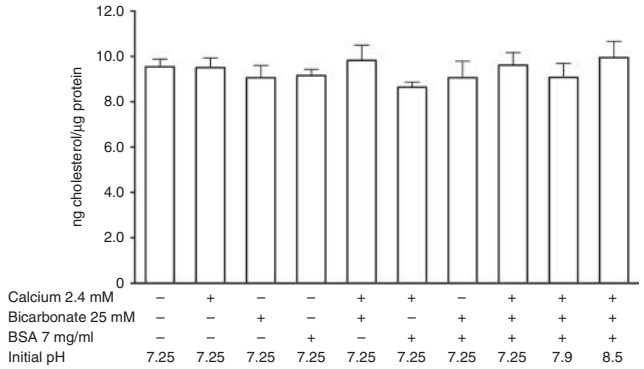

Figure 3 Effect of calcium, BSA, bicarbonate, and medium pH on sperm cholesterol content. Spermatozoa (10×10^6 sperm/ml in 500 µl) were incubated for 4 h, lysed, and the amount of cholesterol measured and normalized to its protein content (ng cholesterol/µg protein). The experiment was carried out using four ejaculates from four different stallions ($n=4$). No statistically significant differences were found among treatments ($P>0.05$); the error bars represent the S.E.M.

treatments when compared with other treatment groups. In 1 mM MβCD, fluorescence was limited to remnants of the equatorial band, and, in 3 mM MβCD, fluorescence was essentially eliminated. These findings support those of the cholesterol quantification assay (Fig. 1), in that treatment with MβCD showed a dose-dependent reduction in the amount of equine sperm cholesterol, whereas BSA had no effect.

Effect of calcium, BSA, bicarbonate, and medium pH on sperm cholesterol efflux

To evaluate whether the cholesterol-acceptor ability of BSA in equine sperm was influenced by medium pH, calcium, or bicarbonate, the cholesterol quantification assay was performed on sperm incubated in basal MW, MW+Bic, MW+Ca, MW+BSA, or combinations of these additions at initial pH 7.25, and in MW+++ at initial pH 7.9 and pH 8.5 (Fig. 3). No significant differences were found in cholesterol content among treatments ($P>0.05$), indicating that none of the tested factors affected cholesterol efflux in equine sperm.

Effect of albumin concentration and type on sperm cholesterol content

To evaluate whether cholesterol efflux required different, possibly species-specific, serum albumin, or higher quantities of serum albumin, we assessed sperm cholesterol content after treatment with these factors. There was no reduction in the cholesterol content associated with incubation of equine sperm with BSA 7030 (used in the above studies), BSA 9418, hSA, eSA, or delipidated eSA (LD-eSA), at either 7 or 20 mg/ml, whereas incubation with 3 mM MβCD induced highly significant cholesterol depletion (Fig. 4). Incubation of sperm with non-delipidated eSA was associated with a significant increase in measured cholesterol.

When 5 µl of media (without sperm) containing either 7 or 20 mg/ml of the different albumins were tested for cholesterol content, non-delipidated eSA was associated with a highly significant, dose-dependent increase in measured cholesterol levels, which was not present for delipidated eSA (LD-eSA), BSA 7030, or hSA (Fig. 5). Incorporation of cholesterol from the medium by sperm in this treatment may explain the increase in cholesterol content observed in sperm incubated in the presence of non-delipidated eSA. BSA 9418 showed a minor but significant increase in cholesterol between the 7 and 20 mg/ml solutions.

Effect of medium components, pH, and MβCD addition on sperm ROS production

ROS production has been shown to be associated with cholesterol oxidation and cholesterol efflux from the sperm plasmalemma (Boerke *et al.* 2013) and overall with sperm capacitation. As neither the three components, BSA, bicarbonate, and calcium, alone nor high pH induced a significant decrease in the cholesterol content of equine sperm, we tested whether these components alone or in combination were associated with increased sperm ROS production (Fig. 6). Incubation in MW+Bic resulted in the highest proportion of live sperm producing ROS (31.5%±4.9; mean±S.E.M.). Surprisingly, the addition of calcium and/or BSA resulted in a significant decrease in ROS production compared with incubation with MW+Bic alone (4.3%±0.6 and 5.1%±1.3 for the MW+Bic+Ca and MW+Bic+BSA treatments respectively; $P<0.05$) or with incubation in MW alone (16.9%±4.3 vs 6.4%±1.0 and 2.1%±0.6

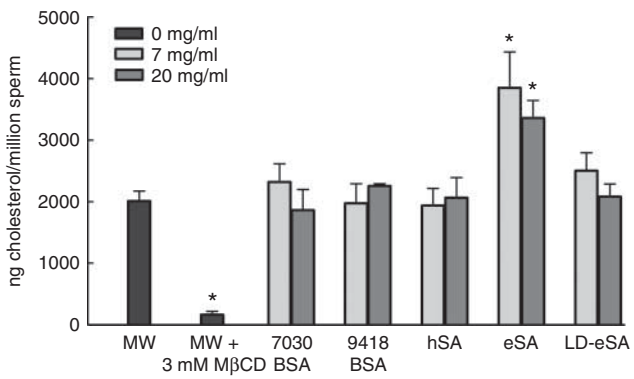

Figure 4 Effect of albumin concentration and type on sperm cholesterol content. Spermatozoa (10×10^6 sperm/ml in 500 µl) were incubated with different albumin sources and concentrations (7 or 20 mg/ml) for 4 h in MW+Bic+Ca; after the incubation the sperm were lysed, and the amount of cholesterol measured and normalized to the final sperm number (ng cholesterol/million sperm). The experiment was carried out using three ejaculates from three different stallions ($n=3$). Values bearing * differ significantly ($P<0.05$) from the MW treatment; the error bars represent the S.E.M.

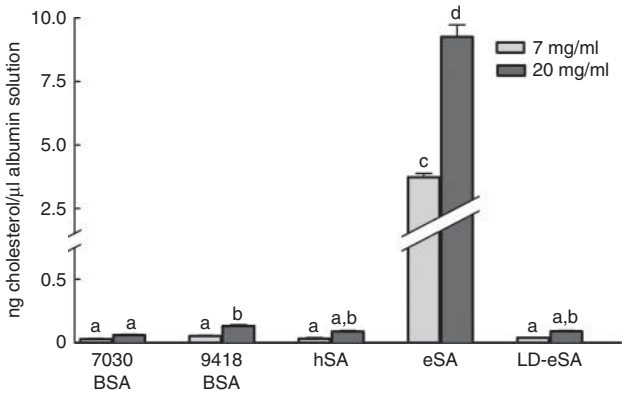

Figure 5 Cholesterol content of media containing different albumins in solution. Cholesterol concentrations (ng/per µl albumin solution) of assay preparations made with 5 µl MW containing 7 or 20 mg/ml of the different tested albumins (hSA, human serum albumin; eSA, equine serum albumin; LD-eSA, delipidated equine serum albumin). Bars bearing different letters differ significantly ($P<0.05$) and the error bars represent the S.E.M.

for MW+Ca and MW+BSA respectively), although in this case only the addition of BSA was statistically significant ($P<0.05$). Neither MW+++ at any of the pH values tested nor MW+MβCD at any of the concentrations used resulted in a significant increase in ROS production.

Protein tyrosine phosphorylation

The protein tyrosine phosphorylation (PY) status of equine sperm was assessed by the increase in the percentage of sperm showing fluorescence, indicating phosphorylation of tyrosine residues, along the tail on immunocytochemistry (Fig. 7). PY was consistently increased when bicarbonate was added to the media either alone (MW+Bic; 43.8%±10.1%) or in combination with BSA (MW+Bic+BSA; 42.3%±6.4%) or calcium (MW+Bic+Ca; 33.0%±8.8%) compared with MW alone (9.7%±2.3%; Fig. 7). When calcium, bicarbonate, and BSA were added (MW+++ pH 7.25), an increase in PY was observed but it was not significantly different from that for MW, due to the high variability existing between stallions in PY induction in these media, as reported previously (Gonzalez-Fernandez *et al*. 2012). Induction of PY was pH dependent, as found previously (Gonzalez-Fernandez *et al*. 2012), with MW+++ at pH 7.9 and 8.5 showing the greatest prevalence of PY (50.3%±7.3% and 59.7%±4.2% respectively). Addition of MβCD (0.1–3 mM) to MW did not result in a significant increase in PY compared with MW (Fig. 7).

Heterologous binding assay

To evaluate the capability of equine sperm incubated under different conditions to bind to bovine ZP, sperm were incubated in MW, MW+Bic+Ca, and MW+++ at pH 7.25, or MW+++ at pH 7.9; sperm–ZP co-incubation was tested in those same media for the homologous treatments. In addition, sperm were also incubated in MW+++, pH 7.25, with 0.1 or 1 mM MβCD, or in MW+++, pH 7.9, and were co-incubated with bovine oocytes in MW+++, pH 7.25. Sperm from each stallion tested (IP, XA and JC) responded similarly to the different media (Fig. 8), although the absolute number of sperm bound to bovine ZP differed significantly among stallions. For stallion IP, the experiment was repeated five times and a total of 252 oocytes per treatment were evaluated (34–39 per treatment). For stallion XA, the experiment was repeated five times and a total of 252 oocytes were evaluated (32–38 per treatment). For these stallions, the number of sperm bound to the ZP was low enough to quantify. For stallion JC, the experiment was performed four times and a total of 180 oocytes were assessed (24–26 per treatment). For this stallion, the number of sperm bound to the ZP in some treatments was too high to quantify, and thus, representative photomicrographs are shown in Fig. 8 and statistical analyses were not performed. For all stallions, incubation in MW or in MW+Bic+Ca did not induce appreciable sperm binding to the ZP; however, addition of BSA (MW+++) induced a marked increase in the number of sperm bound to the ZP compared with that for MW or MW+Bic+Ca (significant for quantified stallions (IP and XA; $P>0.05$). In stallion IP, an additional significant increase in sperm–ZP binding was observed in sperm incubated in MW+++ supplemented with 1 mM MβCD ($P<0.01$); however, this was not true for Stallion XA, and no difference could be appreciated between these treatments in stallion JC.

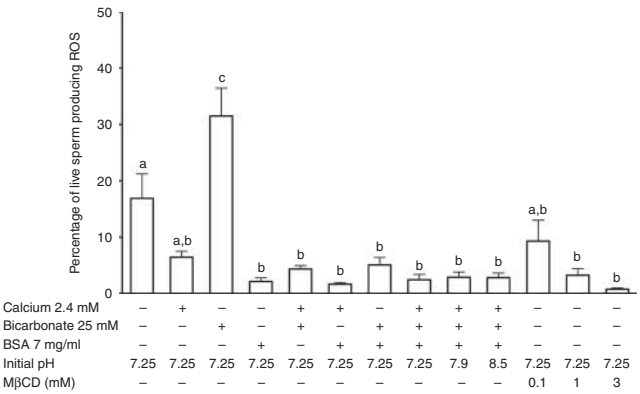

Figure 6 Effect of media components, pH, and MβCD addition on sperm ROS production. Sperm were incubated at a concentration of 10×10^6 million/ml at 37 °C in air for 4 h in the designated media. One hundred µl were diluted in 900 µl of PBS, incubated with 5 µM of DCFDA for 30 min at 37 °C in the dark, and 1 µM of PI was added in the last 5 min. ROS production in live cells was measured using a flow cytometer. Three ejaculates from three different stallions were used ($n=3$); bars bearing different letters differ significantly ($P<0.05$) and the error bars represent the S.E.M.

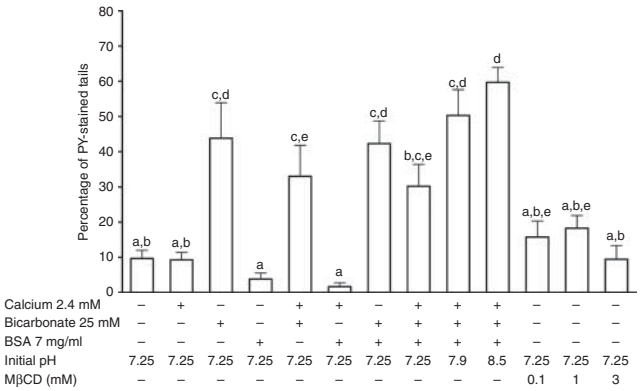

Figure 7 Percentage of sperm tails showing positive phosphotyrosine labeling. Sperm were incubated at a concentration of 10×10^6 million/ml in the given media at 37 °C in air for 4 h. Two ejaculates from three different stallions were used ($n=6$). One hundred sperm per sample were evaluated using a fluorescence microscope (100×) and the percentage of stained tails was calculated. Bars bearing different letters differ significantly ($P<0.05$); the error bars represent the S.E.M.

Discussion

The results of this study demonstrate that, in equine sperm, exposure to standard capacitating conditions (medium containing BSA, bicarbonate, and calcium) results in neither ROS production nor cholesterol efflux, even under conditions (increased medium pH) associated with increased PY (Figs 1, 2, 3, 4, 5, 6, and 7). This is in direct contrast to findings in murine (Visconti *et al.* 1999*b*), porcine (Flesch *et al.* 2001), and human sperm (Osheroff *et al.* 1999).

Flesch *et al.* (2001) reported that albumin-mediated cholesterol extraction from boar sperm was dependent upon bicarbonate; in combination, bicarbonate and BSA stimulated a marked decrease (up to 30%) in the cholesterol content of porcine sperm, and presence of BSA, calcium, and bicarbonate induced a vivid change in cholesterol distribution, as detected by filipin staining. This finding is in agreement with that of Boerke *et al.* (2013), who reported that bicarbonate or other pro-oxidants induced ROS production, which, in turn, stimulated cholesterol oxidation and promoted cholesterol efflux in porcine and murine sperm. By contrast, we found no change in cholesterol distribution or content in equine sperm subjected to 4 h of incubation in the presence of bicarbonate, calcium, and BSA, alone or in combination, nor did we find a decrease in the cholesterol content associated with the treatment that significantly promoted ROS production (MW+Bic) (Figs 1, 2, 3, and 6).

The lack of decrease in membrane cholesterol content in equine sperm exposed to BSA could be attributed to an insufficient albumin concentration or the need of a different albumin source; it has previously been reported that different albumin preparations and source (bovine vs human) vary in their ability to support human sperm capacitation (Ravnik *et al.* 1990, 1993). However, we found no effect of different albumin sources (human, equine, or bovine) or concentrations (0, 7 or 20 mg/ml; Figs 4 and 5) on the cholesterol content of equine sperm. In fact, the non-delipidated eSA increased the sperm cholesterol content. Our results are consistent with those of Go & Wolf (1985), which demonstrated that, in mice, cholesterol-loaded BSA increased sperm cholesterol content, inhibiting capacitation and decreasing fertilization rates *in vitro*. The results of our experiments indicate that non-delipidated albumins should be avoided when working with equine sperm to prevent cholesterol transfer.

The highest levels of ROS production were found in sperm incubated in MW+Bic or MW (Fig. 6). Surprisingly, BSA and calcium, alone or in combination, significantly inhibited ROS production in both MW+Bic and MW media (Fig. 6). These findings are in contrast to those in boar (Matas *et al.* 2010) and human sperm (O'Flaherty *et al.* 2005), in which capacitating conditions (media containing bicarbonate, BSA, and calcium) increased ROS production and PY. To the best of our knowledge, inhibitory effects of calcium and BSA on ROS production during capacitation have not been reported previously in sperm of any species.

As we have previously reported (Gonzalez-Fernandez *et al.* 2012), when bicarbonate was added to the medium under the conditions of this study (10×10^6 sperm/ml in 500 µl and incubation in air), an increase in PY was observed (Fig. 7). We have previously found that this increase in PY was attributable to the increase in pH associated with incubation of bicarbonate-containing medium in air (e.g., at initial pH 7.25, the final pH of bicarbonate-containing medium after 4 h of incubation in air is 7.7–8.0 (Gonzalez-Fernandez *et al.* 2012)). Correspondingly, a significant increase in the percentage of PY-stained sperm was observed in this study in MW+++ when the initial medium pH was raised (Fig. 7). However, MW+++ at high pH was associated neither with cholesterol depletion nor with enhanced ROS production (Figs 3 and 6). This finding is notable, as Visconti *et al.* (1999*b*) found that, under standard conditions, PY in mouse sperm was dependent upon cholesterol efflux. Thus, the increase in PY found in equine sperm in response to high pH does not seem to reflect other ongoing capacitation-related changes. These results show that relationships among ROS production, PY, and cholesterol efflux during capacitation in equine sperm may be regulated differently than are those of other domestic species, in which these events are intimately related (Aitken & Nixon 2013). Supporting this theory, while standard capacitating medium under increasing pH induces equine sperm PY (McPartlin *et al.* 2008, Gonzalez-Fernandez *et al.* 2012), this was not associated with effective IVF (McPartlin *et al.* 2009). Overall, this suggests that PY induction is not a

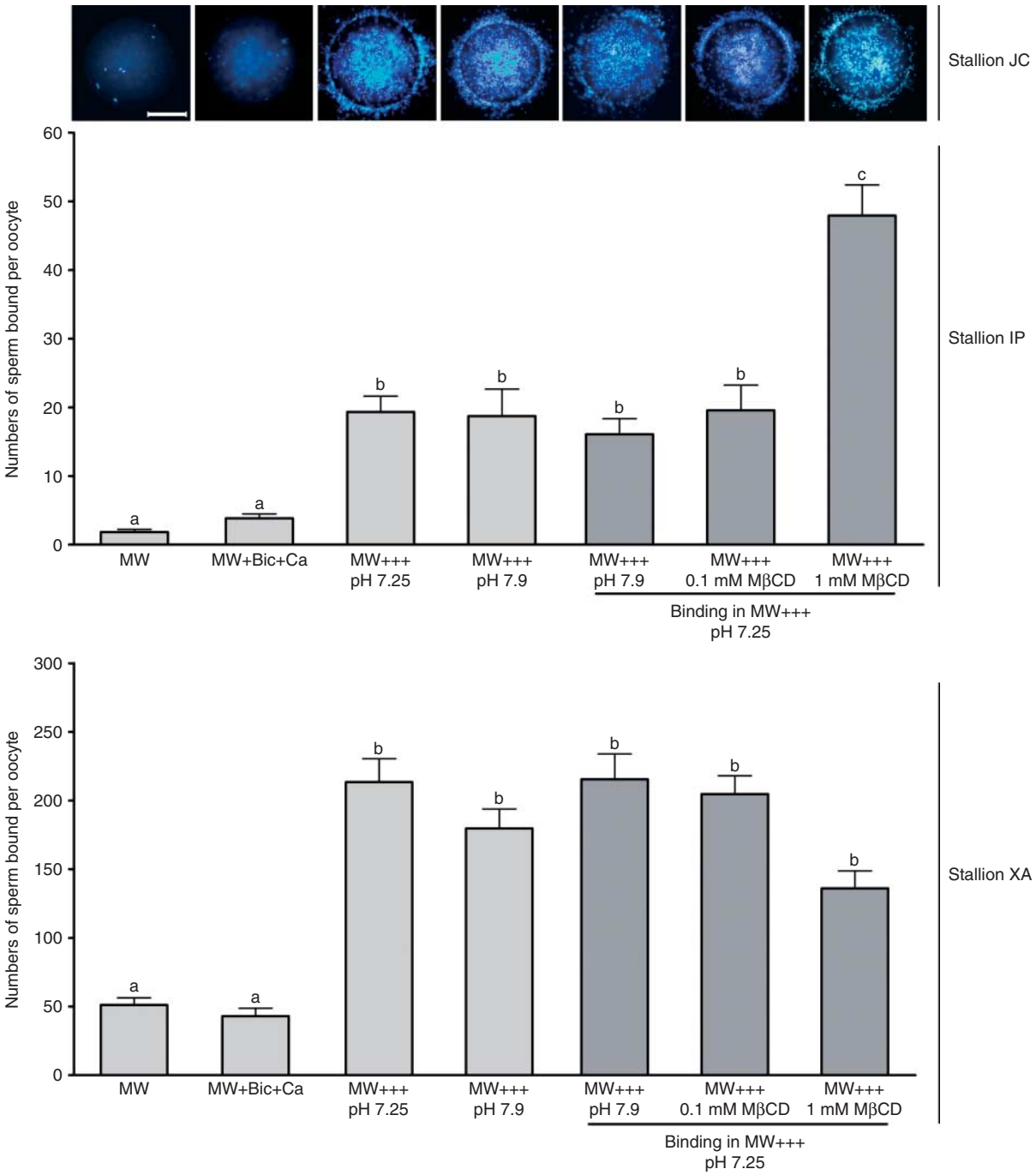

Figure 8 Number of equine sperm bound to bovine ZP. Equine sperm (1×10^6 sperm/ml) and bovine oocytes (five to seven per droplet) were co-incubated for 2 h after sperm capacitation. The values given represent the number of equine sperm bound to bovine ZP for stallion IP and XA; bars bearing different superscripts differ significantly ($P<0.05$); the error bars represent the S.E.M. The microphotographs are representative of the number of sperm bound to bovine ZP for stallion JC. A minimum of 24 oocytes were evaluated per treatment for each stallion. Sperm–oocyte complexes were stained using Hoechst 33342 and observed at 20× magnification; the bar represents a scale of 100 μm.

reliable indicator of capacitation (acquisition of fertilization ability) in equine sperm.

The only treatment that consistently induced a significant decrease in sperm cholesterol content was MW with MβCD at 1 and 3 mM (Figs 1 and 2). Incubation with MβCD has been shown to extract cholesterol from sperm plasmalemma of different mammalian species (Visconti *et al.* 1999*c*, Bromfield *et al.* 2014). Consistent with our findings, Boerke *et al.* (2013) found in porcine sperm that cholesterol efflux mediated by MβCD is ROS independent, unlike BSA-mediated cholesterol extraction, which requires the

formation of oxysterols. In addition, MβCD extracts cholesterol from the entire sperm membrane, while BSA does not affect cholesterol content of the detergent-resistant membrane fraction (Boerke *et al*. 2008). MβCD-induced cholesterol loss in porcine sperm was associated with an increase in PY (Boerke *et al*. 2013). By contrast, we found that, in equine sperm, MβCD-induced cholesterol efflux was not related to a significant increase in PY compared with MW alone (Fig. 7).

While our results show that BSA appears to be associated neither with cholesterol efflux nor with PY induction in equine sperm, it is possible that BSA has other, non-cholesterol-related effects that could potentiate capacitation in equine sperm, as BSA has been shown to increase sperm intracellular alkalinization (Zeng *et al*. 1996, Huang *et al*. 2005) and to trigger calcium influx through the CatSper ion channel in mouse sperm (Xia & Ren 2009). Incubation with BSA did increase binding of equine sperm to bovine ZP in our heterologous binding assay. The increase in ZP binding associated with the presence of BSA does not appear to be related to extraction of cholesterol from the sperm (Figs 1, 2, 3, and 4) or to a rise in ROS. Albumin causes equine sperm to become 'sticky', as presence of BSA is associated with sperm agglutination (Bromfield *et al*. 2014), and thus, the increase in zona binding may be non-specific. However, equine sperm–bovine ZP binding can be functional, as acrosome reaction and penetration through bovine ZP have been shown to take place (Sinowatz *et al*. 2003). In our experiments, we observed an individual variability among stallions in the number of sperm attached to bovine ZP, as described previously (Balao da Silva *et al*. 2013). Additionally, incubation with MβCD did not affect sperm binding in two stallions, but in one stallion (IP), incubation with 1 mM MβCD in MW+++ increased sperm binding over MW+++ alone. Only 0.1 and 1 mM MβCD were tested in our experiments due to a marked loss of motility of equine sperm when exposed to 3 mM MβCD; this effect was not related to a significant loss of sperm viability (membrane integrity), as determined by PI staining (Supplementary Figure 1, see section on supplementary data given at the end of this article). Interestingly, stallion IP had the lowest baseline sperm binding of the three stallions evaluated; it is possible that, perhaps due to different membrane compositions among stallions (Garcia *et al*. 2011), a too rigid membrane failed to express areas to which BSA could bind to cause 'stickiness'; the effect of MβCD in this stallion highlights the stallion-to-stallion variability found in other capacitation-related events such as PY (Gonzalez-Fernandez *et al*. 2012). Bromfield *et al*. (2014) reported that MβCD improved binding of equine sperm to bovine ZP; however, individual variability was not appreciated in that study, as the authors pooled ejaculates from three stallions.

In conclusion, our results show that neither serum albumin nor bicarbonate, either alone or in combination, is associated with cholesterol efflux from equine sperm. Bicarbonate alone induced a rise in ROS production and an increase in PY. Addition of calcium or BSA dramatically blunted ROS production, but not PY. Increasing pH induced an increase in PY that was not related to cholesterol efflux, increased ROS production, or improved sperm–ZP binding. To the best of our knowledge, this is the first report revealing that incubation with BSA is not associated with cholesterol efflux from equine sperm and that ROS production does not occur under standard capacitating conditions (presence of bicarbonate, calcium, and BSA). The addition of BSA increased the percentage of ZP binding in all stallions tested, independent of PY induction, ROS production, or cholesterol efflux. Thus, this study demonstrates apparent species-specific differences in conditions for sperm membrane cholesterol efflux and in the relationship between ROS and cholesterol efflux, and capacitation-related events for the first time. Our findings corroborate previous reports that equine sperm do not undergo functional capacitation under standard capacitating conditions, and bring into question the validity of PY as a parameter of functional capacitation in equine sperm. More work is required to understand the molecular changes undergone by equine sperm in preparation for fertilization, and to increase our understanding of the roles of the different medium components that may support a repeatable equine IVF protocol.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0457.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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