

—Original Article—

Hyaluronan improves neither the long-term storage nor the cryosurvival of liquid-stored CD44-bearing AI boar spermatozoa

Manuel ÁLVAREZ-RODRIGUEZ^{1)*}, Alejandro VICENTE-CARRILLO^{1)#} and Heriberto RODRIGUEZ-MARTINEZ¹⁾

¹⁾Department of Clinical and Experimental Medicine (IKE), BKH, Obstetrics and Gynecology, Faculty of Medicine & Health Sciences, Linköping University, SE-581 15 Linköping, Sweden

#Present: Evidensia Valla Djursjukhus Linköping, Linköping, Sweden

Abstract. Hyaluronan (hyaluronic acid, HA) apparently improves sperm survival *in vitro* and *in vivo* (oviduct), maintaining sperm motility and inducing capacitation, but not acrosome exocytosis, either by direct action as a macromolecule or via CD44 membrane receptors. This study explored ejaculated, liquid-extended pig spermatozoa to ascertain (i) the presence (Western blotting) and specific location (immunocytochemistry) of the CD44 receptor, using a specific monoclonal commercial antibody; (ii) whether the CD44 receptor changed location when exposed to bicarbonate, a capacitating trigger, *in vitro*; and (iii) whether the addition of HA, of molecular size comparable to that produced in the oviduct sperm reservoir (0.0625 to 2.0 mg/ml; 0 HA: control), to semen extenders would improve sperm liquid storage *in vitro* or cryosurvival post-freezing. Variables tested were sperm velocity and progressive motility (Qualisperm™), sperm viability and acrosome status, membrane integrity and early destabilization, mitochondrial activation, and superoxide production (flow cytometry). The CD44 receptor presence in ejaculated, liquid-stored AI boar spermatozoa, as confirmed by a porcine-specific monoclonal antibody, maintained its membrane location under *in vitro* capacitation-inducing conditions. HA exposure to 24-, 48-, or 72-h liquid-stored (17–20°C) spermatozoa lowered sperm velocity in membrane-intact spermatozoa, but increased mitochondrial superoxide production. Finally, HA addition during cooling did not improve cryosurvival but did increase mitochondrial activation and membrane destabilization in surviving cells. These results confirm the existence of a CD44 receptor in pig spermatozoa, but the usefulness of adding HA for long-term storage or cryopreservation of liquid-stored, extended boar semen remains in question, thereby warranting further non-empirical analyses of HA-sperm membrane interactions.

Key words: CD44 receptor, Hyaluronan, Spermatozoa

(J. Reprod. Dev. 64: 351–360, 2018)

Hyaluronan (hyaluronic acid, HA) is a ubiquitous non-sulfated glycosaminoglycan (GAG) built by an unbranched linear chain of repeating N-acetyl-D-glucosamine and D-glucuronic acid disaccharide units, which displays a surprising wide range of structural, physiological, and pathological roles, creating and filling up extracellular matrixes (See recent review by Rodriguez-Martinez *et al.* [1]). Hyaluronan is also present in genital organs and their secretions [2], including seminal plasma [3–6], the oviduct and oviduct fluid [7], and the space between the granulosa cells of newly ovulated cumulus-oocyte-complexes (COC, [8]). The connective tissue and the epithelia lining the lumen of the ductus epididymis, the seminal vesicle, the prostate, and the bulbourethral gland all contain HA, mostly at the apical membrane [4]. Moreover, HA was even localized in the luminal content of the sexual accessory glands, which confirms the origin of the HA present in seminal plasma [3, 5, 6]. *In vitro*, HA

improves sperm motility in a dose-dependent manner [9, 10]. It also prevents sperm capacitation of boar spermatozoa *in vitro* (as seen using a chlortetracycline [CTC] assay), without inducing acrosome exocytosis or cell death [11, 12], which is a desirable effect during IVF-conditions, considering the proven synergistic effect of the capacitating exogenous bicarbonate and HA [13, 14].

In the oviduct, HA has been studied in relation to the sperm reservoir established after insemination, a reservoir that is functional during the entire pre- and peri-ovulatory phase [15]. The concerted evidence from experimental findings, albeit without explanation of the underlying mechanisms, concluded that immersion in tubal fluid HA of intermediate length (0.12–1 × 10⁶ Da) specifically produced by the HA-synthase, Has-3, in the oviductal epithelial cells [16] was beneficial for sperm survival, perhaps simply by delaying the permissive capacitation process [17, 18].

Considering the above HA properties and the fact that the spermatozoon suffers membrane destabilization when semen is extended for the preparation of artificial insemination (AI) doses and further processed by the removal of decapacitating factors or the inclusion of bicarbonate [11, 12], HA has been tested as an additive to improve sperm survival. In highly extended, liquid-stored pig semen, the addition of HA at 50 and 100 µg/ml to the classical semen extender Beltsville Thawing Solution (BTS) seemed to delay sperm capacitation after 3 days of cooling [19]. Similar studies were done with cryopreserved semen,

Received: October 26, 2017

Accepted: May 17, 2018

Published online in J-STAGE: June 8, 2018

©2018 by the Society for Reproduction and Development

Correspondence: M Álvarez-Rodríguez (e-mail: manuel.alvarez-rodriguez@liu.se)

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

considering the stabilizing effects of HA on the plasma membrane [20] and its capacity as an antioxidant [21, 22]. Results were extremely variable, mostly due to the use of non-physiological dosages (well above the levels present in seminal plasma or oviductal fluid) and disparate experimental designs [23, 24]. Moreover, when we initially investigated the HA size and doses similar to those found in the porcine oviduct fluid, exogenous HA did not appear effective for cryosurvival after conventional freezing [25]. Yet, such HA effects may occur due to its direct action as a macromolecule, for instance by modifying the viscosity of the media. Alternatively, the effects could be issued via specific membrane receptors.

Hyaluronan modulates cell responses by acting as a ligand to specific HA membrane receptors [26], among which the transmembrane CD44 receptor is the most commonly recognized [27]. The CD44 receptor is present on most epithelial cells, including those of the pig pre-ovulatory oviductal sperm reservoir [16] as well as granulosa and cumulus cells [28]. The CD44 receptor acts both as an adhesion receptor for cell attachment on an HA substratum and as a mediator of the transduction of intracellular signals leading to changes in cell proliferation, survival, and differentiation [29]. Spermatozoa from human or bovine and possibly porcine species seem to depict a functionally active, HA-adhesive form of the CD44 receptor in their plasma membrane where HA would bind (See revision by Rodriguez-Martinez *et al.* [1] and references therein). However, although most cell types express the CD44 receptor, not all cells bind HA, which calls for further exploration of the presence and location of the CD44 receptor in pig spermatozoa, particularly in those extended for liquid-storage and used for AI or those eventually cryopreserved.

This study therefore used ejaculated, liquid-extended pig spermatozoa to investigate (i) the presence and specific location of the CD44 receptor using a specific monoclonal commercial antibody, (ii) whether the CD44 receptor changes location in response to the capacitating trigger bicarbonate *in vitro*, and (iii) whether the addition of HA to semen extenders at molecular size and doses comparable to those recorded *in vivo* would improve conventional sperm liquid storage in the 17–20°C temperature range for up to 72 h or cryosurvival using a conventional freezing method.

Materials and Methods

Reagents

All reagents were obtained from Sigma-Aldrich (Sweden), unless otherwise stated. For Western blotting (WB) and immunocytochemistry (ICC) analyses, the monoclonal antibody 60224-1-Ig and its specific blocking peptide CD44 fusion protein Ag7633 were purchased from Nordic BioSite (Proteintech Europe, Manchester, UK), while secondary antibodies were purchased from Abcam (Cambridge, UK) or LI-COR Biosciences (Lincoln, NE, USA). Fluorochromes for flow cytometry, excluding Hoechst 33342 (H33342, Sigma-Aldrich, Sweden), that were used in all staining combinations to define DNA-containing events and discard debris were obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA), including YO-PRO-1 iodide (YO-PRO-1), PNA-Alexa Fluor 488 (*Arachis hypogaea* lectin (peanut)), propidium iodide (PI), MitoTracker Deep Red (MIT), and MitoSOX Red (MSOX).

Sources of spermatozoa

Ejaculated pig spermatozoa were obtained as commercial AI doses (12 batches) from Quality Genetics, Sweden, extended to 2.5×10^9 total spermatozoa/dose, with each batch built as pools of ejaculates from 3 boars of proven fertility, extended in Durasperm™ [Jørgen Kruuse A/S, Langeskov, Denmark]), and stored at the 17–20°C temperature range recommended for AI purposes. All experiments were performed at the Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden in accordance with the relevant animal research regulations of the European Union (EC-Directive 2010/63/EU) and in compliance with current Swedish legislation (SJVFS 2015:24). The experimental protocol, including postmortem collection of pig seminal vesicles, had been previously reviewed and approved by the Local Ethical Committee for Experimentation with Animals at Linköping, Sweden (permit nr 74-12 and nr ID-1400).

Ejaculated human spermatozoa, used as a positive control for detection of the CD44 receptor, were obtained from anonymous, fertile donors after obtaining informed written consent at the Reproductive Medicine Center (RMC), Region Östergötland, Linköping, Sweden (Ethical Permission Number Dnr 2015/387-31).

Experimental design

Ejaculated, AI extended pig spermatozoa ($n = 12$ batches) were studied by WB of isolated sperm membrane proteins for the presence of the CD44 receptor, while ICC at a light microscopy level was used to specifically locate the CD44 receptor in the plasma membrane of intact spermatozoa. Human spermatozoa, subjected to gradient density cleansing (PureSperm, Nidacon, Göteborg, Sweden), and proteins from boar seminal vesicles were used as positive control for the presence of the somatic CD44 receptor [30]. The liquid-stored boar spermatozoa were further exposed to capacitation-inducing conditions *in vitro* (high concentrations of the capacitating trigger bicarbonate) to determine the eventual changes in the location of the CD44 receptor according to WB and ICC. Moreover, the extended spermatozoa were kept at 17–20°C for up to 72 h and challenged *in vitro* by the agonist HA at concentrations ranging from 0.0625 to 2 mg/ml, at 38°C for 60 min with 24-, 48-, or 72-h storage. The same HA dosages enriched a lactose-egg yolk extender (LEY) used for the conventional chilling and freezing of 72-h liquid-stored spermatozoa. Sperm velocity, progressive motility, viability, membrane intactness and destabilization, acrosome integrity, mitochondrial activation, and superoxide production were assessed after 10, 30, or 60 min of *in vitro* incubation or at 30 min post-thawing at 38°C.

CD44 receptor presence and location

Western blot: Sperm and seminal vesicle proteins were extracted by incubating the spermatozoa and portions of the seminal vesicle gland in RIPA buffer (Sigma-Aldrich) at 4°C for 40 min. The extracted samples were centrifuged at $13,000 \times g$ for 10 min, and the supernatant was collected. Proteins were quantified using a DC Protein assay kit (Bio Rad, Hercules, CA, USA), following manufacturer instructions. Protein suspensions (2.5 µg protein/µl) were denatured by heating at 70°C for 10 min. Aliquots (10 µl) of each protein suspension were loaded into NuPAGE 4–12% Bis-Tris SDS-PAGE gels (Life Technologies, Carlsbad, CA, USA). Electrophoresis was

performed at 180 V for 90 min, followed by transfer of the proteins to polyvinylidene difluoride (PVDF) membranes (Invitrolon PVDF filter paper sandwich, Life Technologies) at 125 mA for 90 min. The membranes were blocked at room temperature for 60 min with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA, USA) containing 0.1% Tween-20 (Sigma-Aldrich) (PBST). After three washes of 10 min in PBST, one membrane was incubated at 4°C overnight with the primary monoclonal (1:500 dilution, anti-CD44 antibody 60224-1-Ig; mouse monoclonal antibody to CD44; species specificity: pig; Nordic BioSite, Proteintech Europe, Manchester, UK). The specificity of the monoclonal antibody was tested in another membrane by previous co-incubation (RT with agitation for 30 min) of the primary antibody in excess presence (1:5 ratio) of its specific blocking peptide (CD44 Fusion Protein Ag7633, Nordic BioSite, Proteintech Europe, Manchester, UK). Positive controls were provided by human spermatozoa and pig seminal vesicles (somatic control). The day after, the membrane was washed 3 times in PBST and incubated for 60 min with a dilution 1:15,000 of the secondary antibody (goat anti-mouse IRDye 800 CW (925-32210, LI-COR Biosciences, Lincoln, NE, USA)) followed by extensive washing in PBST. The membranes were scanned using the Odyssey CLx (LI-COR Biosciences, Lincoln, NE, USA), and images of the blots were obtained using the Image Studio 4.0 software (LI-COR Biosciences, Lincoln, NE, USA).

Immunocytochemistry

Human (positive control) and porcine spermatozoa were fixed in 4% paraformaldehyde at room temperature (r.t.) for 20 min. Cell suspensions were then centrifuged at $1,200 \times g$ for 6 min, and the pellet was resuspended in PBS, pH 7.3 to prepare smears on poly-L-lysine slides (LSM, Thermo Scientific, Germany). The smears were allowed to dry at r.t., washed 3 times for 5 min each with PBS, and blocked with 5% BSA in PBS at 4°C for 120 min. After three washes in PBS for 5 min each, the slides were incubated at 4°C overnight with the primary monoclonal antibody (see WB protocol) diluted 1:100 in 1% BSA-PBS. The smears were then washed 3 times in PBS for 5 min each before incubation in the dark at room temperature for 75 min with a 1:1,000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG H&L (ab150113, Abcam, Cambridge, UK) in 1% BSA-PBS. The smears were washed extensively and then mounted with Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA, USA). Negative controls were performed using the blocking peptide (CD44 Fusion Protein Ag7633, Nordic BioSite, Proteintech Europe, Manchester, UK), as described in the WB protocol. Results were collected using a Zeiss Axioscop microscope (Zeiss, Germany) equipped with an epifluorescence module and a CCD Camera (QIMAGING, Canada) at $1,000 \times$ magnification. At least 200 cells were counted per replicate. Immunolabeling was present in > 95% of the spermatozoa and absent in the negative controls.

Distribution of the CD44 receptor in pig spermatozoa before and after induced capacitation

Boar spermatozoa from the original AI-dose sperm suspension (see above) were re-extended in modified BTS (without the calcium chelator EDTA but with 35 mM NaHCO₃) and incubated at 38°C

for 30 min to induce sperm capacitation [31]. The presence and distribution of the CD44 receptor was assessed by WB and ICC before and after incubations with 35 mM NaHCO₃.

Challenge to hyaluronan in vitro

A stock solution of 4 mg/ml of hyaluronan (HA) with an intermediate molecular size ($0.12-1 \times 10^6$ Da, Sigma-Aldrich Cat nr 53747), which corresponded to the size of the HA produced *in vivo* by the oviductal epithelial lining of the female pig sperm reservoir, [16] was made in an extender (Durasperm™), kept frozen (-20°C), and thawed immediately before use. Six 1:2 serial dilutions of HA (4, 2, 1, 0.5, 0.25, and 0.125 mg/ml) combined for a final volume of 500 ml. The osmolality of the media with or without (control) the serial concentrations of HA was measured using a Fiske® Micro Osmometer, Model 210 (Fiske associates; Norwood, Massachusetts, USA). Subsequently, 500 µl of a suspension of 75–98% of viable spermatozoa (48×10^6 spermatozoa/ml) were added to 500 µl of each dilution or the extender only (control) and then carefully mixed to reach a working sperm concentration of 24×10^6 spermatozoa/ml. The final HA concentrations were 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/ml. The HA-supplemented sperm suspensions were placed on a shaking plate inside an incubator at 38°C, and 2 aliquots were taken per time-point for analyses at 10, 30, and 60 min: 25 µl for flow cytometry and 10 µl for sperm velocity/progressive motility analyses. The exogenous HA did not significantly modify the osmolality of the media used to incubate spermatozoa ranging from 333 mOsm/l in the control (no HA) to 344 mOsm/l in the highest HA concentration.

Sperm freezing with an HA-enriched LEY extender

Spermatozoa were further extended after 72 h of AI-dose preparation, using a conventional LEY extender (Lactose 310 mM-20% egg yolk) enriched with HA (cat. nr 53747, Sigma-Aldrich) in dosages starting at 2.0 (HA2) mg/ml or following 50% decreasing concentration steps ((1.0 (HA1), 0.5 (HA05), 0.250 (HA0250), 0.125 (H0125), 0.0625 (HA0625)) until 0 (HA0, control). All samples were slowly cooled ($\approx -0.25^\circ\text{C}/\text{min}$ to $+5^\circ\text{C}$) before being mixed 2:1 with the LEYGO freezing extender (LEY + 3% glycerol + 1.5% Orvus WA (963-1000, Preservation Equipment, UK)) to reach a final concentration of 1×10^9 sperm/ml. Spermatozoa were then packaged at $+5^\circ\text{C}$ in 0.5 ml plastic straws, and frozen in a programmable bio-freezer (IceCube 14 M-A; Minitube International, Tiefenbach, Germany) using the following curve: $-6^\circ\text{C}/\text{min}$ from $+5^\circ\text{C}$ to -5°C , holding for crystallization 1 min at -5°C , followed by $-60^\circ\text{C}/\text{min}$ from -5°C to -140°C . The straws were then plunged into liquid nitrogen (LN₂, -196°C) for a minimum of 1 week of storage until thawing for analyses. The straws were thawed in circulating water at 38°C for 15 sec and directly extended 1:4 in pre-warmed (38°C) BTS until analyses.

Sperm assessment

Sperm velocity and progressive motility: Sperm velocity ($\mu\text{m}/\text{sec}$) and proportions of progressive sperm motility were assessed using an upright Zeiss Axio Scope A1 light microscope equipped with a $10 \times$ phase contrast objective (Carl Zeiss, Stockholm, Sweden) connected via a CMOS camera (UEye, IDS Imaging Development Systems GmbH, Ubersulm, Germany) to a computer holding the

Qualisperm™ sperm analysis software (Biophos SA, Lausanne, Switzerland). Semen droplets (24×10^4 sperm in $10 \mu\text{l}$) were placed on a pre-warmed Menzel-Gläser pre-cleaned microscope slide (size: $76 \times 26 \text{ mm}$; ThermoFisher Scientific, Waltham, MA, USA) covered by a pre-warmed coverslip (size: $18 \times 18 \text{ mm}$; VWR, Stockholm, Sweden), on a thermal plate (Temp Controller 2000-2, Pecon GmbH, Erbach, Germany) kept at 38°C . The Qualisperm™ technology is based on fluorescence correlation spectroscopy analysis of single particles (sperm) in confocal volume elements, yielding a regression fluctuation algorithm. Individual sperm are projected on a pixel grid of the CMOS camera, and the algorithm calculates the number of fluctuations in each pixel by correlation function of the sperm numbers and translation classes. From the correlation function, the speed (velocity) distribution is determined as a primary variable, and recalculations of the proportion of spermatozoa depicting progressive sperm motility are thereafter obtained. This system benefits from a high-throughput screen (usually 4 fields per minute), analyzing $> 2,000$ sperm/field, and has been validated for several species, including porcine [32].

Flow cytometry

Stock solutions of the fluorochromes were prepared in milliQ water (PI and H33342) and in Dimethyl sulfoxide (DMSO, SIGMA-ALDRICH, Sweden) (YO-PRO-1, MIT, MSOX). All stock solutions were kept at -20°C (except when kept at $+4^\circ\text{C}$ for H33342) and brought to RT immediately before diluting them in BTS for its use. Working solutions of the fluorochromes were prepared in BTS: $4.5 \mu\text{M}$ H33342, $1 \mu\text{g/ml}$ PNA-AlexaFluor488, $2.4 \mu\text{M}$ PI, 200 nM YO-PRO-1, 100 nM MIT, and $5 \mu\text{M}$ MSOX. Sperm suspensions (1×10^6 sperm/ml) were analyzed in a Gallios™ flow cytometer (Beckman Coulter, Bromma, Sweden) equipped with standard optics: a violet laser (405 nm) in 2 colors, argon laser (488 nm) in 5 colors, and HeNe-laser (633 nm) in 3 colors. The filter configuration was as follows: Blue: FL1 550SP 525BP (YO-PRO-1, PNA-Alexa Fluor 488, CellROX), FL2 595SP 575BP (MSOX), FL3 655SP 620/30 (PI), FL4 730SP 695/30 - alt 675BP, FL5 755LP; Red: FL6 710SP 660BP (MIT), FL7 750SP 725/20, FL8 755LP; Violet: FL9 480SP 450/50 (H33342), FL10 550/40. The instrument was controlled via Navios software (Beckman Coulter, Bromma, Sweden). Analyses of acquired data were performed using the Kaluza software (Beckman Coulter, Bromma, Sweden) on a separate PC. In all cases, we assessed 25,000 events per sample, with a flow rate of 500 cells/sec.

The intactness of the plasma membrane integrity was assessed using YO-PRO-1 and PI to yield the percentage of viable spermatozoa with an intact membrane (*PI- YO-PRO-*) and spermatozoa that were viable but showed early destabilization changes in the plasma membrane (*PI- YO-PRO+*). Acrosome intactness was assessed in viable spermatozoa with a triple stain: PNA-AlexaFluor488, PI, and H33342 (*PI- PNA-AlexaFluor488-*). Mitotracker Deep Red (MIT) was used to address the mitochondrial status, yielding the proportion of viable sperm with active (*PI- MIT+*) or inactive mitochondria (*PI- MIT-*). The MitoSOX mitochondrial superoxide indicator was used to yield the proportion of viable spermatozoa producing superoxides (*PI- MSOX+*).

Statistical analysis

The variables collected were statistically analyzed in the R

statistical environment (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0). Results are shown as means and standard error of the mean (SEM), unless otherwise stated. Normal distribution and homoscedasticity of the data were analyzed using analytical tests, and further analyses of the post-thawing data were carried out using linear mixed-effects models, with the arcsin transformation of data in cases of non-normal distribution (total and progressive motility). Two models were built, with the type of extender as the fixed effect and ejaculated sperm as the random variable. Significant fixed effects were further analyzed using multiple comparisons of means with Tukey contrasts.

Results

The CD44 receptor was present in boar spermatozoa

The WB analysis was performed to enable the specific detection of the full-sized CD44 receptor using the specific porcine monoclonal anti-CD44 antibody 60224-1-Ig (Fig. 1A). A conspicuous band was detected at 85 kDa in both human (Lane 2) and porcine spermatozoa; the latter was either uncapacitated (Lane 3) or following induction of capacitation *in vitro* (Lane 4), as well as in the somatic control (seminal vesicle, Lane 5). This CD44 receptor band was removed by treatment with the specific blocking peptide CD44 fusion protein Ag7633 (Fig. 1B, Lanes 1–5), thus confirming the specificity of the antibody 60224-1-Ig and the presence of a full-sized CD44 receptor in porcine spermatozoa.

On ICC, porcine spermatozoa depicted the CD44 receptor on the plasma membrane over the post-acrosomal region, neck, and midpiece (Fig. 2B–C). Human spermatozoa showed clear CD44 receptor-immunolabeling over the post-acrosome area of the sperm head (Fig. 2A). Negative controls by treatment with the specific blocking peptide CD44 fusion protein Ag7633 (inserts A'–C') were clearly lacking immune labeling (Fig. 2A–C).

The CD44 receptor maintained boar sperm head localization after bicarbonate-induced capacitation

The induction of capacitation in pig spermatozoa modified neither the presence (WB, Fig. 1A, compare Lanes 3 and 4) nor the distribution (ICC) of the CD44 receptor in the membrane of boar spermatozoa (Fig. 2B–C), although the labeling was more intense in the non-capacitated spermatozoa.

In vitro, HA did not affect membrane intactness but decreased sperm velocity and progressive motility and mitochondrial activity, leading to a clear increase in superoxide production over time

The mean results of the HA challenge *in vitro* (10 – 60 min , 0.0625 to 2.0 mg/ml) are expressed as the deviation from baseline (Control: 0) following the incubation of AI doses with different storage times (for 24, 48, or 72 h) and depicted in Fig. 3A–F. Supplementary data (Mean \pm S.E.M) are presented in Supplementary Table 1 (online only).

Sperm velocity decreased for all incubation times, with the lowest velocity registering when exposed to 2 mg/ml HA (HA2). In contrast, after 30 min of incubation, HA0.125 increased in velocity relative to control levels. Storage time (24 – 72 h) did not appear to affect the results (Fig. 3A). In general, the lower concentration of

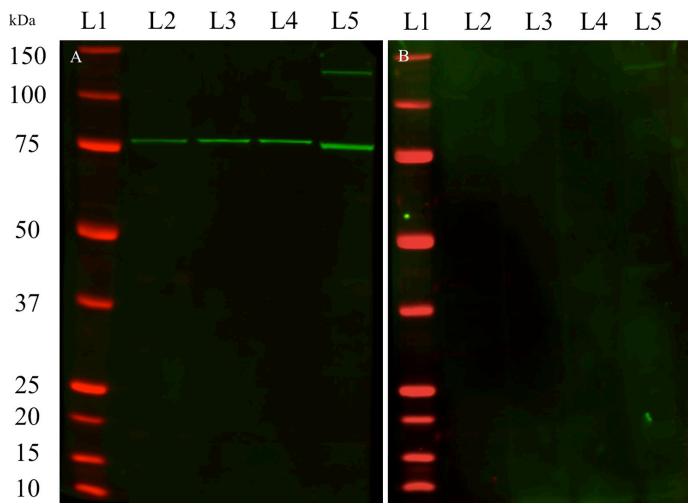


Fig. 1. Detection of the CD44 receptor by Western blotting (WB) in human and pig spermatozoa. In A, using a porcine-specific anti-CD44 monoclonal antibody (60224-1-Ig, Nordic BioSite, Proteintech), the CD-44 receptor was detected in pig and human spermatozoa at 85 kDa. Gel B depicts co-incubation of this monoclonal primary antibody with its specific blocking peptide (Ag7633, Nordic BioSite, Proteintech Europe, Manchester, UK), which eliminated the 85 kDa band of the full-sized CD-44 receptor in all cells/tissues. L1: ladder, L2: human, L3: non-capacitated boar spermatozoa, L4: capacitated boar spermatozoa, L5: boar seminal vesicle (somatic positive control).

HA (0.065 mg/ml) induced a decrease in progressive motility, an effect that was also observed for the higher concentration of HA (Supplementary Table 1).

HA exposure (HA0.125, HA0.5, and HA1) significantly maintained membrane integrity, as observed in as little as 10 min (Fig. 3C). Over time, this positive effect of HA 0.125 persisted for up to 30 min, as compared to controls. Sperm storage time had an effect on the results, with the older spermatozoa (72 h post-initial extension) not being positively affected by HA, irrespective of dose. Membrane destabilization was not affected by low HA doses, but was evidently increased by higher doses. Notably, the oldest spermatozoa (72 h) were less protected by the HA (Fig. 3D). Acrosome integrity was better maintained by the lower HA dosages, irrespective of sperm age (Fig. 3B). HA exposure was unable to maintain the proportions of activated mitochondria, an effect that was not modified by the sperm age or the length of exposure (Fig. 3E). HA-exposed spermatozoa showed a significant increase (highest with HA2) in mitochondrial superoxide production across the incubation times and irrespective of sperm age (Fig. 3F). All parameters were statistically correlated after incubation ($P < 0.001$) (Supplementary Table 2: online only).

Enrichment of LEY extender with HA (0.0625 to 2.0 mg/ml) did not improve the cryosurvival of boar AI semen doses liquid-stored for 72 h, but it appeared to be protective for membrane intactness

Spermatozoa stored at 17–20°C for 72 h, $83 \pm 3.7\%$ of which were motile (mean \pm SEM), were used for the cryopreservation trial. Cryosurvival in the controls reached $21 \pm 5.4\%$ of motile

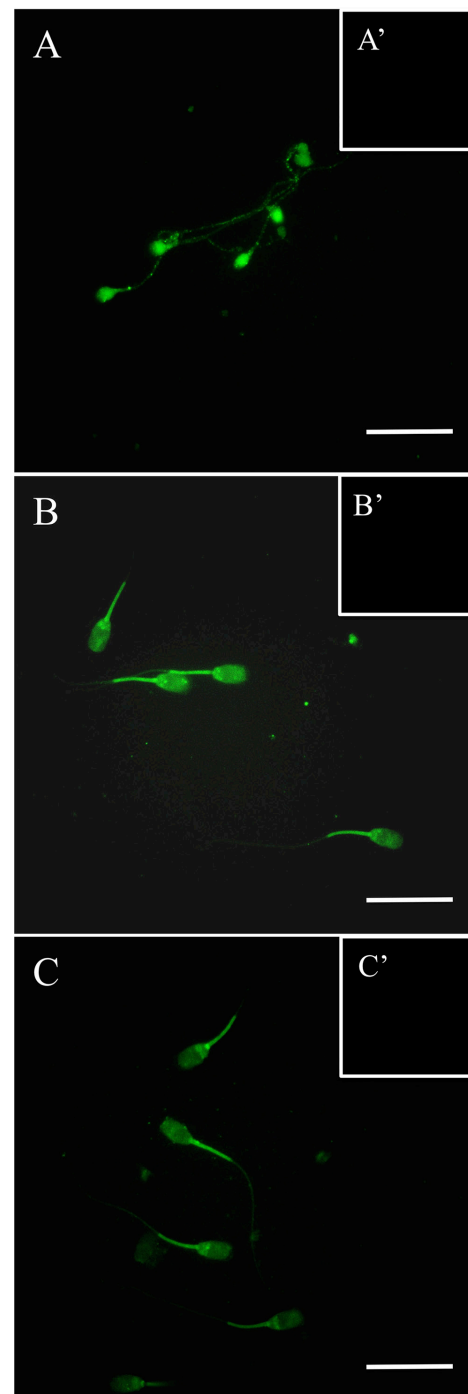


Fig. 2. Localization of the CD44 receptor on immunocytochemistry (ICC) analysis in human (A) and pig (B: uncapacitated, C: capacitated) spermatozoa using a monoclonal antibody (60224-1-Ig, Nordic BioSite, Proteintech Europe, Manchester, UK). The insets in the top-right corner in each figure (marked A'–C') depict ICC negative control images (where the antibody was neutralized by treatment with the specific blocking peptide CD44 fusion protein Ag7633 (Nordic BioSite, Proteintech Europe, Manchester, UK)). Human spermatozoa (positive control) had clear CD44-immunostaining over the post-acrosome area of the sperm head (Fig. 2A). In pig spermatozoa (Fig. 2B; 2C), the CD44 receptor appeared consistently localized on the plasma membrane over the post-acrosomal region, neck, and midpiece. Confocal laser scanning microscopy, scale bar: 10 μ m.

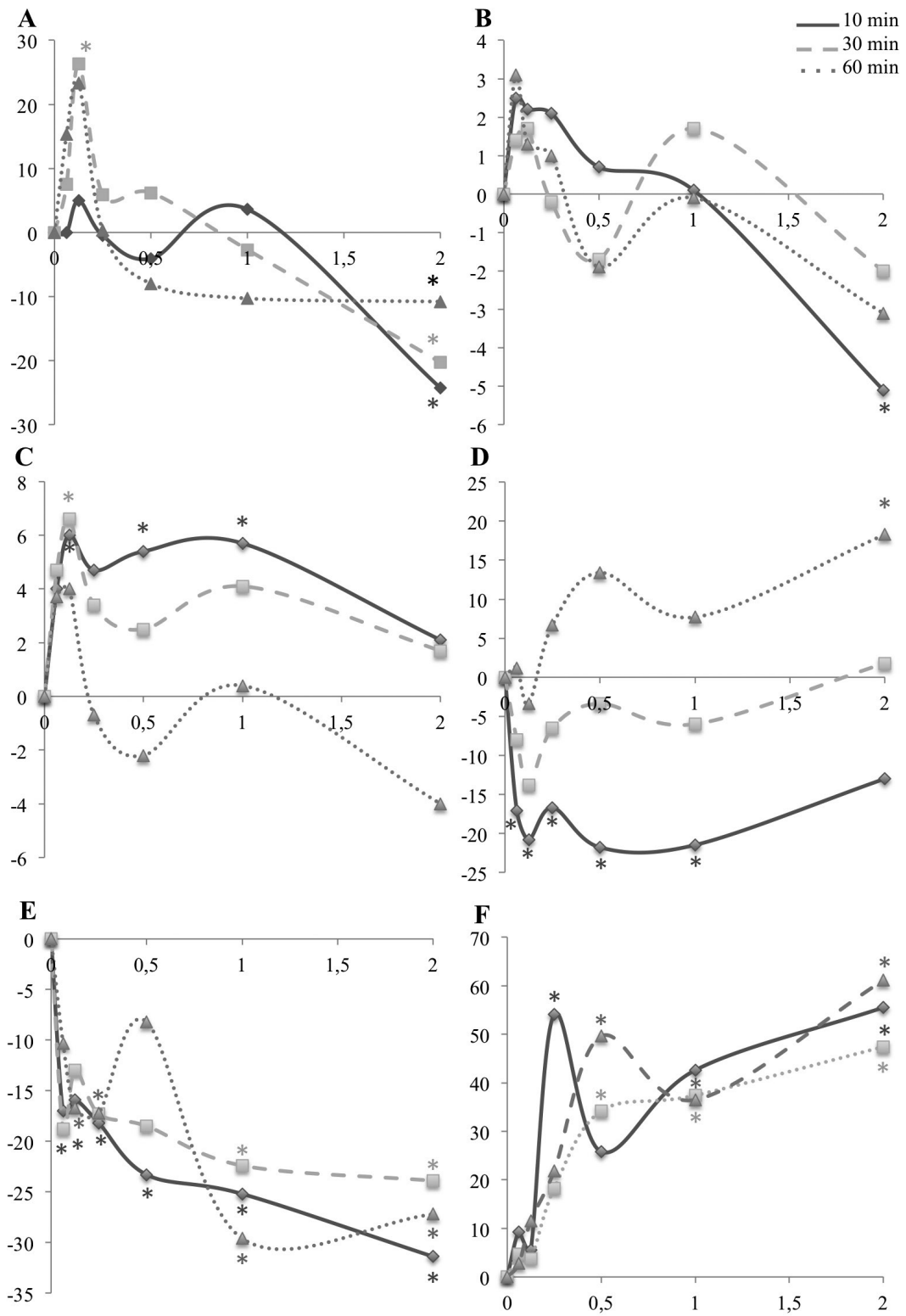


Fig. 3. Dose effects, expressed as a deviation from baseline (0%, control), of hyaluronan (HA) addition (0.0625 to 2.0 mg/ml, 0 HA: Control) on pig sperm velocity (A, µm/sec) and the percentages of viable spermatozoa with intact acrosomes (B, H33342/PI/PNA-AlexaFuor488), with intact plasma membrane (C, YO-PRO-1-/PI-), depicting early membrane destabilization changes (D, YO-PRO-1+/PI-), having active mitochondria (E, PI-/MIT+), or showing mitochondrial superoxide production (F, PI-MSOX+) when incubated for 10, 30, or 60 min at 38°C; n = 9 batches. Incub: incubation time. * Indicates differences (P < 0.05), relative to the control, for each parameter and incubation time.

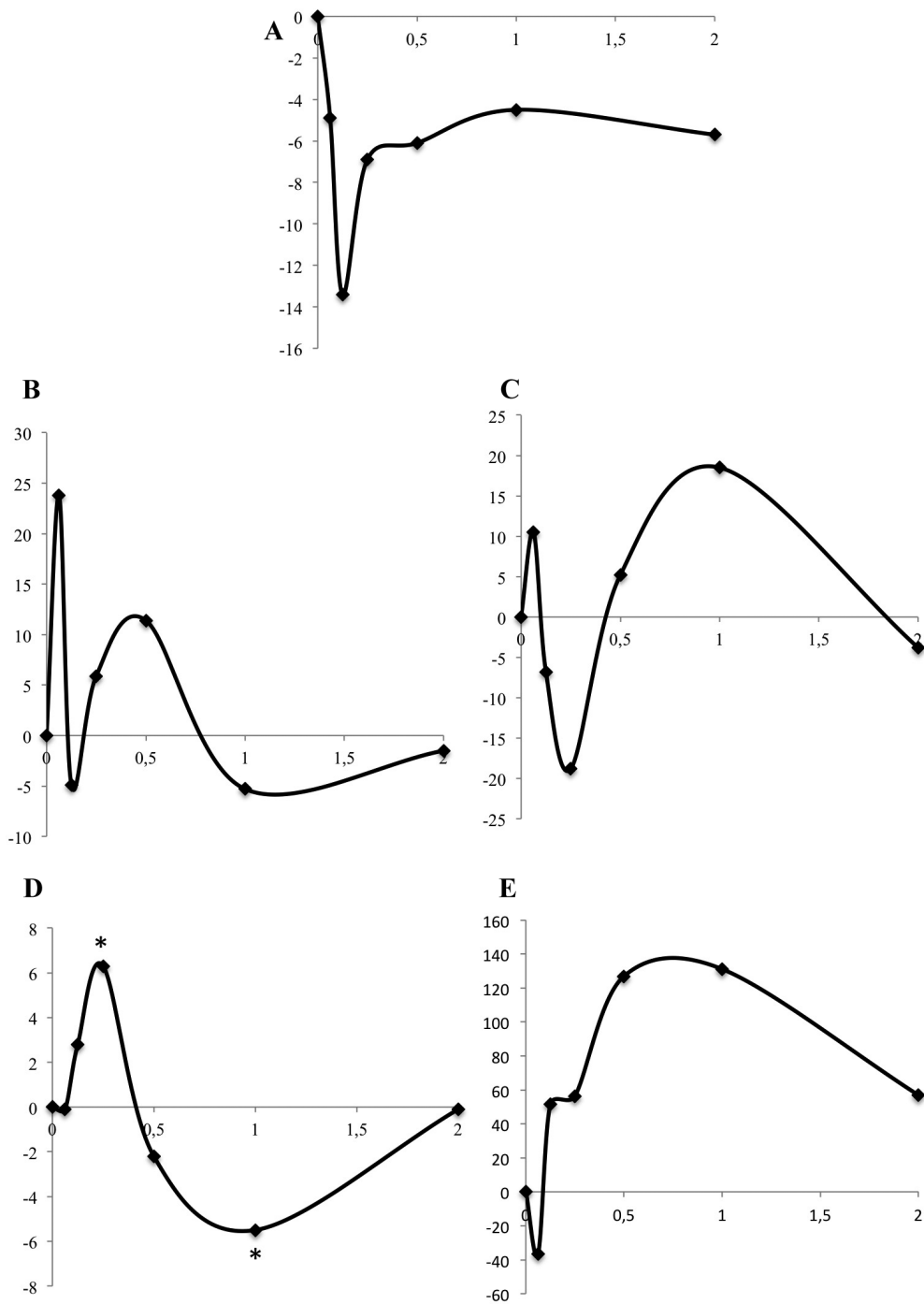


Fig. 4. Post-thaw effects of hyaluronan (HA), expressed as a deviation from baseline (0%, control) added to the pre-freezing LEY extender (0.0625 to 2.0 mg/ml, 0 HA: control) to 72-h extended spermatozoa on sperm velocity (A, $\mu\text{m}/\text{sec}$) and the percentages of viable spermatozoa with intact acrosomes (B, H33342/PI/PNA-AlexaFuor488), with intact plasma membrane (C, YO-PRO-1-/PI-), depicting early membrane destabilization changes (D, YO-PRO-1+/PI-), or having active mitochondria (E, PI-/MIT+), incubated at 38°C for 30 min; n = 9 batches. Results are shown as means. * Indicates differences ($P < 0.05$), relative to the control, for each parameter and incubation time.

spermatozoa. HA enrichment of the LEY extender during cooling did not improve sperm velocity, which actually decreased compared to controls (Fig. 4A). Membrane integrity was increased with medium

HA doses (0.5–1.0 mg/ml), but was not protected by exposure to 0.125–0.25 mg/ml HA concentrations nor the highest dosages (2 mg/ml) (Fig. 4C). These findings were mirrored when membrane

destabilization was assessed (Fig. 4D): a medium HA dose (1.0 mg/ml) was the most protective of ($p < 0.05$) the sperm membranes of surviving spermatozoa (Fig. 4C, D). Acrosome integrity had variable results with HA higher than 0.5 mg/ml (Fig. 4B). On the other hand, the mitochondria of the surviving spermatozoa that were chilled in the presence of HA (irrespective of dose) showed an apparent, but not significant, increase in mitochondrial activation (Fig. 4E). Interestingly, all sperm that were alive after cryopreservation were positive for mitochondrial superoxide production, with 100% of the living spermatozoa presenting a high level of superoxide production in the mitochondria, irrespective of the experimental group. Among all the post-thaw variables, only membrane integrity and membrane destabilization, both analyzed using a YO-PRO-1 staining-based method, correlated significantly ($P < 0.001$) (Supplementary Table 3: online only).

Discussion

The results of the present study, using a porcine-specific monoclonal anti-CD44 receptor antibody, confirmed that a full-sized CD44 receptor is present in the membrane of extended and liquid-stored boar ejaculated spermatozoa as well as in human spermatozoa [33]. The porcine location was maintained under capacitation-inducing conditions *in vitro* (high bicarbonate levels) but at a presumably lower intensity of immunostaining. A 60-min-long exposure of membrane-intact spermatozoa stored for 24, 48, or 72 h at 17–20°C to HA of intermediate molecular size lowered their velocity and progressive motility and increased their production of mitochondrial superoxide. The addition of HA to liquid-stored spermatozoa for 72 h during chilling using the LEY extender did not improve their cryosurvival. The spermatozoa that survived the freezing-thawing process showed increased mitochondrial activation and membrane destabilization, thereby putting the usefulness of exogenous HA for long-term storage or cryopreservation of liquid-stored, extended boar semen into question.

The macromolecule HA, a simple, negatively charged polysaccharide GAG, has been isolated and identified in the seminal plasma of several species [3–6] as well as in the cervix, the oviductal reservoirs, and the cumulus cells surrounding the oocyte [4, 7, 8]. Owing to this presence, purely empirical trials have been performed to investigate a pre-considered beneficial effect of exogenous HA on the long-term storage of extended, liquid semen [19] or the prevention of the deleterious effects of cryopreservation, which are notably evident in pig semen [23]. However, these studies did not solve the matter of whether the effect obtained was caused by the physical properties of the macromolecule (for instance changing the viscosity of the medium) or by a specific signaling with mediated stimulation of any of the specific HA receptors already determined to exist in nature [26]. Among the 4 well described HA receptors (LYVE-1: lymphatic vessel endothelial receptor 1; RHAMM: receptor for hyaluronan-mediated motility; TLR-4: toll-like receptor-4, and CD44 receptor: antigen, a type of transmembrane glycoprotein), the CD44 receptor is present on most epithelial cells including the pig pre-ovulatory functional sperm reservoir [16], granulosa and cumulus cells [28], as well as in spermatozoa [34], as CD44 receptor.

Boar semen designated for AI breeding is highly extended for

liquid storage for 24–72 h (as with BTS). The semen intended for cryopreservation/storage uses either the liquid-stored semen or the extended ejaculate, when the laboratories for freezing the semen are located far away from the boars. In either case, the spermatozoa are deprived of the low concentrations of hyaluronan present in the seminal plasma, which is either diluted or even eliminated by centrifugation. However, the inseminated spermatozoa (either liquid-stored or post-thawed) still encounter the HA of intermediate molecular size ($0.12\text{--}1 \times 10^6$ Da, which specifically corresponds to the one used in this study) that is present in the lumen of the oviductal sperm reservoir of the inseminated female [7]. This HA is produced by the HA-synthase, Has-3, in the sperm reservoir of the pig [16].

Since the expected results of trials using varying amounts, molecular weights, and sources of exogenous HA as salts (hyaluronate or hyaluronic acid) were based on its properties as an extracellular macromolecule, it is not surprising that the results varied significantly among trials, with either no effect [25] or positive effects [20–22]. Clearly, the use of supra-physiological doses, well above those found in the oviduct fluid or the seminal plasma further complicated the interpretation of results, particularly in light of the fact that the proportions found in these fluids where spermatozoa bathe during sperm transport are rather low [35]. If HA is to be efficient *in vivo* (or *in vitro*), it should hypothetically act via the HA receptors at the sperm plasma membrane.

The present study is, to the best of our knowledge, the first to determine the location of the full-sized HA-CD44 receptor in specific domains of the plasmalemma of boar spermatozoa. This study also indicates that the induction of sperm capacitation by exposure to high levels of sodium bicarbonate, a well-known destabilizer of the lipids in pig sperm membranes, did not modify the location of the CD44 receptor, although it may decrease the intensity of the immunostaining. Exogenous HA of physiological molecular size and at doses similar to those found in fluids where spermatozoa are present (as in tubal fluid or the oocyte cumulus vestment) has been proven to prevent sperm capacitation in pigs [19] without leading to the exocytosis of the acrosome [12]. Noteworthy, each of the cells that is in contact with the HA, whether epithelial or spermatozoa, contains the CD44 receptor, which is the most relevant HA receptor [16, 34].

Seminal plasma causes a transient inflammatory reaction in the cervix and uterus of pigs (and other species, including humans) that serves to cleanse the lumen of foreign spermatozoa and other accompanying cells (as in leukocytes or epithelial cells), microorganisms, and seminal plasma proteins [36]. Notably, spermatozoa residing in the oviductal sperm reservoir, a site of ubiquitous, active production of HA by the epithelium, are not attacked by the female immune system [15, 18, 36]. This seemingly protective mechanism could be related to the non-antigenic nature of HA. As a macromolecule, HA may simply act like a “covering cloud”, allowing spermatozoa to remain “undetectable” by the immune system of the female. However, other mechanisms may be present, considering that implants delivering HA induce the production of the anti-inflammatory cytokine IL-10 by the female genitals [37]. This likely HA-mediated immunological interplay that is initiated by the deposition of semen may help to unveil the mechanisms by which the female immune system tolerates foreign cells throughout pregnancy, including the trophoblast and the placenta.

The hyaluronan-binding method, commonly used for selection of “sperm maturity” *in vitro*, results in the selection of a sub-population of spermatozoa with intact chromatin [38], irrespective of their morphology [39]. Whether this “sperm maturity” status is only related to the presence of the CD44 receptor, which enables the recognition of HA, remains to be explored. Thus, the present study sought to determine if a range of quasi-physiological dosages of well-described, commercial HA could affect the attributes of long-term (24–72 h) stored ejaculated spermatozoa, prepared as AI-doses of liquid semen. Hyaluronic acid in concentrations between 0.05 and 0.1 mg/ml has apparently delayed sperm capacitation during the long storage of liquid boar semen at 15°C [19], although the effects were not evaluated as to whether they were caused by binding to the CD44 receptor or were merely a physical action of the HA macromolecule. Our results are in agreement, although we obtained higher values of sperm velocity and membrane integrity after incubation with 0.125 mg/ml at 38°C, up to 60 min, which are conditions that should have mimicked those during sperm transport/pre-fertilization within a female. Concentrations higher than 0.5 mg/ml have proven to cause a significant increase in superoxide production and ROS species by the incubated spermatozoa, which would jeopardize their survival by the deterrence of membrane phospholipids [40]. Caution is thus recommended when using HA as additive at dosages considered out of physiological range.

The freezing and thawing of mature motile spermatozoa did not appear to alter the functional HA binding sites they presented [41]. Therefore, HA should also be able to elicit physiological or therapeutic effects during cooling or thawing. The addition of HA (0.004 to 0.012 mg/ml) to boar spermatozoa during cryopreservation has been tested and was found to be beneficial for cryosurvival [24], again without consideration of the effects exerted by the physical properties of the HA macromolecule (increased viscosity, water retention properties, etc.) or acting via binding to HA cell receptors. In the present study, however, the effects were not beneficial at all and the most likely factor involved is the age of the spermatozoa subjected to conventional freezing. The spermatozoa used were ejaculated spermatozoa that had been stored in liquid form for at least 72 h in a commercial extender. It is well known that the source and age of spermatozoa impacts cryosurvival [23, 42]. However, the motility of the extended spermatozoa was high (80%), even after 72 h of extension/storage, and we considered it optimal to expose these spermatozoa to HA, despite the fact that they could be considered stressed by the duration of storage in a simple extender. Further post-thawing *in vitro* HA incubation was omitted since we were interested in the effects provoked by the addition of HA early in the cryopreservation process (during cooling). According to the results, it became evident that the addition of HA to a suspension of CD44-bearing spermatozoa was not sufficiently protective of membrane intactness, possibly due to the enormous increase in superoxide/ROS production, which essentially affected all cells. Although the range of HA concentrations in our study was quite wide (from 0.0625 to 2 mg/ml), viability and mitochondrial activation presented increasing curves up to 1 mg/ml, with a decrease to control values for the maximum concentration (2 mg/ml), which can be considered a supra-physiological value of hyaluronic acid. We hypothesized that such bimodality, obtained in some parameters at physiological

and supra- physiological doses, could be due to selective blocking of the CD44 receptor, highlighted by an increase in viscosity and/or accumulation of solutes, particularly when extracellular ice is forming during the dehydration of the sperm cells.

Sperm freezing and thawing induces a decrease in membrane fluidity with considerable inter-male variation [20], probably caused by the varying amounts of long-chain poly-unsaturated fatty acids (PUFA) in the sperm plasma membrane among sires [42]. Oxidative attacks to these PUFA leading to lipid peroxidation could be corrected by physiological levels of ROS, thus facilitating the preservation of normal sperm function [43]. Previous *in vitro* studies on boar spermatozoa have shown that incubated spermatozoa (control group) may be 75% positive for superoxide production [39]. In the present study, all the spermatozoa (alive and dead) tested positive for this parameter, which is most likely associated with the deleterious effect of the 15°C long-term storage up to 72 h. Moreover, none of the exogenous HA concentrations tested were able to decrease the production of sperm superoxides. The effects were even more dramatic during cryopreservation.

In conclusion, the full-sized CD44 receptor is present in highly extended, ejaculated boar spermatozoa, keeping its membrane location under capacitation-inducing conditions *in vitro* (higher bicarbonate levels). HA exposure to liquid-stored spermatozoa for up to 72 h (doses from 0.0625 to 2 mg/ml), generally lowers sperm velocity and progressive motility in membrane-intact spermatozoa but increases their mitochondrial production of superoxide, similar to what is seen among spermatozoa surviving conventional freezing, yet with markedly increased mitochondrial activation and membrane destabilization. Therefore, the usefulness of the addition of HA during the storage or cryopreservation of liquid-stored, extended boar semen remains questionable, and further non-empirical analyses of HA-sperm membrane interactions are warranted, including *in vitro* penetration tests [44] with physiological concentrations between 0.125 and 1.0 mg/ml.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgements

We would like to thank Stefan Koch and Celia Escudero-Hernandez for their assistance in the WB analysis. The study was made possible by grants from The Swedish Research council VR, Stockholm (Grant 521-2011-6553), the Swedish Research Council FORMAS (Grants 221-2011-512 and 2017-00946), Stockholm, as well as by FORSS (Forskningsrådet i Sydöstra Sverige, Grant 473121) and Lions Forskningsfond 2016-postdoc, Linköping, Sweden.

References

1. Rodriguez-Martinez H, Tienthai P, Atikuzzaman M, Vicente-Carrillo A, Rubér M, Alvarez-Rodríguez M. The ubiquitous hyaluronan: Functionally implicated in the oviduct? *Theriogenology* 2016; **86**: 182–186. [Medline] [CrossRef]
2. Laurent C, Hellström S, Engström-Laurent A, Wells AF, Bergh A. Localization and quantity of hyaluronan in urogenital organs of male and female rats. *Cell Tissue Res* 1995; **279**: 241–248. [Medline] [CrossRef]
3. Geipel U, Kropf J, Krause W, Gressner AM. The concentration pattern of laminin, hyal-

- uronan, and aminoterminal propeptide of type III procollagen in seminal fluid. *Andrologia* 1992; **24**: 205–211. [Medline] [CrossRef]
4. Tammi R, Rönkkö S, Ågren UM, Tammi M. Distribution of hyaluronan in bull reproductive organs. *J Histochem Cytochem* 1994; **42**: 1479–1486. [Medline] [CrossRef]
 5. Binette JP, Ohishi H, Burgi W, Kimura A, Suyemitsu T, Seno N, Schmid K. The content and distribution of glycosaminoglycans in the ejaculates of normal and vasectomized men. *Andrologia* 1996; **28**: 145–149. [Medline] [CrossRef]
 6. Laudat A, Guechot J, Foucault P, Giboudeau J, Palluel AM. Fibronectin and hyaluronic acid in seminal fluid: relationship with sperm count and percentage of typical forms. *Pathol Biol (Paris)* 1997; **45**: 462–466 (in French). [Medline]
 7. Tienthai P, Kjellén L, Pertoft H, Suzuki K, Rodríguez-Martínez H. Localization and quantitation of hyaluronan and sulfated glycosaminoglycans in the tissues and intraluminal fluid of the pig oviduct. *Reprod Fertil Dev* 2000; **12**: 173–182. [Medline] [CrossRef]
 8. Yokoo M, Kimura N, Abe H, Sato E. Influence of hyaluronan accumulation during cumulus expansion on in vitro porcine oocyte maturation. *Zygote* 2008; **16**: 309–314. [Medline] [CrossRef]
 9. Huszar G, Willetts M, Corrales M. Hyaluronic acid (Sperm Select) improves retention of sperm motility and velocity in normospermic and oligospermic specimens. *Fertil Steril* 1990; **54**: 1127–1134. [Medline] [CrossRef]
 10. Shamsuddin M, Rodríguez-Martínez H, Larsson B. Fertilizing capacity of bovine spermatozoa selected after swim-up in hyaluronic acid-containing medium. *Reprod Fertil Dev* 1993; **5**: 307–315. [Medline] [CrossRef]
 11. Rodríguez-Martínez H, Han Y, Song X, Funahashi H, Niwa K. Additive effects of sodium hyaluronate as inducer of capacitation of boar spermatozoa in vitro. *Proc 5th Int Conf Pig Reprod (ICPR)*, Holland, June 2–4 1997; **1**: 142.
 12. Rodríguez-Martínez H, Larsson B, Pertoft H. Evaluation of sperm damage and techniques for sperm clean-up. *Reprod Fertil Dev* 1997; **9**: 297–308. [Medline] [CrossRef]
 13. Suzuki K, Eriksson B, Shimizu H, Nagai T, Rodríguez-Martínez H. Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized in vitro. *Int J Androl* 2000; **23**: 13–21. [Medline] [CrossRef]
 14. Suzuki K, Asano A, Eriksson B, Niwa K, Nagai T, Rodríguez-Martínez H. Capacitation status and in vitro fertility of boar spermatozoa: effects of seminal plasma, cumulus-oocyte-complexes-conditioned medium and hyaluronan. *Int J Androl* 2002; **25**: 84–93. [Medline] [CrossRef]
 15. Rodríguez-Martínez H. Role of the oviduct in sperm capacitation. *Theriogenology* 2007; **68**(Suppl 1): S138–S146. [Medline] [CrossRef]
 16. Tienthai P, Kimura N, Heldin P, Sato E, Rodríguez-Martínez H. Expression of hyaluronan synthase-3 in porcine oviductal epithelium during oestrus. *Reprod Fertil Dev* 2003; **15**: 99–105. [Medline] [CrossRef]
 17. Rodríguez-Martínez H, Tienthai P, Suzuki K, Funahashi H, Ekwall H, Johannisson A. Involvement of oviduct in sperm capacitation and oocyte development in pigs. *Reprod Suppl* 2001; **58**: 129–145. [Medline]
 18. Rodríguez-Martínez H, Saravia F, Wallgren M, Tienthai P, Johannisson A, Vázquez JM, Martínez E, Roca J, Sanz L, Calvete JJ. Boar spermatozoa in the oviduct. *Theriogenology* 2005; **63**: 514–535. [Medline] [CrossRef]
 19. Yeste M, Briz M, Pinart E, Sancho S, García-Gil N, Badia E, Bassols J, Pruneda A, Bussalleu E, Casas I, Bonet S. Hyaluronic acid delays boar sperm capacitation after 3 days of storage at 15°C. *Anim Reprod Sci* 2008; **109**: 236–250. [Medline] [CrossRef]
 20. Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Effect of hyaluronan supplementation on boar sperm motility and membrane lipid architecture status after cryopreservation. *Theriogenology* 2004; **61**: 63–70. [Medline] [CrossRef]
 21. Najafi A, Najafi MH, Zanganeh Z, Sharafi M, Martínez-Pastor F, Adeldust H. Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. *Reprod Domest Anim* 2014; **49**: 934–940. [Medline] [CrossRef]
 22. Sariözkan S, Tuncer PB, Büyükleblebici S, Bucak MN, Cantürk F, Eken A. Antioxidative effects of cysteamine, hyaluronan and fetuin on post-thaw semen quality, DNA integrity and oxidative stress parameters in the Brown Swiss bull. *Andrologia* 2015; **47**: 138–147. [Medline] [CrossRef]
 23. Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. *Zygote* 2004; **12**: 117–124. [Medline] [CrossRef]
 24. Qian L, Yu S, Zhou Y. Protective effect of hyaluronic acid on cryopreserved boar sperm. *Int J Biol Macromol* 2016; **87**: 287–289. [Medline] [CrossRef]
 25. Alvarez-Rodríguez M, Rodríguez-Martínez H. Exogenous hyaluronan does not improve cryosurvival of boar spermatozoa. *19th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR)*. Varna, Bulgaria, September, 2015. Oral communication. *Reprod Domest Anim* 2015; **50** S3 pp33.
 26. Thorne RF, Legg JW, Isacke CM. The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events. *J Cell Sci* 2004; **117**: 373–380. [Medline] [CrossRef]
 27. Day AJ, Prestwich GD. Hyaluronan-binding proteins: tying up the giant. *J Biol Chem* 2002; **277**: 4585–4588. [Medline] [CrossRef]
 28. Yokoo M, Tienthai P, Kimura N, Niwa K, Sato E, Rodríguez-Martínez H. Localisation of the hyaluronan receptor CD44 in porcine cumulus cells during in vivo and in vitro maturation. *Zygote* 2002; **10**: 317–326. [Medline] [CrossRef]
 29. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* 2004; **4**: 528–539. [Medline] [CrossRef]
 30. Castellà Fernández E. Estudio de la expresión de CD44 en procesos preneoplásicos y neoplásicos de esófago, estómago y páncreas. *Doctoral Thesis 2000*; ISBN846890036. <http://hdl.handle.net/10803/4227>.
 31. Vicente-Carrillo A, Álvarez-Rodríguez M, Rodríguez-Martínez H. The CatSper channel modulates boar sperm motility during capacitation. *Reprod Biol* 2017; **17**: 69–78. [Medline] [CrossRef]
 32. Tejerina F, Buranaamnuay K, Saravia F, Wallgren M, Rodríguez-Martínez H. Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized instruments. *Theriogenology* 2008; **69**: 1129–1138. [Medline] [CrossRef]
 33. Bains R, Adeghe J, Carson RJ. Human sperm cells express CD44. *Fertil Steril* 2002; **78**: 307–312. [Medline] [CrossRef]
 34. Vicente-Carrillo A, Rodríguez-Martínez H. The hyaluronan membrane receptor CD44 maintains sperm head localization after bicarbonate-induced capacitation. *Abstract for Poster presentation in the 18th International Congress on Animal Reproduction (ICAR)*, Tours (France), (2016)-06-26/30.
 35. Frunza I, Cernescu H, Korodi G. Physical and chemical parameters of boar sperm. *Actualities in Animal Breeding and Pathology*, Conference, 2008 April 17–18. *Lucr St. Med Vet vol. XLI* 2008; **634**, Timisoara.
 36. Rodríguez-Martínez H, Kvist U, Ernerudh J, Sanz L, Calvete JJ. Seminal plasma proteins: what role do they play? *Am J Reprod Immunol* 2011; **66**(Suppl 1): 11–22. [Medline] [CrossRef]
 37. Boltyky PL, Vernon RB, Falk BA, Preisinger A, Gooden MD, Nepom GT, Gebe JA. IL-10 induction from implants delivering pancreatic islets and hyaluronan. *J Diabetes Res* 2013; **2013**: 342479. [Medline] [CrossRef]
 38. Yagci A, Murk W, Stronk J, Huszar G. Spermatozoa bound to solid state hyaluronic acid show chromatin structure with high DNA chain integrity: an acridine orange fluorescence study. *J Androl* 2010; **31**: 566–572. [Medline] [CrossRef]
 39. Petersen CG, Massaro FC, Mauri AL, Oliveira JB, Baruffi RL, Franco JG Jr. Efficacy of hyaluronic acid binding assay in selecting motile spermatozoa with normal morphology at high magnification. *Reprod Biol Endocrinol* 2010; **8**: 149–155. [Medline] [CrossRef]
 40. Álvarez-Rodríguez M, Vicente-Carrillo A, Rodríguez-Martínez H. Exogenous individual lecithin-phospholipids (phosphatidylcholine and phosphatidylglycerol) cannot prevent the oxidative stress imposed by cryopreservation of boar sperm. *Vet Med Surg* 2017; **1**: 1.
 41. Nijs M, Creemers E, Cox A, Janssen M, Vanheusden E, Castro-Sanchez Y, Thijs H, Ombelet W. Influence of freeze-thawing on hyaluronic acid binding of human spermatozoa. *Reprod Biomed Online* 2009; **19**: 202–206. [Medline] [CrossRef]
 42. Waterhouse KE, Hofmo PO, Tverdal A, Miller RR Jr. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction* 2006; **131**: 887–894. [Medline] [CrossRef]
 43. Aitken RJ. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* 1995; **7**: 659–668. [Medline] [CrossRef]
 44. Vasan SS. Semen analysis and sperm function tests: How much to test? *Indian J Urol* 2011; **27**: 41–48. [Medline] [CrossRef]