


RESEARCH

Extracellular vesicles from seminal plasma to improve fertilizing capacity of bulls

Anna Lange-Consiglio¹ , Emanuele Capra², Noemi Monferini¹, Simone Canesi¹, Giampaolo Bosi¹, Marina Cretich³, Roberto Frigerio³, Valentina Galbiati⁴, Federica Bertuzzo⁵, Francesco Cobalchini⁵, Fausto Cremonesi¹ and Bianca Gasparrini⁶

¹Dipartimento di Medicina Veterinaria e Scienze Animali (DIVAS), Università degli Studi di Milano, Lodi, Italy

²Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche IBBA CNR, Lodi, Italy

³Istituto di Scienze e Tecnologie Chimiche 'Giulio Natta', Consiglio Nazionale delle Ricerche SCITEC-CNR, Milan, Italy

⁴Università degli Studi di Milano, Laboratory of Toxicology (DiSFeB), Milan, Italy

⁵Intermizoo National Bull Centre of Vallecchia, Caorle, Venezia, Italy

⁶Dipartimento di Medicina Veterinaria e Produzioni Animali (DMVPA), Università degli Studi di Napoli Federico II, Naples, Italy

Correspondence should be addressed to F Cremonesi: fausto.cremonesi@unimi.it

Abstract

Extracellular vesicles (EVs) contained in seminal plasma, vehicle RNA, proteins, and other molecules able to influence the biological function of sperm. The aim of this study was to improve the fertilizing capacity of male gametes of low-fertility bulls using EVs isolated by ultracentrifugation from the seminal plasma of a bull of proven fertility. After a dose–response curve study, 10×10^6 sperm of low-fertility bulls were co-incubated for 1 h with 400×10^6 EVs/mL. In addition, it has been verified that the incorporation of EVs, which takes place in the sperm midpiece, is maintained for 5 h and even after cryopreservation. Subsequently, the spermatozoa of low-fertility bulls, with EVs incorporated, were used for the *in vitro* production of embryos. The rate of blastocyst at seventh day yield *in vitro*, with the use of sperm with EVs incorporated, increased by about twice the yield obtained with the same sperm in the absence of EVs: bulls having an average embryonic yield of $6.41 \pm 1.48\%$, $10.32 \pm 4.34\%$, and $10.92 \pm 0.95\%$ improved their yield to $21.21 \pm 1.99\%$, $22.17 \pm 6.09\%$, and $19.99 \pm 5.78\%$, respectively ($P < 0.05$). These encouraging results suggest that it might be possible to keep breeding bulls with poor fertility. Further studies will be needed to evaluate the *in vivo* fertility of sperm treated with EVs and understand how the content of EVs is involved in the sperm–vesicle interaction and in the improved sperm performance.

Lay summary

Sperm can fertilize eggs after they mature as they move through the tube in the testes. As they move, the sperm communicate with the lining of the tubes, thanks to small sacs which are made by the tube itself. These sacs contain many molecules that may play a part in the mechanisms that help sperm fertilize eggs. In veterinary medicine, as with humans, there are fertile and less-fertile individuals. It is possible that the sacs of the semen from a bull which is known to be fertile are different to those from a bull with low fertility. For this reason, sacs from bulls with proven fertility were mixed with sperm from the less-fertile bulls to test in the laboratory how the sperm was able to fertilize eggs and produce embryos. The results show that, in the laboratory, the number of embryos produced is doubled. This suggests it would be possible to improve the fertility of people who are less fertile.

Keywords: ▶ extracellular vesicles ▶ spermatozoa ▶ *in vitro* fertilization ▶ bull

Reproduction and Fertility (2022) **3** 313–327

Introduction

The male subfertility is a major limiting factor of the efficiency of livestock production, leading to severe economic losses. Furthermore, a high individual variability in fertilizing ability is recorded in cattle (Ward *et al.* 2003), with a proportion of high-merit bulls failing to give full-term pregnancies (Parkinson 2004). There are some studies that demonstrated links between the paternal environment, fertility, and fecundity (Lane *et al.* 2014, Braun *et al.* 2017, Glazer *et al.* 2017). Inside the seminal plasma, there are extracellular vesicles (EVs) that could be important vehicles of endogenous molecules that, with exogenous factors (chemicals, environmental stressor, etc.), can modify the constitution and biological function of sperm (Lane *et al.* 2014, Schjenken *et al.* 2020, Rimmer *et al.* 2021).

The EVs are produced by nearly all cell types and, based on their origin, can differ in size, morphology, composition, and biological function (Simon *et al.* 2018, Doyle *et al.* 2019). They are classified as microvesicles if produced by budding of plasma membrane or as exosomes if released by exocytosis by multivesicular bodies (Camussi *et al.* 2010). They are present in all biological fluids (urine, blood, saliva, cerebral spinal fluid, seminal plasma, etc.) and contain lipids, signaling proteins, messenger RNA, and regulatory small non-coding RNAs (Simon *et al.* 2018, van Niel *et al.* 2018). The EVs are involved in the intercellular signaling and communication to exchange information, with a crucial role in the maintenance of physiological homeostasis and the propagation of pathological disease (Stahl *et al.* 2019).

In the male reproductive tract, EVs have been isolated from testes, epididymis, vas deferens, and accessory glands (Manin *et al.* 1995, Sahlen *et al.* 2010, Stewart *et al.* 2019, Rimmer *et al.* 2021). All these EVs contribute to the complex pool of EVs isolated from seminal plasma that is the body fluid that contains the most abundant amount of EV populations (Höög *et al.* 2015). Some studies support the role of EVs present in the seminal plasma in assisting sperm to reach functional maturity and influence the physiology of female reproductive tract cells to support reproductive success, although their specific functions remain controversial (Park *et al.* 2011, Ronquist *et al.* 2013, Sullivan *et al.* 2013, Kim *et al.* 2015, Machtinger *et al.* 2016, Murdica *et al.* 2019a).

The EVs discovered in the seminal plasma have been distinguished based on their origin and have acquired a different nomenclature: for example, vesicles of epididymal origin are called epididymosomes and vesicles of prostatic

origin are called prostasomes. Epididymosomes are released from the epididymal epithelium by apocrine secretion into the lumen, and they were first documented in hamsters but have since been identified in all mammals studied, including mice, rats, bulls, rams, and humans (Sullivan *et al.* 2013). The epididymosomes can interact with transiting spermatozoa and deliver cargo that influences not only sperm maturation but also capacitation, acrosome reaction, fertilization, embryonic development, and long-term health of the offspring (Ellerman *et al.* 2006, Sullivan *et al.* 2013, Martin-DeLeon *et al.* 2015, Saewu *et al.* 2017, Nixon *et al.* 2019, Gaikwad *et al.* 2020).

The prostasomes represent the most abundant population of EVs in the seminal plasma that, in addition to epididymosomes, contains other kinds of EVs probably deriving from other somatic cells of the male reproductive tract (Tamessar *et al.* 2021). The prostasomes regulate sperm membrane fluidity, preventing capacitation and acrosomal reactions from occurring early (Carlini *et al.* 1997, Arienti *et al.* 1998, Brouwers *et al.* 2013, Aalberts *et al.* 2014). In addition, they also have antioxidant and antibacterial properties that give greater protection to ejaculated spermatozoa (Sullivan *et al.* 2013).

Given the critical contribution of EVs to gamete function, it is perhaps not surprising that their dysregulation is linked with infertility (Stegmayr *et al.* 1982, Carlsson *et al.* 2004, Park *et al.* 2011, Bai *et al.* 2018, Gabrielsen & Lipshultz 2019) that can be influenced by determining factors including age, body mass, lifestyle, and exposure to environmental factors (Lane *et al.* 2014).

There is evidence that EVs of the male reproductive tract are fundamental regulators of reproductive success. Indeed, it was already reported that EVs from the seminal plasma influence human sperm function (Arienti *et al.* 1999) and that EVs from normozoospermic men improve sperm motility and trigger capacitation of vasectomized men (Murdica *et al.* 2019a). EVs from boar seminal plasma were reported to be essential for maintaining sperm motility, membrane integrity, and antioxidant abilities as well as for inhibiting premature capacitation (Du *et al.* 2016). One explanation of the influence of EVs on sperm function could be related to their ability to transfer specific microRNAs (miRNAs) inside the spermatozoa, thus affecting their performances. Seminal plasma EVs were observed to contain different miRNAs potentially able to influence sperm cryotolerance in boar semen (Pedrosa *et al.* 2021). Specific sperm-borne miRNAs were observed to be positively related to the first cleavage rate, blastocyst cell number, and division number in bovine species (Alves *et al.* 2019). In this context, it is conceivable that the cargo

of EVs of seminal plasma could influence the fertility of semen and that EVs from bull of proven fertility could improve the semen quality of low-fertility bulls. To test this hypothesis, at first EVs from the seminal plasma of bull of proven fertility were isolated and the EV incorporation into sperm of low-fertility bulls at different times and after cryopreservation was evaluated. Then, the effectiveness of EVs in improving the semen quality of bulls of proven low fertility was evaluated after *in vitro* fertilization (IVF), comparing the *in vitro* outcome of low-fertility semen before and after treatment with EVs.

Materials and methods

Reagents, semen samples, and ovaries

All reagents were purchased from Sigma-Aldrich, while test tubes and culture plates were purchased from Euroclone (Milan, Italy).

Raw and cryopreserved semen samples of Holstein-Friesian bulls (*Bos taurus*) were obtained from a bull artificial insemination centre (Intermizoo National Bull Centre of Vallecchia Caorle, Venezia, Italy).

Ovaries were collected at a slaughterhouse called INALCA (Lody, Italy).

Ethics

The experimental design and animal treatments do not require any Ethical Animal Care. Ovaries were collected from cows slaughtered in a slaughterhouse (INALCA) under national food hygiene regulations. Frozen semen straws were obtained from a bull artificial insemination Center.

Experimental design

The study was conducted through several experiments.

In Experiment 1, EVs were isolated from raw seminal plasma from three bulls of proven fertility, characterized by NanoSight, to determine the average concentration and size, western blotting (WB) and electron transmission microscopy, and labeling with fluorochromes.

In Experiment 2, EV incorporation into sperm was assessed using EVs labeled with PKH-26 fluorochrome by performing a dose–response curve. Subsequently, based on the results, sperm were incubated with 400×10^6 EVs/mL for 1 h, and retention of incorporated EVs was assessed over time (until 12 h) and following cryopreservation.

In Experiment 3, the EVs isolated from the bull that gave the highest blastocyst rate were incorporated into raw

spermatozoa of low-fertility bulls that were tested for *in vitro* embryo production before and after EV incorporation.

Each experiment was replicated three times.

Animal selection

The bulls of proven fertility were called A1, A2, and A3. The low-fertility bulls were called B1, B2, and B3. The characteristics of semen samples of all bulls are related to the last 25 collections for A1, A2, and A3 and 15 collections for B1, B2, and B3. The evaluation of semen was performed by CASA system. The instrument setting for computerized semen analysis is summarized in Table 1 and the semen characteristics are summarized in Supplementary file 1 (see section on [supplementary materials](#) given at the end of this article), showing that bulls with proven fertility have higher progressive motility and kinetic parameters than low-fertility bulls.

In addition, Bull Centre provided the fertility data of each bull and the production, functionality, and type (PFT) index, as shown in Supplementary file 2.

The fertility values are obtained on data registered in Italy regarding the animal used on this study and edited by a comparison made between the animals. An average of the bulls is thus identified based on pregnancies on fertilization interventions, setting the average at 100 with a s.d. of 5 points. Each point above the average will be 101, 102, 103, and so on. Any point below the average will be 99, 98, 97, and so on. So, the fertility in the field is expressed considering the average 100, with values lower than 100 indicating low fertility.

The PFT index is a selection index of Italian Holstein and allows to select high-producing and healthy cows. This index considers the production data (for example milk fat yield and protein), functional data (for example somatic

Table 1 Instrument setting for computerized semen analysis.

Parameter	Value
Number of frames to analyze	20 frames
Sampling frequency	30 frames s ⁻¹
Minimum sampling point for motility	1 point
Minimum sampling point for velocity	3 points
Maximum velocity	150 μm s ⁻¹
Threshold velocity	8 μm s ⁻¹
Minimum sampling point for calculating AHL	7 points
Minimum velocity for calculating AHL	20 μm s ⁻¹
Minimum linearity for calculating AHL	3.5 μm s ⁻¹
Pixel scale	0.688 μm per pixel
Cell size range	4–15 pixels

AHL, amplitude of lateral head displacement.

cell score, functional feet, and legs), and type (Biffani *et al.* 2002, Fuerst & Gredler 2009).

Experiment 1

Isolation of EVs from seminal plasma

Three ejaculates of each proven fertility bull (once a week) were used to isolate EVs by ultracentrifugation that was carried out as previously described (Ding *et al.* 2021) with some modification. To collect the EVs present in seminal plasma, no filtration by 0.22 micrometers was performed. Briefly, semen of bulls of proven fertility was centrifuged at 600 *g* for 20 min to eliminate the spermatozoa. The supernatant was centrifuged again at 4000 *g* for 20 min to eliminate any cellular debris. The obtained seminal plasma was ultracentrifuged twice at 100,000 *g* for 60 min at 4°C. The ultracentrifuge was an L-60 Beckman Coulter Life Sciences (Milan, Italy) with a type 50.2 Ti Fixed Angle Rotor Beckman Coulter Life Sciences and with polycarbonate bottle assembly 70 mL (Cod. Cat. #355622).

The pellet of EVs was resuspended in 200 μ L of PBS and stored at -80°C until further analyses.

NanoSight characterization of concentration and size of EVs

EVs were characterized for size and concentrations by NanoSight NS300 (equipped with a sCMOS camera and 532 nm diode laser: Malvern Scientific, Richmond, UK). Videos were acquired and processed by nanoparticle tracking analysis (NTA) software version 3.4, based on the manufacturer's recommendation. Ambient temperature was recorded manually and did not exceed 25°C. EV samples were diluted in filtered PBS to a final volume of 1 mL (ratio 1:1000). Samples were analyzed within 15 min of the initial dilution with a delay of 10 s between sample introduction and the start of the measurement. Ideal measurement concentration was found by pre-testing the ideal particle per frame value (20–120 particle/frame). Three videos of 60 s were recorded at camera level 11–12, while a syringe pump with a constant flow was injected into the sample. From each video, mean, mode, median, and particle concentration, expressed in particles per milliliters, were calculated by the software with a threshold of 5.

Western blotting

Isolated EVs in Laemmli buffer were treated for 5 min at 95°C; 8 μ L of reduction buffer were added to 32 μ L of EVs. The sample was separated by SDS-PAGE (4–20%, Mini-Protean TGX Precast protein gel, Bio-Rad) and transferred onto a nitrocellulose membrane (Bio-Rad, Trans-Blot

Turbo). Blocking step was performed to saturate nonspecific sites – 1 h with 5% (w/v) bovine serum albumin (BSA) in T-TBS (tris-buffered saline: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.5% Tween 20). Membranes were incubated overnight at 4°C with anti-CD63 (1:1000; BD Pharmingen, San Jose, CA, USA), anti-Alix (1:1000, Santa Cruz), anti-TSG101 (1:1000, Novus Bio, Centennial, CO, USA), and anti-calnexin (1:1000, Santa Cruz). After washing with T-TBS, membranes were incubated with the horseradish peroxidase-conjugated (Jackson ImmunoResearch) secondary antibodies diluted 1:3000 for 1 h. After washing, the signal was detected using Bio-Rad Clarity Western ECL Substrate (Bio-Rad) and imaged using a Chemidoc XRS+ (Bio-Rad).

Transmission electron microscopy

EVs were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffered solution at pH 7.4 for 1 h at room temperature. After washing in the same buffer, samples were post-fixed in 1% OsO₄ and 1.5% potassium ferrocyanide in 0.1 M cacodylate for 1 h in dark condition on ice. After several washings in distilled water, samples were stained with 0.5% uranyl acetate in water overnight at 4°C and, finally, were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, and 96% for 5 min each and washed three times with absolute ethanol for 10 min each). The samples were infiltrated with ethanol and resin (Araldite-Epon) at volumetric proportions of 1:1 for 2 h and then in 100% Epon twice for 1 h each and, finally, polymerized at 60°C for 48 h. Sectioning was performed using an Ultracut E microtome (Reichert, Vienna, Austria). Sections of 70 nm were collected on 300-mesh uncoated copper grid and observed with a Zeiss LEO 912ab Energy Filtering transmission electron microscopy (TEM) operating at 120 kV. Digital images were acquired using a CCD-BM/1K system operating with the iTEM (Olympus Soft Imaging Solutions).

Labeling EVs from seminal plasma of bulls of proven fertility by PKH-26 fluorochrome

The EVs were labeled with PKH-26 that is a red fluorochrome lipophilic that binds to the membranes (Fischer & Mackensen 2003). The labeling protocol requires that the 1×10⁸ EVs are diluted with the diluent C present in the PKH-26 dye kit to reach a final volume of 1 mL. A solution consisting of 1 mL of diluent C and 2 μ L of PKH-26 were added to the suspension of EVs which was then incubated for 15 min at 38.5°C. At the end of the reaction, 7 mL of Roswell Park Memorial Institute (RPMI) medium were added to the suspension, which was centrifuged again at

100,000 *g* for 1 h at 4°C. At the end of the centrifugation, the supernatant was removed, and the labeled EV pellet was resuspended in 200 μ L of RPMI medium and stored at -20°C until its use. This fluorescent dye when excited at a wavelength of 550–560 emits red.

Experiment 2

Experiment 2 is summarized in Fig. 1.

Dose-response curve and evaluation of incorporation times of EVs in spermatozoa

To evaluate the concentration and the best time of incorporation of the EVs, a dose-response study was performed by co-incubation of spermatozoa of each low-fertility bull (1B, 2B, and 3B) and EVs isolated from seminal plasma of proven bull that gave the higher blastocyst rate *in vitro* (bull 1A). Each experiment was replicated three times.

Raw semen of low-fertility bulls was used for this experiment. Live and morphologically normal spermatozoa were selected by centrifugation at 300 *g* for 30 min on a discontinuous density gradient (45/90) using a sterile colloidal silica suspension (Percoll®). The

supernatant was removed, and the live sperm pellet present on the bottom was resuspended to obtain a concentration of 10×10⁶ sperm/mL in Sperm Tyrode’s–albumin–lactate–pyruvate (TALP) medium. This medium is composed of 100 mM NaCl, 3 mM KCl, 0.4 mM NaH₂PO₄, 0.4 mM MgCl₂–6H₂O, 25 mM NaHCO₃, 100 mM HEPES (3785), 12.6 mM pyruvate, 10 mg/mL gentamicin, 3.6 mM sodium lactate, 6 mg/ml BSA, and 7.35 mg/mL CaCl₂.

The experiment was performed by co-culture of labeled EVs and semen using a dose-response curve. In petri dishes of 35 mm of diameter, drops of 70 μ L each of Sperm TALP containing 100,000 spermatozoa and different doses of EVs/mL (50, 100, 150, 200, 300, and 400×10⁶) were prepared. The suspension of spermatozoa and EVs was divided in six petri dishes and incubated for different times (1, 2, 3, 4, 5, and 6 h) in an incubator at 38.5°C with 5% of CO₂, to evaluate the shortest time in which the best incorporation takes place and the optimal concentration detectable by confocal microscopy. Samples of the same raw semen were incubated in Sperm TALP for 6 h and used as control to evaluate motility parameters by the CASA system.

Fifteen minutes before the end of each incubation, 10 μ g/mL of Hoechst 33342 fluorescent dye was added

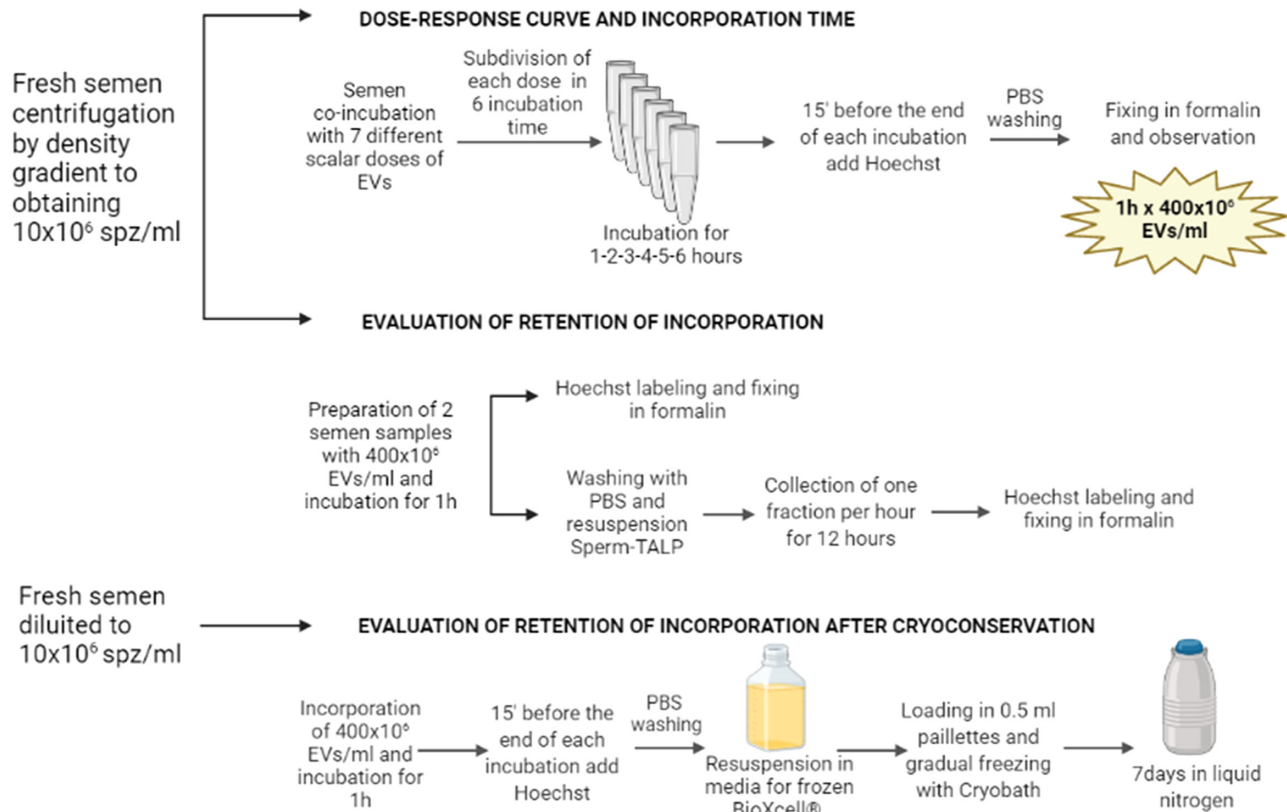


Figure 1 Schema of incorporation tests, created by bioender.com.

to allow visualization of the sperm heads under a confocal microscope. At the end of each incubation, the spermatozoa motility was evaluated by the CASA system. At the end of each time point, each semen drop was washed by centrifugation at 100 *g* for 10 min, the supernatant was removed, and the pellet was resuspended in the same volume of PBS at 38.5°C and further centrifuged at 100 *g* for 10 min. The sperm pellets were then fixed in 10% formalin and stored at +4°C until the observation by a FV300 confocal laser scanning microscope (Olympus).

Evaluation of retention of incorporation over time

Having identified the optimal incorporation time (1 h) and the optimal concentration (400×10^6 EVs/mL, as will be reported in the 'Results' section), the evaluation of the maintenance of the incorporation of the EVs over time was studied. Each experiment was replicated three times.

Samples of semen were prepared following the protocol previously described for the incorporation of the EVs. At the end of 1 h incubation with EVs, a sample of semen was stained with Hoechst and fixed in formalin for confocal microscopic evaluation to verify the successful incorporation of the EVs after 1 h. A second sample was washed by centrifugation to remove the EVs that were not incorporated. Subsequently, it was incubated at 38.5°C in Sperm TALP for the next 12 h to understand the permanence of the EVs in the spermatozoon over time.

At each hour, fractions of this sample were stained with Hoechst and fixed in formalin, according to the previously described protocol, up to the time of observation with a confocal microscope.

Evaluation of retention of incorporation after cryopreservation

With this experiment, it was evaluated whether the EVs, incorporated in spermatozoa during co-incubation, remained incorporated in the spermatozoa even after cryopreservation.

Each experiment was replicated three times.

Samples of raw semen of each low-fertility bull were prepared following the protocol previously described for the incorporation of the EVs. Fifteen minutes before the end of the incubation, 10 µg/mL of Hoechst 33342 fluorescent dye was added. At the end of the incubation, centrifugation was carried out at 100 *g* for 10 min at room temperature, the supernatant was removed, and the sperm pellet was washed with PBS and centrifuged again at 100 *g* for 10 min. The pellet was resuspended first in Sperm TALP and then in BioXcell® freezing medium (IMV Technologies,

Piacenza, Italy). Bovine semen freezing medium was added to semen in a ratio 1:1. The BioXcell freezing medium was previously prepared according to the indicated protocol, by diluting the medium 1:5 with ultrapure distilled water, and was kept at 30°C. Thereafter, this dilution was kept for 20 min at room temperature before the loading of semen into 0.5 mL straws. Control straws were made with the same semen in the absence of EVs.

According to the freezing protocol, the straws were kept at +4°C for 4.5 h, and then, with the use of CryoBath, the temperature dropped from +4°C to -10°C in 5°C per minute and from -10°C to -122°C in 6°C per minute. Once the temperature reached -122°C, the straws were immersed in liquid nitrogen (-196°C).

The straws were kept in liquid nitrogen for 7 days and then thawed in the water bath at 37°C for 30 s. The incorporation after freezing was observed by a confocal microscope.

Experiment 3

In vitro embryo production

The *in vitro* embryo production was carried out according to our standard procedures (Perrini *et al.* 2018), as reported in Fig. 2. Two studies were carried out: at first, an *in vitro* embryo production was performed with cryopreserved semen of bulls of proven fertility to detect the bull with better *in vitro* performance. Since the EV incorporation was studied on raw spermatozoa of low-fertility bulls, in the second study, the EVs of the most *in vitro* fertile bull were used for incorporation in raw spermatozoa of all low-fertility bulls that were tested for *in vitro* embryo production before and after EV incorporation. The incorporation of EVs in spermatozoa of low-fertility bulls was carried out 1 h before the start of fertilization. The rate of embryo production refers to the percentage of blastocysts on the seventh day.

Collection of oocytes

Ovaries were collected from slaughtered Holstein-Friesian cows (*Bos taurus*) with unknown history (age, genealogy, and physiological status). Ovaries were transported to the laboratory in sterile saline solution (0.9% NaCl) supplemented with 150 mg/L kanamycin and maintained at 30°C. Oocytes were retrieved by aspiration of 3–5 mm diameter follicles with 18 G needles. Cumulus–oocyte complexes were selected and washed three times in pre-incubated TCM 199–HEPES buffer supplemented with 10% fetal calf serum (FCS).

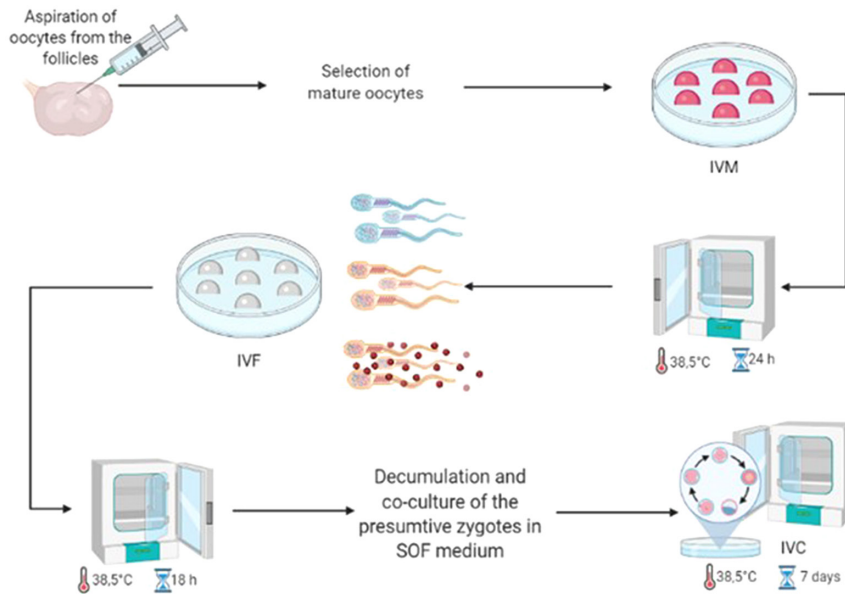


Figure 2 *In vitro* embryo production steps, created by biorender.com.

***In vitro* maturation**

In vitro maturation was performed under standard conditions for 24 h in TCM 199 Earl's salt medium supplemented with 10% FCS, 5 µg/mL LH (Lutropin, Vetoquinol, France), 0.5 µg/mL FSH (Folltropin, Vetoquinol), 0.2 mM sodium pyruvate, 5 µg/mL gentamycin, and 1 mg/mL estradiol 17β. Cultures were performed in 70 µL droplets (up to 20 oocytes/droplet) of the medium under mineral oil, at 38.5°C in 5% CO₂.

***In vitro* fertilization**

IVF was performed in TALP medium containing 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine, 20 µg/mL heparin, 114 mM NaCl, 3.2 mM KCl, 0.4 mM NaH₂PO₄, 10 mM sodium lactate, 25 mM NaHCO₃, 0.5 mM MgCl₂·6H₂O, 2.0 mM CaCl₂·2H₂O, 6 mg/mL BSA, 5 µg/mL gentamicin, and 0.2 mM sodium pyruvate. Frozen-thawed semen (at 37°C in water bath for 30 s) from a single proven fertility bull or raw semen of all low-fertility bulls was prepared by Percoll density gradient (Sigma Aldrich) (45/90%). Semen was thawed at 37°C for 30 s, placed on the top of the Percoll gradient, and centrifuged for 30 min at 300 g. The semen suspension was diluted in the appropriate volume of fertilization medium to obtain a final concentration of 10⁷ spermatozoa per milliliters.

An aliquot of 10 µL of semen was co-incubated with matured oocytes for 18 h at 38.5°C in 5% CO₂. Culture was performed in 70 µL droplets (up to 20 oocytes/droplet) of the medium under mineral oil. At the end of gamete co-culture, cumulus cells were completely removed, and cumulus-free presumptive zygotes were randomly

transferred into different culture systems and cultured up to day 7.

***In vitro* culture**

The standard medium for *in vitro* culture (IVC) was synthetic oviductal fluid with amino acids (SOFaa; Holm *et al.* 1999) composed of 1.1 M NaCl, 72 mM KCl, 12 mM KH₂PO₄, 7.4 mM MgSO₄, 50 mM DL-lactate, 250 mM NaHCO₃, 260 mM phenol red, 100 mM sodium pyruvate, 178 mM CaCl₂·2H₂O, 125 mM HEPES sodium salt, 30.8 mM glutamine, 500 mM glycine, 84.2 mM alanine, 100× minimum essential medium non-essential, 100× basal medium Eagle, 2.8 mM myo-inositol, 340 mM trisodium citrate, 2% FCS, 0.005 g/mL BSA, 0.2 mM sodium pyruvate, and 5 µg/mL gentamicin.

IVC was performed for 7 days in 5% O₂, 5% CO₂, and 90% N₂ in a humidified atmosphere at 38.5°C. Cultures were performed in 70 µL droplets (up to 20 oocytes/droplet) of the medium. The medium was renewed on days 3 and 6 during the culture period.

On day 7, blastocysts from the control and EV groups were collected from the IVC drops.

Statistical analysis

The statistical analysis was carried out with the GraphPad InStat 3.10 software. The total motility and progressive motility at different incubation time of sperm non-treated (-EVs) and treated with EVs (+EVs) were analyzed with Student's *t*-test. Fertilization and blastocyst rate of sperm non-treated (-EVs) and treated with EVs (+EVs) were

analyzed with ANOVA. The data distribution was normal, and variances were homogenous.

The post-hoc tests were Bonferroni and Tukey to compare all pairs of columns.

The significance value was $P < 0.05$.

Results

Experiment 1

Concentration and size of isolated EVs

EVs were characterized according to MISEV2018 guidelines, by NTA, TEM, and WB (Théry *et al.* 2018).

The analysis carried out at NanoSight shows that the average concentration of EVs was $4.88 \times 10^{11} \pm 2.17 \times 10^{11}$ /mL, and the size was 177.4 ± 5.89 nm.

The concentration and size of the EVs isolated from the seminal fluid of fertile bulls 1A, 2A, and 3A obtained by NanoSight are shown in Fig. 3A. The concentration was $3.22 \times 10^{11} \pm 1.82 \times 10^{10}$, $4.08 \times 10^{11} \pm 1.60 \times 10^{10}$, and $7.34 \times 10^{11} \pm 3.51 \times 10^{10}$ EVs/mL and the EV size was 184.2 ± 66.8 , 174.5 ± 60 , and 173.8 ± 59.9 nm, respectively, in bulls 1A, 2A, and 3A.

The concentration of EVs in bull 3A was higher ($P < 0.05$) compared to the other two bulls.

WB showed the presence of specific EVs markers such as CD63, Alix, and TSG101 and a negligible contamination

of calnexin (Fig. 3B). Observation by electron microscope revealed that preparations contained EVs (Fig. 3C).

Experiment 2

Dose-response curve and evaluation of incorporation times of EVs in spermatozoa

By confocal microscopy, it was verified that the optimal concentration of EVs that is incorporated by the male gametes is 400×10^6 EVs/mL and that the incorporation takes place since the first hour of spermatozoa-EV co-incubation, as shown in Fig. 4. It can be observed that the EV incorporation is in the sperm midpiece which appears red, as EVs were labeled with PKH-26, while the heads of spermatozoa appear blue by Hoechst fluorescent dye.

For each concentration of EVs tested using the dose-response curve, all spermatozoa were able to uptake EVs in the sperm midpiece, but the incorporation was uniform only at a concentration of 400×10^6 EVs/mL (Figure 5A). Hence this concentration was used for all the experiments. Further, lower concentrations showed an irregular uptake, detectable as red dots (Fig. 5B). In the z-stack image obtained by confocal microscopy, it is possible to see EVs included in the thickness of the tail and not resting on the membrane (Fig. 5C). The image is a merge of 16 focal planes in a range of $1.05 \mu\text{m}$.

During this study, at each time point, the sperm total motility and progressive motility were evaluated by

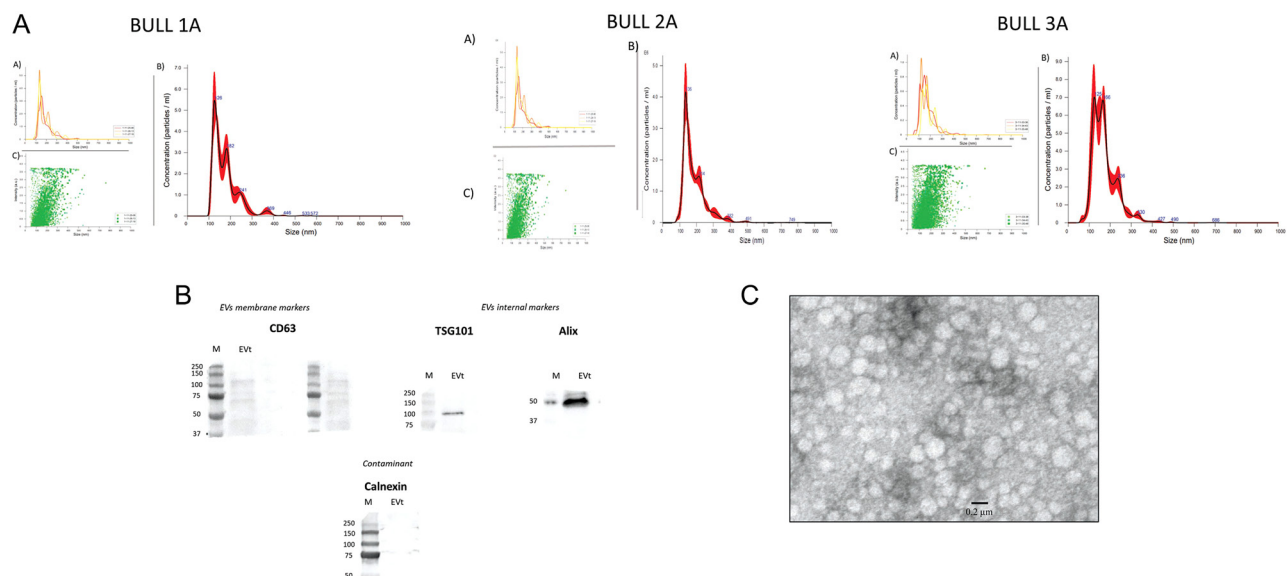


Figure 3 (A) Results of the NanoSight analysis of bulls 1A, 2A, and 3A. (a) Finite track length adjustment (FTLA) identifies the concentration/size of the particles isolated from the seminal plasma. The different colors of the curves identify the measurements in triplicate for each sample. (b) The average obtained by FTLA. (c) The intensity of light scattering plotted as a function of the particle size. (B) Western blot for EV markers and contaminants: internal markers (TSG101 and Alix); membrane markers (CD63); and calnexin as marker of cell contamination in EV preparation. (C) Transmission electron Microscopy analysis of EVs inside the seminal plasma revealed typical morphologies characteristic of vesicle (scale bar: $0.2 \mu\text{m}$).

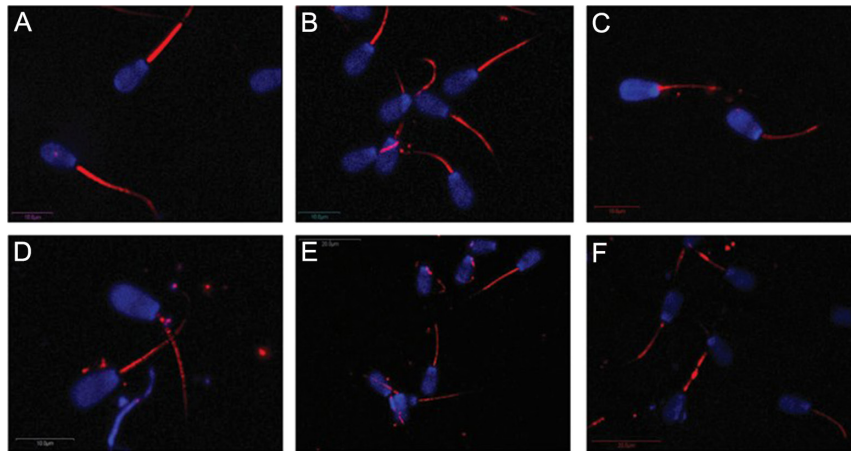


Figure 4 Study of incorporation of EVs over time. (A) Image acquired with a confocal microscope of the spermatozoa after 1 h of co-incubation with the EVs. (B) Spermatozoa after 2 h of co-incubation with EVs. (C) The incorporation after 3 h of co-incubation. (D) Incorporation of the EVs after 4 h and (E) after 5 h, and (F) shows how the incorporation of the EVs into the spermatozoa also occurs at 6 h of co-incubation. Magnification 100×.

the CASA system. The data are summarized in Table 2. Treatment with EVs did not affect sperm motility; indeed, the total motility decreased at 5 h and 6 h of co-incubation both in non-treated semen (control) and in semen co-incubated with EVs. The rate of progressive motility of EV-treated spermatozoa improved at 2 and 3 h of co-incubation, to progressively return to the initial values of the raw semen. Furthermore, at 3 h incubation, the progressive motility of EV-treated spermatozoa was higher ($P < 0.05$) than that of control spermatozoa ($P < 0.05$).

Considering that the percentage of total motility of the semen does not change within 3 h of co-incubation of the

semen with the EVs, in the subsequent experiments, it was decided to use only 1 h of sperm–EV co-incubation because this could favor a shorter handling time of the semen in the case of processing for cryopreservation.

Evaluation of retention of incorporation over time and after cryopreservation

Following the results obtained in the previous experiment, the incorporation takes place successfully already at 1 h of co-incubation of the semen with the EVs and persists for 5 h after washing with the EVs remaining embedded in the sperm midpiece (Fig. 6A).

The incorporation of the EVs within the intermediate tract and in the tail of the spermatozoa was also maintained following cryopreservation (Fig. 6B).

Experiment 3

In vitro embryo production

Among bulls of proven fertility, bull 1A gave higher ($P < 0.001$) blastocyst rate after IVF than bulls 2A and 3A (38.3 ± 0.5 , 22.6 ± 2.5 , and 32.7 ± 0.1 , respectively).

Therefore, the EVs isolated from the seminal plasma of bull 1A were used for their incorporation into the spermatozoa of low-fertility bulls.

Treatment of sperm with EVs increased by about two-fold the fertilization rate and the blastocyst rate *in vitro*, compared to the same sperm in the absence of EVs ($P < 0.05$; Fig. 7 and Table 3).

Discussion

This study was designed to evaluate whether co-incubation with EVs isolated from seminal plasma of bulls of proven fertility improves sperm function of low fertility bulls.

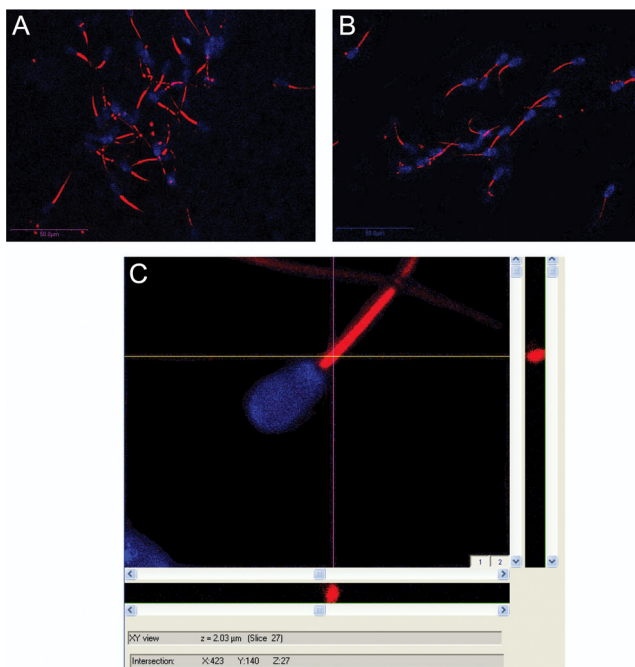


Figure 5 Dose–response curve. (A) At 400×10^6 EVs/mL, the incorporation was uniform. (B) Incorporation with red dots at lower concentration. (C) z-Stack image obtained by confocal microscopy. EVs are included in the thickness of the tail and not resting on the membrane.

Table 2 Total motility and progressive motility at different incubation time of sperm non-treated (-EVs) and treated with EVs (+EVs).

Time (h)	% Total motility		% Progressive motility	
	-EVs	+EVs	-EVs	+EVs
0	86.12 ± 3.26 ^a	86.12 ± 3.26 ^a	68.23 ± 3.02	68.23 ± 3.02 ^a
1	88.15 ± 2.17 ^a	89.29 ± 3.65 ^a	70.32 ± 2.44	69.90 ± 2.29 ^a
2	88.44 ± 2.72 ^a	87.56 ± 3.33 ^a	71.55 ± 3.46	74.90 ± 3.47 ^b
3	85.52 ± 3.15 ^a	81.92 ± 10.03 ^a	70.32 ± 3.19 ^x	85.04 ± 5.08 ^y ^b
4	81.47 ± 2.49 ^a	85.72 ± 6.66 ^a	68.99 ± 3.42	68.60 ± 6.20 ^a
5	75.13 ± 4.52 ^b	73.13 ± 16.22 ^b	67.65 ± 3.36	53.65 ± 14.58 ^a
6	76.72 ± 4.34 ^b	79.44 ± 14.84 ^b	65.42 ± 3.67	63.42 ± 14.14 ^a

^{a,b}Values with different superscripts within columns are significantly different ($P < 0.05$). ^{x,y}Values with different superscripts between columns are significantly different ($P < 0.05$).

The bulls of proven fertility enrolled in this study had higher ejaculate volume, progressive motility, and kinetic parameters than low-fertility bulls but similar concentration. A diagnosis of hypo-fertility cannot be carried out only with the spermogram, then the classification of these bulls as low-fertility was confirmed by progeny data provided from Bull Centre and by our *in vitro* embryo production study. Our results showed that bull 1A was the one with the highest blastocyst rate; therefore, EVs of this bull were used to assess the efficacy to improve *in vitro* fertility of low-fertility semen.

The first step of this study was the isolation of EVs from the seminal plasma of proven bulls. Compared to the protocol of isolation as described by Ding *et al.* (2021), no filtration by 0.22 µm was performed to obtain both kinds of EVs: microvesicles (from plasma membrane) and exosomes (from endosomes) based on nomenclature defined by Théry *et al.* (2018). In this way, we were supposed to be in a situation more similar to the alive one. A characterization on isolated EVs by TEM, NanoSight, and WB was performed to confirm the isolation of EVs with a size in a range from 173.8 to 184.2 nm, overlapping the range from 50 nm to 200 nm of Ding *et al.* (2021).

Then, the EV incorporation was assessed by incubating sperm with EVs labeled with the PKH-26 dye and by confocal

microscopy analysis. The dose-response curve showed that the best concentration was 400×10^6 EVs/mL because, at this concentration, the spermatozoa show uniform red staining and not red dots as in lower concentration. The incorporation took place at the shortest incubation time (1 h) in the sperm midpiece. Our results are in line with a previous report showing optimal incorporation in cat spermatozoa co-incubated with oviductal or epididymal EVs for 1 h (Ferraz *et al.* 2019, Rowilson *et al.* 2021), while male gametes from mouse and pig models required a co-incubation time greater than 2 h (Al-Dossary *et al.* 2013, Alcântara-Neto *et al.* 2020). This suggests that the binding of the EVs with the spermatozoa could depend on the source of the spermatozoa, the kind of EVs, the concentration, and the experimental conditions in which the co-incubation was carried out.

During the incorporation study, the total motility did not change until 5 h post-incubation, while the progressive motility significantly improved at 2–3 h to return to initial values at increasing times. At 3 h of incubation, the progressive motility of EV-treated sperm was significantly higher compared to non-treated sperm used as control. It was previously reported that boar spermatozoa co-incubated with exosomes obtained from

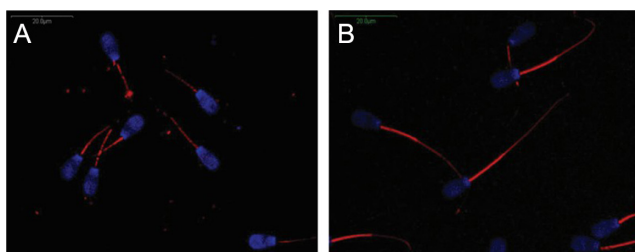


Figure 6 Maintenance of incorporation of EVs (A) at 5 h of incubation, after washing from medium supplemented with EVs, and (B) after a week of cryopreservation. Magnification 60×.

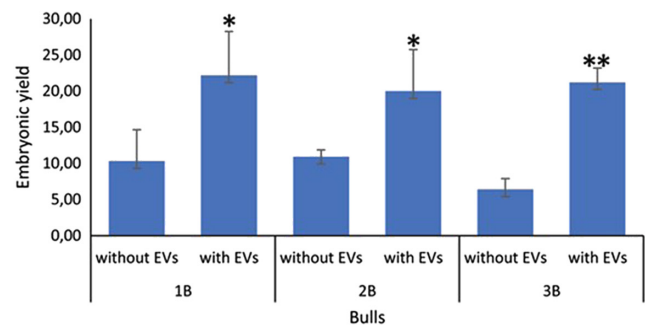


Figure 7 Embryonic yield of low-fertility bulls before and after incorporation of EVs. * represents $P < 0.05$; ** represent $P < 0.005$.

Table 3 Fertilization and blastocyst rate of sperm non-treated (-EVs) and treated with EVs (+EVs).

	-EV fertilization rate (%)	+EV fertilization rate (%)	-EVs blastocyst rate (%)	+EVs blastocyst rate (%)
Bull 1B	19.48 ± 2.61 ^a	41.41 ± 4.02 ^b	10.32 ± 4.340 ^A	22.17 ± 6.09 ^B
Bull 2B	20.91 ± 2.87 ^a	40.14 ± 3.57 ^b	10.92 ± 0.95 ^A	19.99 ± 5.78 ^B
Bull 3B	15.22 ± 1.98 ^a	44.32 ± 4.13 ^b	6.41 ± 1.48 ^A	21.21 ± 1.99 ^B

^{a,b}Values with different superscripts between columns are significantly different ($P < 0.05$). ^{A,B}Values with different superscripts between columns are significantly different ($P < 0.05$).

seminal plasma maintain their motility even after 10 h of co-incubation (Du *et al.* 2016), suggesting that proteins inside the EVs are involved in the motility of spermatozoa. The fact that EVs are incorporated in the tract that generates the movement in the spermatozoon, or in the intermediate portion, is undoubtedly suggestive of a role on motility. Not knowing the content of the EVs and the molecules that are involved, then, we cannot confirm this hypothesis; we can, however, affirm that through the CASA system the sperm progressivity after 1 h of incubation with EVs increased and only at the fourth hour it decreased. In the subsequent experiments, as the incorporation of EVs did not vary with time and the total motility was constant between the first and the third hour of co-incubation, it was decided to use only 1 h of sperm-EV co-incubation to have a shorter handling time of the semen in the case of processing for cryopreservation.

Another experiment of this study aimed to evaluate the maintenance of the incorporation of EVs by the sperm over time. In fact, for potential use, it is important to ensure that the EVs incorporated in the intermediate tract after 1 h of incubation remain incorporated. The fluorescent signal was detected until 5 h after incorporation. We hypothesize that after 5 h of internalization, the spermatozoa start the phagocytosis of EVs for the release of their contents. This phagocytosis probably results in the destruction of the EV membranes and, consequently, in the loss of fluorescence signal.

Another interesting finding of this work, for the potential applications, was the demonstration that EV incorporation persists in sperm following cryopreservation. This positive outcome led us to think that semen from low-fertility bulls could be improved with EVs from bulls of proven fertility because the incorporation time is only 1 h and, subsequently, the semen can be cryopreserved and used for *in vivo* fertilization or IVF. In this way, it would be possible to transmit the genetic potential of bulls which, due to age or other factors that arise later, are characterized by low fertility.

In the last experiment, raw spermatozoa from low-fertility bulls were co-incubated for 1 h with EVs from the

seminal plasma of bull 1A (which was found to have the highest rate of embryonic yield *in vitro* among the bulls of proven fertility tested) and later they were used for the IVF to verify the possible increase of their embryonic yield. The low-fertility bulls were tested with and without EVs to compare the rate of fertilization and blastocysts formed on the seventh day of IVC. Interestingly, the results show that incubation with EVs from bull 1A significantly increased the blastocyst rate of low-fertility bulls. The results obtained, by comparing the embryonic yields of each bull with and without the use of EVs, are very promising, as the yield increased by almost two-fold.

Many studies have demonstrated that EVs can transfer various molecules to sperm (Jodar 2019, Chen *et al.* 2020) regulating not only a series of physiological activities of mature sperm (Arienti *et al.* 1999, Pons-Rejraji *et al.* 2011, Du *et al.* 2016, Murdica *et al.* 2019b) but also the development of offsprings (Chan *et al.* 2020).

Our results confirm the functional role of plasma EVs derived from proven fertility bulls, but other study will be necessary to understand the cargo of molecules contained in these EVs to realize which molecules may have improved the motility of the semen and the embryonic yield. This is the only paper on the effect of isolated EVs from seminal plasma of bull on spermatozoa of low-fertility animals. Although we assume that the composition of EVs deriving from seminal plasma can vary between species, and that the results obtained in other species are not attributable to the bovine species, at this moment we can only compare our data with that existing in the literature.

In mouse, culture of epididymal sperm with prostasomes was demonstrated to affect sperm motility and fertilization success *in vitro* and *in vivo* (Tamessar *et al.* 2021). EVs from normozoospermic men improve sperm motility and trigger capacitation of vasectomized men (Murdica *et al.* 2019a). Exosomes from boar seminal plasma are essential for maintaining sperm motility, membrane integrity, and antioxidant abilities (Du *et al.* 2016). The improved fertility of low-fertility bulls *in vitro* through their exposure to EVs from seminal plasma of bulls of proven fertility has certainly impacted the livestock industry,

as it also allows enrollment of high-merit bulls with low-fertility in reproduction programs.

The EVs used in this study belong to a heterogeneous family, derived from different organs of the male genital tract. It would also be interesting to know from which trait, tissue, or cell type of the reproductive system the EVs derive and characterize their actual content. To date, little is known about the content of EVs and how the molecules they carry can influence the vitality and functions of spermatozoa. It can be assumed that the improvement in the *in vitro* embryo rate given by the EVs to the spermatozoa of low-fertility bulls may be conditioned by proteins contained in the vesicles. It was previously reported that proteins P25b and GLI pathogenesis-related 1 like 1, known to intervene in the sperm interaction with the oocyte, are localized at the level of the sperm head and are acquired by the gamete through the fusion of the epididymosomes in bovine and mouse (Frenette *et al.* 2004, Gaikwad *et al.* 2019). It is also possible that the proteins involved in sperm motility improve the fertilization capacity of the sperm, allowing them to move more skillfully between the cells of the cumulus. Bovine epididymosomes contain several proteins that participate in the acquisition of sperm motility, fertilization ability, and protection against reactive oxygen species (Frenette *et al.* 2004, 2006a,b, Vernet *et al.* 2004). These proteins include a 55-kDa hyaluronan-hydrolyzing protein and sperm adhesion molecule 1, hyaluronidases that intervene during the penetration of the sperm through the cumulus cells. These molecules are released during the acrosomal reaction or are present in the plasma membrane, playing a fundamental role in the hydrolysis of hyaluronic acid and favoring the movement of the tail spermatozoon (Kimura *et al.* 2009). Other proteins such as macrophage migration inhibitory factor and aldo-keto-reductase family 1 member b (AKR1B1) are also involved in the motility of spermatozoa and accumulate in the dense fibers of the intermediate tract (Eickhoff *et al.* 2004); moreover, AKR1B1 also has the task of maintaining the spermatozoa in a state of quiescence during epididymal transit (Machtiger *et al.* 2016). In addition to proteins inside the EVs, there are also proteins on the membrane of the EVs that interact with target cells promoting adhesion and incorporation. These molecules are cluster differentiation (CD) 9, CD81, and other tetraspanins, which are present on EVs and on the head and intermediate portion of spermatozoa (Caballero *et al.* 2010, 2013).

The EVs also contain small regulatory RNAs such as miRNAs that could influence sperm fertility. The comparison of EVs in seminal plasma of boars with different sperm motility showed variation in several miRNAs including

miR-222. This miRNA was reported to be transferred into sperm by semen EVs and regulate the maintenance of sperm viability reducing apoptosis (Ding *et al.* 2021). Recently, in addition to protein and miRNAs inside the EVs, their phospholipids are becoming an important research area. Based on accumulating evidence, phospholipids of EVs are regulators of biological processes and not simply membrane component. Several phospholipids have important roles in EV biogenesis, identification, and cell function regulations (Chang *et al.* 2022). From our results, it is possible to affirm that the sperm motility after 1 h of incubation with EVs increases and that *in vitro* fertility of semen improve, probably for effect of EVs. The improvement of fertilization rate could explain the improvement of embryonic rate, but at this moment, we have no information about the molecules inside the EVs, or on their membrane, which may have contributed to the fertility improvement achieved in this study. Then, further studies on molecules that regulate sperm motility and fertility will be necessary.

Fertility is a complex parameter to define, given the complexity of the molecules involved. Our study is a good starting point to underline that EVs from seminal plasma are involved in the fertilizing ability of spermatozoa.

Conclusions

It was demonstrated that bovine sperm incorporate EVs after 1 h incubation and retain EVs for up to 5 h and, more importantly, after cryopreservation. It was also proven that semen from low-fertility bulls can be improved by treatment with EVs isolated from seminal plasma of bulls of proven fertility. Indeed, the use of EVs incorporated in male gametes has given positive results as regards the improvement of the fertilizing abilities of spermatozoa *in vitro*. This could be due to molecules present in the EVs, or on their surface, that assist the gametes during maturation, migration to the oocyte, gamete interaction, and fertilization. These results can be considered a starting point for strategies to improve the fertility of breeding low-fertility bulls. Further studies are, however, needed to also evaluate the *in vivo* fertility of sperm treated with EVs and understand how the content of EVs is involved in the sperm-vesicle interaction and in the improved sperm performance.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/RAF-22-0037>.

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.

Funding

This study was supported by grants from Università degli Studi di Milano, Milano, Italy.

Author contribution statement

A L C and B G conceptualized the project; E C, M C, R F, and V G performed isolation, characterization, and labeling of EVs; F B and F C provided semen; A L C, F C, N M, and S C performed all experimental studies; G B performed confocal microscopy analyses; A L C wrote the manuscript; B G and E C provided intellectual input. All authors approve the final submission of this manuscript.

Acknowledgements

The authors thank Prof. Emanuela Corsini (DiSFeB, Università degli Studi di Milano, Milan, Italy) for their assistance in isolation of EVs.

References

- Aalberts M, Stout TAE & Stoorvogel W** 2014 Prostatomes: extracellular vesicles from the prostate. *Reproduction* **147** R1–R14. (<https://doi.org/10.1530/REP-13-0358>)
- Alcántara-Neto AS, Schmaltz L, Caldas E, Blache MC, Mermillod P & Almiñana C** 2020 Porcine oviductal extracellular vesicles interact with gametes and regulate sperm motility and survival. *Theriogenology* **155** 240–255. (<https://doi.org/10.1016/j.theriogenology.2020.05.043>)
- Al-Dossary AA, Strehler EE & Martin-DeLeon PA** 2013 Expression and secretion of plasma membrane Ca²⁺-ATPase 4a (PMCA4a) during murine estrus: association with oviductal exosomes and uptake in sperm. *PLoS ONE* **8** e80181. (<https://doi.org/10.1371/journal.pone.0080181>)
- Alves MBR, de Arruda RP, De Bem THC, Florez-Rodriguez SA, Sá Filho MF, Belleannée C, Meirelles FV, da Silveira JC, Percin F & Celeghini ECC** 2019 Sperm-borne miR-216b modulates cell proliferation during early embryo development via K-RAS. *Scientific Reports* **9** 10358. (<https://doi.org/10.1038/s41598-019-46775-8>)
- Arienti G, Carlini E, Polci A, Cosmi EV & Palmerini CA** 1998 Fatty acid pattern of human prostatic lipid. *Archives of Biochemistry and Biophysics* **358** 391–395. (<https://doi.org/10.1006/abbi.1998.0876>)
- Arienti G, Carlini E, Nicolucci A, Cosmi EV, Santi F & Palmerini CA** 1999 The motility of human spermatozoa as influenced by prostatomes at various pH levels. *Biology of the Cell* **91** 51–54. (<https://doi.org/10.1111/j.1768-322X.1999.tb01083.x>)
- Bai R, Latifi Z, Kusama K, Nakamura K, Shimada M & Imakawa K** 2018 Induction of immune-related gene expression by seminal exosomes in the porcine endometrium. *Biochemical and Biophysical Research Communications* **495** 1094–1101. (<https://doi.org/10.1016/j.bbrc.2017.11.100>)
- Biffani S, Samorè AB & Canavesi F** 2002 PFT: the new selection index for the Italian Holstein. Abstract A.N.A.F.I. Italian Holstein Breeders Association. *Interbulletin* **29** 142.
- Braun JM, Messerlian C & Hauser R** 2017 Fathers matter: why it's time to consider the impact of paternal environmental exposures on children's health. *Current Epidemiology Reports* **4** 46–55. (<https://doi.org/10.1007/s40471-017-0098-8>)
- Brouwers JF, Aalberts M, Jansen JW, van Niel G, Wauben MH, Stout TA, Helms JB & Stoorvogel W** 2013 Distinct lipid compositions of two types of human prostatomes. *Proteomics* **13** 1660–1666. (<https://doi.org/10.1002/pmic.201200348>)
- Caballero J, Frenette G & Sullivan R** 2010 Post testicular sperm maturation changes in the bull: important role of the epididymosomes and prostatomes. *Veterinary Medicine International* **2011** 757194. (<https://doi.org/10.4061/2011/757194>)
- Caballero JN, Frenette G, Belleannée C & Sullivan R** 2013 CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. *PLoS ONE* **8** e65364. (<https://doi.org/10.1371/journal.pone.0065364>)
- Camussi G, Deregibus MC, Bruno S, Cantaluppi V & Biancone L** 2010 Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney International* **78** 838–848. (<https://doi.org/10.1038/ki.2010.278>)
- Carlini E, Palmerini CA, Cosmi EV & Arienti G** 1997 Fusion of sperm with prostatomes: effects on membrane fluidity. *Archives of Biochemistry and Biophysics* **343** 6–12. (<https://doi.org/10.1006/abbi.1997.9999>)
- Carlsson L, Nilsson BO, Ronquist G, Lundquist M & Larsson A** 2004 A new test for immunological infertility: an ELISA based on prostatomes. *International Journal of Andrology* **27** 130–133. (<https://doi.org/10.1111/j.1365-2605.2004.00458.x>)
- Chan JC, Morgan CP, Adrian Leu N, Shetty A, Cisse YM, Nugent BM, Morrison KE, Jašarević E, Huang W, Kanyuch N, et al.** 2020 Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. *Nature Communication* **11** 15305-w. (<https://doi.org/10.1038/s41467-020-15305-w>)
- Chang W, Xiao D, Fang X & Wang J** 2022 Phospholipids in small extracellular vesicles: emerging regulators of neurodegenerative diseases and cancer. *Cytotherapy* **24** 93–100. (<https://doi.org/10.1016/j.jcyt.2021.09.013>)
- Chen X, Zheng Y, Lei A, Zhang H, Niu H, Li X, Zhang P, Liao M, Lv Y, Zhu Z, et al.** 2020 Early cleavage of preimplantation embryos is regulated by tRNA (Gln-TTG)-derived small RNAs present in mature spermatozoa. *Journal of Biological Chemistry* **295** 10885–10900. (<https://doi.org/10.1074/jbc.RA120.013003>)
- Ding Y, Ding N, Zhang Y, Xie S, Huang M, Ding X, Dong W, Zhang Q & Jiang L** 2021 MicroRNA-222 transferred from semen extracellular vesicles inhibits sperm apoptosis by targeting BCL2L11. *Frontiers in Cell and Developmental Biology* **9** 736864. (<https://doi.org/10.3389/fcell.2021.736864>)
- Doyle LM & Wang MZ** 2019 Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* **8** 727. (<https://doi.org/10.3390/cells8070727>)
- Du J, Shen J, Wang Y, Pan C, Pang W, Diao H & Dong W** 2016 Boar seminal plasma exosomes maintain sperm function by infiltrating into the sperm membrane. *Oncotarget* **7** 58832–58847. (<https://doi.org/10.18632/oncotarget.11315>)
- Eickhoff R, Baldauf C, Kozyro H_W, Wennemuth G, Suga Y, Seitz J, Henkel R & Meinhardt A** 2004 Influence of macrophage migration inhibitory factor (MIF) on the zinc content and redox state of protein-bound sulphhydryl groups in rat sperm: indications for a new role of MIF in sperm maturation. *Molecular Human Reproduction* **10** 605–611. (<https://doi.org/10.1093/molehr/gah075>)
- Ellerman DA, Myles DG & Primakoff P** 2006 A role for sperm surface protein disulfide isomerase activity in gamete fusion: evidence for the participation of ERp57. *Developmental Cell* **10** 831–837. (<https://doi.org/10.1016/j.devcel.2006.03.011>)
- Ferraz M, Deam CA, Dahal R, Noonan MJ & Songsasen N** 2019 Oviductal extracellular vesicles interact with the spermatozoon's head and mid-piece and improves its motility and fertilizing ability in the

- domestic cat. *Scientific Reports* **9** 1–12. (<https://doi.org/10.1038/s41598-019-45857-x>)
- Fischer K & Mackensen A** 2003 The flow cytometric PKH-26 assay for the determination of T-cell mediated cytotoxic activity. *Methods* **31** 135–142. ([https://doi.org/10.1016/S1046-2023\(03\)00123-3](https://doi.org/10.1016/S1046-2023(03)00123-3))
- Frenette G, Lessard C & Sullivan R** 2004 Polyol pathway along the bovine epididymis. *Molecular Reproduction and Development* **69** 448–456. (<https://doi.org/10.1002/mrd.20170>)
- Frenette G, Thabet M & Sullivan R** 2006a Polyol pathway in human epididymis and semen. *Journal of Andrology* **27** 233–239. (<https://doi.org/10.2164/jandrol.05108>)
- Frenette G, Girouard J & Sullivan R** 2006b Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis. *Biology of Reproduction* **75** 885–890. (<https://doi.org/10.1095/biolreprod.106.054692>)
- Fuerst C & Gredler B** 2009 Genetic evaluation for female fertility traits in Austria and Germany. *Interbull Bulletin* **40** 3–9.
- Gabrielsen JS & Lipshultz LI** 2019 Rapid progression in our understanding of extracellular vesicles and male infertility. *Fertility and Sterility* **111** 881–882. (<https://doi.org/10.1016/j.fertnstert.2019.02.021>)
- Gaikwad AS, Anderson AL, Merriner DJ, O'Connor AE, Houston BJ, Aitken RJ, O'Bryan MK & Nixon B** 2019 GLIPRLL1 Is an IZUMO-binding protein required for optimal fertilization in the mouse. *BMC Biology* **17** 86–86. (<https://doi.org/10.1186/s12915-019-0701-1>)
- Gaikwad AS, Hu J, Chapple DG & O'Bryan MK** 2020 The functions of CAP superfamily proteins in mammalian fertility and disease. *Human Reproduction Update* **26** 689–723. (<https://doi.org/10.1093/humupd/dmaa016>)
- Glazer CH, Bonde JP, Eisenberg ML, Giwercman A, Haervig KK, Rimborg S, Vasard D, Pingorg A, Schmidt L & Bräuner EV** 2017 Male infertility and risk of nonmalignant chronic diseases: a systematic review of the epidemiological evidence. *Seminars in Reproductive Medicine* **35** 282–290. (<https://doi.org/10.1055/s-0037-1603568>)
- Holm P, Booth PJ, Schmidt MH, Greve T & Callesen H** 1999 High bovine blastocyst development in a static in vitro production system using sofaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology* **52** 683–700. ([https://doi.org/10.1016/S0093-691X\(99\)00162-4](https://doi.org/10.1016/S0093-691X(99)00162-4))
- Höög JL & Lötval J** 2015 Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. *Journal of Extracellular Vesicles* **4** 28680. (<https://doi.org/10.3402/jev.v4.28680>)
- Jodar M** 2019 Sperm and seminal RNAs: what roles do they play beyond fertilization? *Reproduction* **158** R113–R123. (<https://doi.org/10.1530/REP-18-0639>)
- Kim BJ, Park DR, Nam TS, Lee SH & Kim UH** 2015 Seminal CD38 enhances human sperm capacitation through its interaction with CD31. *PLoS ONE* **10** e0139110. (<https://doi.org/10.1371/journal.pone.0139110>)
- Kimura M, Kim E, Kang W, Yamashita M, Saigo M, Yamazaki T, Nakanishi T, Kashiwabara S-I & Baba T** 2009 Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in fertilization. *Biology of Reproduction* **81** 939–947. (<https://doi.org/10.1095/biolreprod.109.078816>)
- Lane M, Robker RL & Robertson SA** 2014 Parenting from before conception. *Science* **345** 756–760. (<https://doi.org/10.1126/science.1254400>)
- Machtinger R, Laurent LC & Baccarelli AA** 2016 Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. *Human Reproduction Update* **22** 182–193. (<https://doi.org/10.1093/humupd/dmv055>)
- Manin M, Lecher P, Martinez A, Tournadre S & Jean C** 1995 Exportation of mouse vas deferens protein, a protein without a signal peptide, from mouse vas deferens epithelium: a model of apocrine secretion. *Biology of Reproduction* **52** 50–62. (<https://doi.org/10.1095/biolreprod52.1.50>)
- Martin-DeLeon PA** 2015 Epididymosomes: transfer of fertility-modulating proteins to the sperm surface. *Asian Journal of Andrology* **17** 720–725. (<https://doi.org/10.4103/1008-682X.155538>)
- Murdica V, Giacomini E, Alteri A, Bartolacci A, Cermisoni GC, Zarovni N, Papaleo E, Montorsi F, Salonia A, Viganò P, et al.** 2019a Seminal plasma of men with severe asthenozoospermia contain exosomes that affect spermatozoa motility and capacitation. *Fertility and Sterility* **111** 897.e2–908.e2. (<https://doi.org/10.1016/j.fertnstert.2019.01.030>)
- Murdica V, Cermisoni GC, Zarovni N, Salonia A, Viganò P & Vago R** 2019b Proteomic analysis reveals the negative modulator of sperm function glycodeelin as over-represented in semen exosomes isolated from asthenozoospermic patients. *Human Reproduction* **34** 1416–1427. (<https://doi.org/10.1093/humrep/dez114>)
- Nixon B, De Iulius GN, Hart HM, Zhou W, Mathe A, Bernstein IR, Anderson AL, Stanger SJ, Skerrett-Byrne DA, Jamaluddin MFB, et al.** 2019 Proteomic profiling of mouse epididymosomes reveals their contributions to post-testicular sperm maturation. *Molecular and Cellular Proteomics* **18** (Supplement 1) S91–S108. (<https://doi.org/10.1074/mcp.RA118.000946>)
- Park KH, Kim BJ, Kang J, Nam TS, Lim JM, Kim HT, Park JK, Kim YG, Chae SW & Kim UH** 2011 Ca²⁺ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. *Science Signaling* **4** ra31. (<https://doi.org/10.1126/scisignal.2001595>)
- Parkinson TJ** 2004 Evaluation of fertility and infertility in natural service bulls. *Veterinary Journal* **168** 215–229. (<https://doi.org/10.1016/j.tvjl.2003.10.017>)
- Pedrosa AC, Torres MA, Alkmin DV, Pinzon JEP, Martins SMMK, da Silveira JC & de Andrade AFC** 2021 Spermatozoa and seminal plasma small extracellular vesicles miRNAs as biomarkers of boar semen cryotolerance. *Theriogenology* **174** 60.e72. (<https://doi.org/10.1016/j.theriogenology.2021.07.022>)
- Perrini C, Esposti P, Cremonesi F & Consiglio AL** 2018 Secretome derived from different cell lines in bovine in vitro embryo production. *Reproduction, Fertility, and Development* **30** 658–671. (<https://doi.org/10.1071/RD17356>)
- Pons-Rejraji H, Artonne C, Sion B, Brugnion F, Canis M, Janny L & Grizard G** 2011 Prostasomes: inhibitors of capacitation and modulators of cellular signalling in human sperm. *International Journal of Andrology* **34** 568–580. (<https://doi.org/10.1111/j.1365-2605.2010.01116.x>)
- Rimmer MP, Gregory CD & Mitchell RT** 2021 The transformative impact of extracellular vesicles on developing sperm. *Reproduction and Fertility* **2** R51–R66. (<https://doi.org/10.1530/RAF-20-0076>)
- Ronquist KG, Ek B, Morrell J, Stavreus-Eversd A, Holst BS, Humblot P, Ronquist G & Larsson A** 2013 Prostasomes from four different species are able to produce extracellular adenosine triphosphate (ATP). *Biochimica et Biophysica Acta (BBA): General Subjects* **1830** 4604–4610. (<https://doi.org/10.1111/rda.14033>)
- Rowlison T, Ottinger MA & Comizzoli P** 2021 Exposure to epididymal extracellular vesicles enhances immature sperm function and sustains vitality of cryopreserved spermatozoa in the domestic cat model. *Journal of Assisted Reproduction and Genetics* **38** 2061–2071. (<https://doi.org/10.1007/s10815-021-02214-0>)
- Saewu A, Kadunganattil S, Raghupathy R, Kongmanas K, Diaz-Astudillo P, Hermo L & Tanphaichitr N** 2017 Clusterin in the mouse epididymis: possible roles in sperm maturation and capacitation. *Reproduction* **154** 867–880. (<https://doi.org/10.1530/REP-17-0518>)
- Sahlén G, Nilsson O, Larsson A, Carlsson L, Norlen BJ & Ronquist G** 2010 Secretions from seminal vesicles lack characteristic markers for prostasomes. *Uppsala Journal of Medical Sciences* **115** 107–112. (<https://doi.org/10.3109/03009730903366067>)

- Schjenken JE & Robertson SA** 2020 The female response to seminal fluid. *Physiological Reviews* **100** 1077–1117. (<https://doi.org/10.1152/physrev.00013.2018>)
- Simon C, Greening DW, Bolumar D, Balaguer N, Salamonsen LA & Vilella F** 2018 Extracellular vesicles in human reproduction in health and disease. *Endocrine Reviews* **39** 292–332. (<https://doi.org/10.1210/er.2017-00229>)
- Stahl PD & Raposo G** 2019 Extracellular vesicles: exosomes and microvesicles, integrators of homeostasis. *Physiology* **34** 169–177. (<https://doi.org/10.1152/physiol.00045.2018>)
- Stegmayr B & Ronquist G** 1982 Promotive effect on human sperm progressive motility by prostasomes. *Urological Research* **10** 253–257. (<https://doi.org/10.1007/BF00255932>)
- Stewart J, Hoshino A, Rosenwaks Z & Palermo G** 2019 Understanding the role of seminal fluid exosomes within the male reproductive tract. *Fertility and Sterility* **111** e7–e8. (<https://doi.org/10.1016/j.fertnstert.2019.02.044>)
- Sullivan R & Saez F** 2013 Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction* **146** R21–R35. (<https://doi.org/10.1530/REP-13-0058>)
- Tamessar CT, Trigg NA, Nixon B, Skerrett-Byrne DA, Sharkey DJ, Robertson SA, Bromfield EG & Schjenken JE** 2021 Roles of male reproductive tract extracellular vesicles in reproduction. *American Journal of Reproductive Immunology* **85** e13338. (<https://doi.org/10.1111/aji.13338>)
- Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, *et al.*** 2018 Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for extracellular vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* **7** 1535750. (<https://doi.org/10.1080/20013078.2018.1535750>)
- van Niel G, D'Angelo G & Raposo G** 2018 Shedding light on the cell biology of extracellular vesicles. *Nature Reviews: Molecular Cell Biology* **19** 213–228. (<https://doi.org/10.1038/nrm.2017.125>)
- Vernet P, Aitken RJ & Drevet JR** 2004 Antioxidant strategies in the epididymis. *Molecular and Cellular Endocrinology* **216** 31–39. (<https://doi.org/10.1016/j.mce.2003.10.069>)
- Ward F, Rizos D, Boland MP & Lonergan P** 2003 Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: work in progress. *Theriogenology* **59** 1575–1584. ([https://doi.org/10.1016/S0093-691X\(02\)01202-5](https://doi.org/10.1016/S0093-691X(02)01202-5))

Received 22 March 2022

Received in final form 18 October 2022

Accepted 14 November 2022

Accepted Manuscript published online 14 November 2022

