LAY SUMMARY

Sperm can fertilise 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 fertilise 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.
EXTRACELLULAR VESICLES FROM SEMINAL PLASMA TO IMPROVE FERTILIZING CAPACITY OF BULLS

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ABSTRACT
Seminal plasma contains extracellular vesicles (EVs) that 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 dose-response curve, 10x10⁶ sperm of low-fertility bulls were co-incubated for an hour with 400x10⁶ EVs/ml. In addition, it has been verified that the incorporation of EVs, which takes place in the sperm midpiece, is maintained for 5 hours 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±1.48%, 10.32±4.34% and 10.92±0.95% improved their yield to 21.21±1.99%, 22.17±6.09% and 19.99±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 involve in the sperm-vesicle interaction and in the improved sperm performance.

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. 2020). 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 in microvesicles if produced by budding of plasma membrane or in 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 (mRNAs) and regulatory small non-coding RNAs (sncRNAs) (Simon et
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 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 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-DeLeón 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 seminal plasma that, in addition to epididymosomes, contains other kinds of EVs probably deriving from other somatic cells of male reproductive tract (Tamessar et al. 2020). 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 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). Extracellular vesicles 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 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 seminal plasma of bull of proven fertility were isolated and the EV incorporation in 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 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 (Milan, Italy), while test tubes and culture plates were purchased from Euroclone (Milan, Italy).
Raw and cryopreserved semen samples of Holstein-Friesian bulls (Bos taurus) were kindly provided from Intermizoo National Bull Centre of Vallevecchia (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, Lodi, Italy) under national food hygiene regulations. Frozen semen straws were obtained from an artificial insemination Center (AI 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 average concentration and size, Western Blotting and electron transmission microscopy, and labeling with fluorochromes.

In Experiment 2, EV incorporation into sperm was assessed using EVs labelled with PKH-26 fluorochrome performing a dose response curve. Subsequently, based on the results, sperm were incubated with \(400 \times 10^6\) 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 in raw spermatozoa of low-fertility bulls that were tested for \textit{in vitro} embryo production before and after EV incorporation.

Each experiment was replicated three times.

\textbf{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 (provided from Bull Center) 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, 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 standard deviation 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 etc. 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 cell score, functional feet, and legs) and type (Biffani et al., 2002; Canavesi et al., 2009).

\textbf{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. Briefly: semen of bulls of proven fertility was centrifuged at 600 g for 20 minutes to eliminate the spermatozoa. The supernatant was centrifuged again at 4,000 g for 20 minutes to eliminate any cellular debris. The obtained seminal plasma was ultracentrifuged twice at 100,000 g for 60 minutes at 4°C. The ultracentrifuge was an L-60 Beckman Coulter Life Sciences 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 µl of PBS and stored at -80°C until further analyses. In this protocol no filtration by 0.22 mm filters was not performed to collect all kind of extracellular vesicles present in seminal plasma.

NanoSight characterization of concentration and size of EVs

Extracellular vesicles were characterized for size and concentrations by Nanosight NS300 (equipped with a sCMOS camera and 532 nm diode laser: Malvern scientific, UK). Videos were acquired and processed by NTA software version 3.4, based on the manufacture’s recommendation. Ambient temperature was recorded manually and did not exceed 25°C. EVs’ samples were diluted in filtered PBS to a final volume of 1mL (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 seconds were recorded at camera level 11-12, while a syringe pump with a constant flow injected the sample. From each video, mean, mode, median and particles concentration express in particles per mL, were calculated by the software with a threshold of 5.

Western blotting (WB)

Isolated EVs in Laemmli buffer were treated for 5 minutes at 95°C; 8uL of reduction buffer were added at 32uL of EVs. Sample was separated by SDS-PAGE (4–20%, Mini-Protean TGX Precast protein gel, Bio-Rad) and transferred onto a nitrocellulose membrane (BioRad, Trans-Blot Turbo). Blocking step was performed to saturate nonspecific sites, 1 h with 5% (w/v) BSA in T-TBS (tris-buffered saline: 150 mM NaCl, 20 mM TrisHCl, 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, CA, USA), 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, Tucker, GA, USA) 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+ (BioRad).

**Transmission electron microscopy (TEM)**
Extracellular vesicles 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, 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, 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, Austria). Sections of 70 nm were collected on 300-mesh uncoated copper grid and observed with a Zeiss LEO 912ab Energy Filtering 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 1x10^8 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 two μl of PKH-26 were added to the suspension of EVs which was then incubated for 15 minutes at 38.5°C. At the end of the reaction, 7 mL of Roswell Park Memorial Institute (RPMI) Medium (RPMI) was added to the suspension, which was centrifuged again at 100,000g for 1 h at 4°C. At the end of the centrifugation, the supernatant was removed and the labeled EVs pellet was resuspended in 200 μl of RPMI and stored at -20°C until their 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 minutes 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 10x10^6 sperm/ml in Sperm 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 (bovine serum albumin), 7.35 mg/ml CaCl₂.

The experiment was performed by co-culture of labeled EVs and semen in a dose response curve. In petri dishes of 35mm of diameter, drops of 70 µl each of Sperm-Talp containing 100,000 spermatozoa and different doses of EVs/ml (50, 100, 150, 200, 300 and 400x10^6) were prepared. The suspension of spermatozoa and EVs was divided in 6 petri dishes and incubated for different time (1, 2, 3, 4, 5, 6 hours) 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 six hours and used as control to evaluate motility parameters by CASA system.

Fifteen minutes before the end of each incubation, 10 µg/ml of Hoechst 33342 fluorescent dye was added to allow visualization of the sperm heads under confocal microscope. At the end of each incubation, the spermatozoa motility was evaluated by CASA system. At the end of each time point, each semen drop was washed by centrifugation at 100 g for 10 minutes, the supernatant was removed, and the pellet resuspended in the same volume of PBS at 38.5°C and further centrifuged at 100 g for 10 minutes. 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, Hamburg, Germany)."

**Evaluation of retention of incorporation over time**

Having identified the optimal incorporation time (1 h) and the optimal concentration (400x10^6 EVs/ml, as will be reported in the Results), 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 hours 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 minutes at room temperature, the supernatant was removed, and the sperm pellet was washed with PBS and centrifuged again at 100 g for 10 minutes. The pellet was resuspended first in SPERM TALP and then in BioXcell® freezing medium (IVM Technologies). 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 minutes 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 hours, 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 reached -122°C, the straws were immersed in liquid nitrogen (-196°C).

The straws were kept in liquid nitrogen for seven days, then thawed in the water bath at 37°C for 30 seconds. The incorporation after freezing was observed by 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 one hour 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 (COCs) were selected and washed three times in pre-incubated TCM 199-Hepes buffered supplemented with 10% fetal calf serum (FCS).

**In vitro maturation (IVM)**

In vitro maturation was performed under standard condition 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)**

In vitro fertilization was performed in Tyrode’s-albumin-lactate-pyruvate (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 bovine serum albumin (BSA),, 5 μL/mL gentamicin, 0.2 mM sodium pyruvate. Frozen-thawed semen (at 37°C in water bath for 30 seconds) from a single proven fertility bull or raw semen of all low-fertility bulls were prepared by Percoll density gradient (Amersham Pharmacia Biotec) (45/90%). Semen was thawed at 37°C for 30 seconds, placed on the top of the Percoll gradient and centrifuged for 30 min at 300 x g. The semen suspension was diluted in the appropriate volume of fertilization medium to obtain a final concentration of 10⁷ spermatozoa per mL.

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 (IVC)**

The standard medium for IVC was synthetic oviductal fluid with amino acids (SOFaa; Holm et al. 1999) composed of 1.1 M NaCl, 72 mM KCl, 12 mM KH$_2$PO$_4$, 7.4 mM MgSO$_4$, 50 mM DL-lactate, 250 mM NaHCO$_3$, 260 mM phenol red, 100 mM sodium pyruvate, 178 mM CaCl$_2$-2H$_2$O, 125 mM Hepes sodium salt, 30.8 mM glutamine, 500 mM glycine, 84.2 mM alanine, 100X minimum essential medium (MEM) non-essential, 100X basal medium Eagle (BME), 2.8 mM Myo-Inositol, 340 mM trisodium citrate, 2% FCS, 0.005 gr/mL BSA, 0.2 mM sodium pyruvate, 5 µL/mL gentamicin. *In vitro* culture was performed for 7 days in 5% O$_2$, 5% CO$_2$ and 90% N$_2$ 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. 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 test 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**

Extracellular vesicles were characterized according to MISEV2018 guidelines, by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and Western blotting (WB) (Théry et al., 2018).

The analysis carried out at NanoSight shows that the average concentration of EVs was 4.88x10$^{11}$ ±2.17x10$^{11}$/mL and 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 $184.2 \pm 66.8$, $174.5 \pm 60$ and $173.8 \pm 59.9$ 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. Western blotting showed the presence of specific EVs marker such as CD63, Alix and TSG101 and a negligible contamination of Calnexin (Figure 3B). Observation by electron microscope revealed that preparations contained EVs (Figure 3C).

**EXPERIMENT 2**

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

By confocal microscope, it was verified that the optimal concentration of EVs that is incorporated by the male gametes is $400 \times 10^6$ EVs/mL and that the incorporation takes place since the first hour of spermatozoa-EVs 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 during the dose response curve, all spermatozoa were able to uptake EVs in the sperm midpiece, but only at the concentration of $400 \times 10^6$ EVs/ml the incorporation was uniform (Fig. 5A) and not like red dots as at lower concentration (Fig. 5B), then this amount was used for all experiments. 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 um.

During this study, at each time point, the sperm total motility and progressive motility were evaluated by CASA System. The data are summarized in Table 2. Treatment with EVs did not affect sperm motility; indeed, the total motility decreased at 5h 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 EVs-treated spermatozoa improved at 2 and 3 h of co-incubation, to progressivity return to the initial values of the raw semen. Furthermore, at 3 hours incubation the progressive motility of EVs-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 three hours of co-incubation of the semen with the EVs, in the subsequent experiments, it was decided to use only one hour 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 one hour of co-incubation of the semen with the EVs and persists for 5 hours 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 *in vitro* fertilization than bulls 2A and 3 A (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; Table 3 and Fig. 7).

**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. 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 spermiogram, 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 the 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 seminal plasma of proven bulls. Compared to the protocol of isolation as described by Ding *et al.* (2018), no filtration by 0.22 μm was performed to obtain both kinds of extracellular vesicles: microvesicles (from plasma membrane) and exosomes (from endosomes) based on nomenclature defined by Théry *et al.* (2018). In this way, we supposed to be in a situation more similar to the alive one. A characterization on isolated EVs by transmission electron microscopy, Nanosight, and Western Blotting 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 $400 \times 10^6$ 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, Rowlison et al. 2021), while male gametes from mouse and pig models required a co-incubation time greater than two hours (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 hours 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 seminal plasma maintain their motility even after 10 hours 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 CASA system the sperm progressivity after an hour of incubation with EVs increased and only at 4th h decreases. 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 one hour 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 one hour of incubation, remain incorporated. The fluorescent signal was detected until 5 hours after incorporation. We hypothesize that after 5 hours 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 one hour and, subsequently, the semen can be cryopreserved and used for in vivo or in vitro fertilization. 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 one hour with EVs from seminal plasma from 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 in vitro fertilization 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 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 2-fold.

Many studies have proved 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 offspring after being absorbed by sperm (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. 2020). Extracellular vesicles 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 an impact on livestock industry, allowing to enroll in reproduction programs also high-merit bulls with low-fertility otherwise discarded.

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 \textit{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 (GLIPR1L1), 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 \textit{et al.} 2001, Gaikwad \textit{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 \textit{et al.} 2006ab, Frenette \textit{et al.} 2004, Vernet \textit{et al.} 2004). These include a 55-kDa hyaluronan-hydrolyzing protein (HYAL5) and sperm adhesion molecule 1 (SPAM1), hyaluronidases that intervene during the penetration of the sperm through the cumulus cells, that released during the acrosomal reaction or present in the plasma membrane play a fundamental role in the hydrolysis of hyaluronic acid and favor the movement of the tail spermatozoon (Kimura \textit{et al.} 2009). Other proteins as macrophage migration inhibitory factor (MIF) 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 \textit{et al.} 2004); moreover, AKR1B1 also has the task of maintaining the spermatozoa in a state of quiescence during epididymal transit (Machtinger \textit{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, that are present on EVs and on the head and intermediate portion of spermatozoa (Caballero \textit{et al.} 2011, 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 \textit{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 one hour of incubation with EVs increases, and that \textit{in vitro} fertility of semen improve, probably for effect of EVs. The improvement of fertilization rate could explain the improvement of embryonic rate, but in this moment, we have not information about the molecules inside the EVs, or on their membrane, that 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, gametes 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 involve in the sperm-vesicle interaction and in the improved sperm performance.

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 research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

AUTHOR CONTRIBUTION STATEMENT
ALC and BG conceptualized the project; EC, MC, RF and VG performed isolation, characterization and labeling of EVs; FB and FC provided semen; ALC, FC, NM and SC performed all experimental studies; GB performed confocal microscopy analyses; ALC wrote the manuscript; BG and EC provided intellectual input.
All authors approve the final submission of this manuscript.

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REFERENCES


CAPTIONS

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

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

Figure 3 A) Results of the NanoSight analysis of bull 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) is the average obtained by FTLA. C) diagram relating to the intensity of light scattering plotted as a function of the particle size. B) Western Blot for EVs markers and contaminants: internal markers (TSG101 and Alix); membrane markers (CD63), and Calnexin as marker of cell contamination in EVs preparation. C) Transmission Electron Microscopy analysis of EVs inside the seminal plasma revealed typical morphologies characteristic of vesicle (scale bar: 0.2 µm).

Figure 4 Study of incorporation of EVs over time. A) is an image acquired with a confocal microscope of the spermatozoa after one hour of co-incubation with the EVs. B) represents spermatozoa after two hours of co-incubation with EVs. C) represents the incorporation after 3 hours of co-incubation. D) shows the incorporation of the EVs after 4 hours, E) after 5 hours and F) shows how the incorporation of the EVs into the spermatozoa also occurs at six hours of co-incubation. Magnification 100x.

Figure 5 Dose response curve. A) at 400x10⁶ 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.

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

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

737x471mm (28 x 28 DPI)
In vitro embryo production steps.

120x82mm (150 x 150 DPI)
Figure 3 A) Results of the NanoSight analysis of bull 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) is the average obtained by FTLA. C) diagram relating to the intensity of light scattering plotted as a function of the particle size. B) Western Blot for EVs markers and contaminants: internal markers (TSG101 and Alix); membrane markers (CD63), and Calnexin as marker of cell contamination in EVs preparation. C) Transmission Electron Microscopy analysis of EVs inside the seminal plasma revealed typical morphologies characteristic of vesicle (scale bar: 0.2 µm).
Study of incorporation of EVs over time. A) is an image acquired with a confocal microscope of the spermatozoa after one hour of co-incubation with the EVs. B) represents spermatozoa after two hours of co-incubation with EVs. C) represents the incorporation after 3 hours of co-incubation. D) shows the incorporation of the EVs after 4 hours, E) after 5 hours and F) shows how the incorporation of the EVs into the spermatozoa also occurs at six hours of co-incubation. Magnification 100x.

150x76mm (150 x 150 DPI)
Figure 5 Dose response curve. A) at 400x106 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.
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150x55mm (150 x 150 DPI)
Figure 7 Embryonic yield of low-fertility bulls before and after incorporation of EVs. * represents $P<0.05$; ** represent $P<0.005$. 
100x56mm (150 x 150 DPI)
Table 1: Instrument setting for computerized semen analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of frames to analyse</td>
<td>20 frames</td>
</tr>
<tr>
<td>Sampling frequency</td>
<td>30 frames s(^{-1})</td>
</tr>
<tr>
<td>Minimum sampling poits for motility</td>
<td>1 point</td>
</tr>
<tr>
<td>Minimum sampling point for velocity</td>
<td>3 points</td>
</tr>
<tr>
<td>Maximum velocity</td>
<td>150 µm s(^{-1})</td>
</tr>
<tr>
<td>Treshold velocity</td>
<td>8 µm s(^{-1})</td>
</tr>
<tr>
<td>Minimum sampling points for calculating AHL</td>
<td>7 points</td>
</tr>
<tr>
<td>Minimum velocity for calculating AHL</td>
<td>20 µm s(^{-1})</td>
</tr>
<tr>
<td>Minimum linearity for calculating AHL</td>
<td>3.5 µm s(^{-1})</td>
</tr>
<tr>
<td>Pixel scale</td>
<td>0.688 µm per pixel</td>
</tr>
<tr>
<td>Cell size range</td>
<td>4-15 pixel</td>
</tr>
</tbody>
</table>
Table 2. Total motility and progressive motility at different incubation time of sperm non-treated (-EVs) and treated with EVs (+EVs).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% Total motility</th>
<th>% Progressive motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- EVs</td>
<td>+ EVs</td>
</tr>
<tr>
<td>0</td>
<td>86.12±3.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86.12±3.26&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>88.15±2.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.29±3.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>88.44±2.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.56±3.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>85.52±3.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.92±10.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>81.47±2.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.72±6.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>75.13±4.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.13±16.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>76.72±4.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79.44±14.84&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Values with different superscripts within columns are significantly different (P<0.05).

<sup>x,y</sup> Values with different superscripts between columns are significantly different (P<0.05).
Table 3. Fertilization and blastocyst rate of sperm non treated (- EVs) and treated with EVs (+ EVs).

<table>
<thead>
<tr>
<th></th>
<th>- EVs Fertilization rate (%)</th>
<th>+ EVs Fertilization rate (%)</th>
<th>- EVs Blastocyst rate (%)</th>
<th>+ EVs Blastocyst rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull 1B</td>
<td>19.48±2.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.41±4.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.32±4.34&lt;sup&gt;A&lt;/sup&gt;</td>
<td>22.17±6.09&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bull 2B</td>
<td>20.91±2.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.14±3.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.92±0.95&lt;sup&gt;A&lt;/sup&gt;</td>
<td>19.99±5.78&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bull 3B</td>
<td>15.22±1.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.32±4.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.41±1.48&lt;sup&gt;A&lt;/sup&gt;</td>
<td>21.21±1.99&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> 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).
Supplementary file 1. Characteristics of semen of proven and low-fertility bulls.

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>Average values</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>Average values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume (ml)</strong></td>
<td>8.37±2.69</td>
<td>9.21±2.58</td>
<td>8.62±1.78</td>
<td>8.73±0.43a</td>
<td>8.01±2.08</td>
<td>6.87±1.44</td>
<td>4.52±1.92</td>
<td>6.47±1.78a</td>
</tr>
<tr>
<td><strong>Concentration (mil/ml)</strong></td>
<td>1138.41±384.5</td>
<td>1313.28±324.11</td>
<td>1053.82±209.90</td>
<td>1168.5±132.32a</td>
<td>1036.95±314.89</td>
<td>1288.92±345.09</td>
<td>746.33±423.48</td>
<td>1024.07±271.52a</td>
</tr>
<tr>
<td><strong>Motility (%)</strong></td>
<td>90.75±3.83</td>
<td>89.18±5.26</td>
<td>86.95±5.78</td>
<td>88.96±1.91a</td>
<td>89.29±3.65</td>
<td>81.92±10.03</td>
<td>73.13±16.22</td>
<td>81.45±8.09a</td>
</tr>
<tr>
<td><strong>Progressivity (%)</strong></td>
<td>70.57±5.22</td>
<td>85.04±5.08</td>
<td>75.05±7.16</td>
<td>76.89±7.41a</td>
<td>69.90±4.29</td>
<td>68.36±5.63</td>
<td>53.65±14.58</td>
<td>63.97±8.97b</td>
</tr>
<tr>
<td><strong>VSL (µm/sec)</strong></td>
<td>75.47±18.11</td>
<td>77.95±5.32</td>
<td>84.80±7.76</td>
<td>79.41±4.83a</td>
<td>75.63±17.89</td>
<td>64.00±26.62</td>
<td>70.53±23.03</td>
<td>70.05±5.83b</td>
</tr>
<tr>
<td><strong>STR (%)</strong></td>
<td>81.49±18.52</td>
<td>84.36±14.14</td>
<td>87.16±2.61</td>
<td>84.34±2.84a</td>
<td>79.12±18.34</td>
<td>70.73±28.94</td>
<td>78.44±24.91</td>
<td>76.1±4.66b</td>
</tr>
</tbody>
</table>

Legend: superscript lowercase letters (a,b) represent statistically significant differences (P<0.05).
Supplementary file 2. Progeny data and production, functionality, and type (PFT) index of bulls of proven fertility.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Fertility of semen</th>
<th>PFT</th>
<th>Reliability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>107</td>
<td>+4060</td>
<td>99%</td>
</tr>
<tr>
<td>2A</td>
<td>103</td>
<td>+3896</td>
<td>97%</td>
</tr>
<tr>
<td>3A</td>
<td>103</td>
<td>+3822</td>
<td>99%</td>
</tr>
<tr>
<td>1B</td>
<td>88</td>
<td>+3469</td>
<td>99%</td>
</tr>
<tr>
<td>2B</td>
<td>93</td>
<td>+3461</td>
<td>92%</td>
</tr>
<tr>
<td>3B</td>
<td>95</td>
<td>+4108</td>
<td>94%</td>
</tr>
</tbody>
</table>