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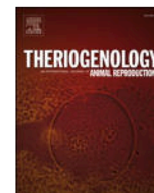
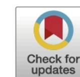


Evaluation of a novel microfluidic chip-like device for purifying bovine frozen-thawed semen for *in vitro* fertilization



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ABSTRACT

The aim of this study was to validate a novel sperm purification device, the VetCount™ Harvester, for use in bovine *in vitro* fertilization (IVF). The device's performance was compared to BoviPure™ gradient centrifugation, a commercially available and accepted routine technique. Semen quality parameters were assessed for frozen-thawed semen from six different bulls ($n = 6$) following sperm purification. For each bull two semen subsamples were purified utilizing BoviPure™ gradient centrifugation and the VetCount™ Harvester, including a third subsample as untreated control. Both treatments significantly increased the proportion of progressively motile sperm cells (84.4 ± 14.1% and 85.1 ± 7.8%, respectively) compared to the untreated semen (41.9 ± 18.8%). BoviPure™ gradient and VetCount™ Harvester selected predominantly viable acrosome intact (VAI) sperm cells with low membrane fluidity and low free intracellular calcium concentration $[Ca^{2+}]_i$ (76.5 ± 4.4% and 78.6 ± 6.0%). Normalizing $[Ca^{2+}]_i$ of VAI sperm cells (non-treated semen: $[Ca^{2+}]_i = 1$) VetCount™ Harvester purified spermatozoa (0.67 ± 0.10) showed significantly lower $[Ca^{2+}]_i$ than BoviPure™ treated sperm (0.84 ± 0.14; $P < 0.05$). Subsequently, the fertilizing ability of the spermatozoa was evaluated performing a competitive fertilization assay. Sperm cells from both treatment groups were fluorescently labelled using different dyes and added in equal amounts to *in vitro* matured oocytes. After 18 h co-incubation, the origin of the fertilizing sperm cell was evaluated via fluorescence microscopy. In two bulls, VetCount™ Harvester selected sperm that fertilized significantly more oocytes then BoviPure™ treated sperm, in another bull it was the opposite. For three bulls no difference was observed.

We conclude that the VetCount™ Harvester selects a high-quality, fertile sperm fraction from frozen-thawed bull semen. However, some considerations have to be kept in mind for the direct use of the isolated sperm fraction in IVF.

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1. Introduction

The importance of *in vitro* embryo production (IVP) in cattle is increasing worldwide [1]. One important step in the IVP process is the fertilization of mature oocytes with good quality bull sperm. For this purpose, mostly frozen thawed semen is used. Therefore, the separation of viable, motile spermatozoa from dead and/or immotile sperm cells and contaminants, such as semen extender or cryoprotectants is required. In addition, the vital sperm cells have to be concentrated in a small volume [2]. Different sperm purification methods have been described in the past and are routinely used for preparing sperm for *in vitro* fertilization as reviewed by Henkel and Schill [3]. The main techniques are based on migration, such as the so called “Swim-up” technique, or density gradient centrifugation (e.g. BoviPure™ gradient) [3]. Although the purpose of these procedures is to purify the viable, motile sperm fraction and thereby enhance the quality of a semen sample, they can negatively affect spermatozoa by different means. In particular, repeated centrifugation steps may lead to sperm damage with decreasing motility and high levels of reactive oxygen species production [4]. In general, sperm purification techniques should be easy to use, cost efficient, yield high sperm recovery rates, and select a clean, good quality sperm fraction while damaging the sperm to the least possible extent [3].

BoviPure™ gradient centrifugation is routinely used to prepare bull semen for *in vitro* fertilization (IVF). BoviPure™ has been specifically developed for purification of cattle semen and is an iso-osmotic salt solution, which contains colloidal, saline-coated silica

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particles [5]. It is commonly used as a two-layer gradient consisting of a 40% solution layer on top of an 80% layer [6–8]. This method is labor intensive and a longer training period is necessary to perform it proficiently, especially for beginners. In the past years, a heterogenous group of (microfluidic) chip devices have been suggested for easy sperm preparation [9–13]. They make use of rheotaxis, thermotaxis, and chemotaxis behavior of sperm, and successfully select sperm with improved morphology [14], DNA integrity [15] and/or motility [16].

In this study we aim to evaluate a novel chip-based sperm separation device, the VetCount™ Harvester (Motility Count ApS, Copenhagen, Denmark), for its suitability of preparing frozen-thawed bull semen for IVF. To this end, we assessed the recovery rate, quality parameters (motility, morphology, acrosome integrity, membrane fluidity, intracellular calcium concentration, and DNA integrity) and the fertilizing ability of spermatozoa after VetCount™ Harvester preparation, and compared it to the commercially available discontinuous BoviPure™ gradient.

2. Material and methods

2.1. Media and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), AppliChem GmbH (Darmstadt, Germany) and ThermoFisher Scientific (Waltham, Massachusetts, USA) unless otherwise stated. Acridine Orange, Fluo-4/AM, Hoechst 33342, Hoechst 33258, and PNA-AlexaFluor®647 were all purchased from ThermoFisher Scientific while Merocyanine 540 was obtained from Sigma-Aldrich. MitoTracker® Red FM and Green FM (ThermoFisher Scientific) were dissolved in dimethyl sulfoxide (DMSO) and stored as stock solutions (1 mM) at −20 °C. Freshly before each experiment working solutions were prepared in WashTALP (305–315 mOsmol), a Tyrode's Albumin-Pyruvate-Lactate (TALP) basic medium (114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 2 mM $NaHCO_3$, 10 mg/L phenol red, 1 mM Na-pyruvate, 3.06 mL/L Na-lactate, 50 mg/L gentamicin) supplemented with 23.8 mM $NaHCO_3$, 0.01 mM HEPES and 6 mg/mL BSA.

2.2. Device description

The VetCount™ Harvester (MotilityCount ApS, Copenhagen, Denmark) is a device the size of a microscope slide which consists of an upper and a lower chamber separated by a microporous (10 μm) membrane. According to the manufacturer's manual, semen is filled in the lower chamber, while medium, suitable for sperm, is placed in the upper chamber (Fig. S1). The VetCount™ Harvester is then incubated for 30 min. During incubation, motile sperm cells actively swim through the pores into the upper chamber and can be directly aspirated after the incubation period has been completed.

2.3. Experimental design

In a first step (Experiment 1), sperm quality before and after semen processing with either a BoviPure™ density gradient or the VetCount™ Harvester was compared. In addition, sperm recovery was assessed for both methods. The quality parameters included motility (CASA), morphology, and flow cytometrically determined DNA integrity (SCSA), viability, acrosome integrity, membrane fluidity, and intracellular calcium concentration.

A side experiment evaluated whether sperm recovery for the VetCount™ Harvester was stable when reducing the semen input from 1 mL to 0.20 mL.

In a second step (Experiment 2), the fertilizing ability of spermatozoa after processing with the VetCount™ Harvester was tested in a competitive *in vitro* fertilization assay against spermatozoa processed by a BoviPure™ density gradient.

2.4. Experiment 1: assessment of sperm parameters

2.4.1. Sperm preparation

Frozen semen samples were obtained from a commercial AI center and the Institute of Farm Animal Genetics. Sperm parameters of frozen-thawed semen of six different bulls ($n = 6$), one ejaculate per bull, were assessed. The bulls were of the breeds Holstein Friesian, German Black Pied cattle (DSN; German: Deutsches Schwarzbuntes Niederungsrind), and Limousin. For each bull 10 straws of frozen semen were thawed for 2 min in a water bath at 35 °C and pooled afterwards. The sperm pool was divided into three subsamples.

Subsample 1 was purified with the VetCount™ Harvester (MotilityCount ApS, Copenhagen, Denmark; hereafter also simply referred to as Harvester) according to the manufacturer's instructions. The Harvester and WashTALP were pre-equilibrated for at least 1 h in an incubator with a humidified atmosphere at 5% CO_2 in air at 38 °C. Utilizing a 1 mL-syringe, 1 mL of semen was injected into the lower chamber of the Harvester. In the upper chamber 800 μL of WashTALP were applied. The loaded Harvester was incubated in a horizontal position for 30 min at 38 °C in a humidified atmosphere at 5% CO_2. At the end of the incubation process, 700 μL sperm-containing medium were aspirated from the upper chamber.

For Subsample 2, two tubes with a discontinuous BoviPure™ (Nidacon International AB, Mölndal, Sweden) gradient (1 ml 40% on 1 ml 80%) were prepared in 15 mL centrifugation tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) directly before thawing the semen. Three hundred microliters of semen were layered on each of the BoviPure™ gradients, followed by centrifugation for 10 min at 300×*g* (room temperature). After centrifugation, the supernatant was removed without disturbing the sperm pellet at the bottom of the tube. Three milliliters pre-equilibrated WashTALP were added and another centrifugation step for 3 min at 300×*g* (room temperature) was carried out. The supernatant was aspirated and discarded down to the 100 μL mark. Two hundred microliter pre-equilibrated WashTALP were added to each tube. Tubes were gently mixed and the content subsequently combined. The BoviPure™ and Harvester treatment were performed simultaneously (Fig. S2).

Subsample 3, i.e. the remainder of the pooled, thawed semen, received no further treatment and served as unprocessed control sample.

Samples for determining DNA fragmentation, sperm morphology, sperm concentration and flow cytometry analysis were taken immediately after thawing (control) and after semen processing with the VetCount™ Harvester or BoviPure™ gradient centrifugation.

2.4.2. Assessment of sperm recovery

Samples were diluted 1:2 to 1:60 in saturated NaCl depending on initial sperm concentration. Sperm concentration was determined using a Neubauer hemocytometer chamber. Minimum sperm count on each side of the chamber for assessing sperm concentration was 100 cells. Sperm recovery rate after processing by BoviPure™ and Harvester was calculated by dividing the total sperm count of the processed semen by the total sperm count of the semen which was initially used for the respective technique.

2.4.3. Assessment of sperm morphology

Samples were fixed in 4% paraformaldehyde and stored at 8 °C until assessment. A total of 200 spermatozoa per sample were

assessed using phase contrast microscopy (X 1000, oil immersion). Scoring was performed according to morphology classifications proposed by Krause (1966) [17]. Scored abnormalities were summarized for those found in the acrosome, head, neck, midpiece, principal piece, and end piece, respectively. For each spermatozoon all defects were recorded.

2.4.4. Assessment of sperm motility

The unprocessed semen (control) was diluted 1:10 in pre-equilibrated WashTALP to yield a sperm concentration permitting CASA-based motility assessment. Control sample and BoviPure™ treated sample were incubated at 38 °C in an incubator with 5% CO_2 in humidified atmosphere. After 5- and 30-min incubation motility was assessed. The 5-min timepoint was chosen to resemble the practical situation in the lab, where BoviPure™ selected spermatozoa are checked for motility within 5 min after purification before being added to matured oocytes. The assessment after 30 min incubation was chosen to resemble an equally long exposure to the WashTALP as for sperm in the Harvester device. Sperm from the Harvester were assessed for motility immediately after retrieval from the upper chamber.

Sperm motility was evaluated using IVOS II, a computer-assisted sperm analysis (CASA) system from Hamilton Thorne (Beverly, MA, USA). All equipment was prewarmed to 37 °C. For analysis, sperm samples (3 μL) were loaded into a Leja-chamber (20 μm depth, 4-chamber slides; Leja, Nieuw Vennep, the Netherlands). Ten fields were analyzed with a frame capture speed of 60 Hz and a recording time of 0.5 s per field. Spermatozoa were classified as motile when they moved more than their head length from their original position. They were classified as progressive motile when straightness (STR) exceeded 30% and the velocity of the average path (VAP) exceeded 15 μm/s. Further information about the CASA settings can be found in Table S1. All data were manually revised by one observer to exclude large egg yolk particles which had been misclassified as sperm cells from the results.

2.4.5. Assessment of DNA fragmentation

Sperm samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Samples were analyzed according to the protocol of Evenson (2022) [18]. In brief, thawed sperm samples were adjusted to 2×10^6 spermatozoa/mL to a total volume of 200 μL in TNE buffer. Four hundred microliter of acid detergent were added to each tube. The tubes were vortexed and the reaction was left for 30 s on ice. Then, 1.2 mL acridine orange staining solution (6 μg/mL) were added and incubated for 2.5 min on ice. After incubation, samples were vortexed briefly and analyzed using a Gallios Flow Cytometer (Beckman Coulter, Krefeld, Germany) with a 488 nm laser (22 mW) and filters for green fluorescence (525/20 nm) and red fluorescence (575/20 nm). Samples were read on low speed. Fluorescence intensities were plotted on linear scales and voltages adjusted to yield an average fluorescence intensity of 120 units for green fluorescence and 30 units for red fluorescence with a reference sample. Data were analyzed using FCS Express (Version 3, De Novo Software; Pasadena, CA, USA). The DNA fragmentation index was calculated as in Evenson (2016) [19]:

$$DFI = \frac{red\ fluorescence}{total\ (red + green)\ fluorescence}$$

The percentage of spermatozoa with moderate to high DNA fragmentation index is reported as %DFI. All samples were prepared and analyzed in duplicates. At the start and end of each flow cytometry session and after every twenty samples, a reference sample with known %DFI was read as biological control.

2.4.6. Simultaneous assessment of viability, acrosome integrity, membrane fluidity, and intracellular Ca^{2+} concentration

The flow cytometer (Cytoflex S; Beckman Coulter, Krefeld, Germany) was equipped with four lasers (violet: 405 nm, 70 mW; blue: 488 nm, 50 mW; yellow: 561 nm, 20 mW; red: 638 nm, 50 mW) and filters for blue (450/45 BP), green (525/40 BP), red (585/42 BP) and far-red fluorescence (660/10 BP). For each sample, signals from 10,000 single spermatozoa, as defined by forward and side scatter characteristics, were recorded at medium flow rate. Data analysis was performed using FCS express software (Version 3, De Novo Software). Spectral overlap was compensated after acquisition.

The assay was carried out in analogy to Umair et al., 2023 [20]. Sperm viability, acrosome integrity, plasma membrane fluidity, and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was assessed using Hoechst 33342 (final concentration: 0.5 ng/mL), Hoechst 33258 (0.5 μg/mL), PNA-Alexa Fluor™ 647 (0.1 μg/mL), Merocyanine 540 (3 μM) and Fluo-4 (1 μM), respectively. Samples diluted in WashTALP were incubated in an incubator at 38 °C in an atmosphere of 5% CO_2-in-air for 15 min. Fluorescent signals were assessed using the following lasers and filters: Hoechst 33342 and Hoechst 33258 (laser: 405 nm; filter 450/45 nm BP), Fluo-4 (488 nm; 525/40 nm BP), Merocyanine 540 (561 nm; 585/42 nm BP), and PNA-Alexa-Fluor™647 (638 nm; 660/10 nm BP). The single sperm population was identified by forward and side scatter characteristics. Additional discrimination of non-sperm events with a size of single spermatozoa and DNA-containing events, i.e. spermatozoa, was achieved with histograms of the combined signal intensity for Hoechst 33342 and 33258. Next, the subset of viable (H33258-negative), acrosome-intact (PNA-negative) spermatozoa (VAI) was defined and further sub-classified. VAI spermatozoa had either low (M540 low) or high plasma membrane fluidity (M540 high) and either a low free $[Ca^{2+}]_i$ (low Fluo-4 fluorescence intensity) or a high free $[Ca^{2+}]_i$ (high Fluo-4 fluorescence intensity). The percentage viable, acrosome intact spermatozoa (VAI) and the percentage viable, acrosome intact spermatozoa with low plasma membrane fluidity and low free $[Ca^{2+}]_i$ are reported. In addition, the geometric mean of the Fluo-4 fluorescence intensity in viable, acrosome intact spermatozoa was assessed. The Fluo-4 fluorescence intensity in viable, acrosome intact sperm varied considerably between bulls. However, the focus of the study was not on inter-bull comparisons, but on a comparison of the impact of semen processing on the free intracellular calcium concentration. Therefore, the data were normalized. To this end, the geometric mean of the Fluo-4 fluorescence intensity in a thawed, non-processed sample (control) was considered as reference point for a given bull and set as 1. Subsequently, all values from a sample after processing with BoviPure™ or the VetCount™ Harvester were normalized by dividing the average value for the fluorescence intensity after processing with either VetCount™ Harvester or BoviPure™ gradient by the value for the fluorescence intensity after thawing.

2.4.7. Effect of volume on recovery rate

Routinely, only one to two straws of 0.25 mL frozen-thawed semen are needed for one IVF session. This is less volume than what is needed for loading the VetCount™ Harvester. Therefore, a small side experiment was carried out in which 1 mL diluted semen, i.e. 200 μL frozen-thawed semen diluted in 800 μL WashTALP, instead of 1 mL pure, frozen-thawed semen was processed with the Harvester. Frozen semen samples were from the same bulls and ejaculates as in the main experiment (n = 6). Sperm concentration was determined before and after Harvester processing.

2.5. Experiment 2: competitive fertilization assay

A competitive fertilization assay was carried out to compare the

fertilizing ability of sperm cells processed with the Harvester directly to that of sperm cells processed by BoviPure™ density gradient centrifugation.

2.5.1. Oocyte collection

Bovine ovaries were collected at a local abattoir and transported in a thermo-insulated box within 2 h to the laboratory. Ovaries arrived with a temperature between 22 °C and 28 °C. They were washed four times in 37 °C physiological saline supplemented with 0.06 g/L Penicillin G and 0.13 g/L Streptomycin sulphate. Follicles with a diameter of 2–5 mm were punctured utilizing an 18-gauge needle attached to a 10-mL syringe. Cumulus-oocyte-complexes (COCs) were harvested and handled in HEPES-TALP (TALP basic medium supplemented with 0.01 mM HEPES and 0.40 mg/mL BSA; 275 to 285 mOsmol, pH 7.2).

2.5.2. In vitro maturation

Cumulus-oocyte-complexes of good quality (homogenous cytoplasm, minimum three compacted cumulus cell layers) were washed once in TCM199 (ThermoFisher Scientific, oNo. 31150022) supplemented with 20 ng/mL epidermal growth factor and 50 μg/mL gentamicin, and then matured in groups of 50–70 COCs in 500 μL of the same medium in a plastic four well dish. Maturation was carried out for 22 h at 38.5 °C in an incubator set to 5% CO_2-in-air in a humidified atmosphere.

2.5.3. Sperm preparation

Semen from six different bulls ($n = 6$) was used. The bulls and semen batches were identical to those used for assessment of sperm recovery and quality. For each bull, 10 straws of cryopreserved semen from one ejaculate were thawed in a water bath at 35 °C for 2 min. The content of 10 straws was pooled and divided into two subsamples. The subsamples were processed simultaneously (Fig. S3).

Subsample 1 was processed with the VetCount™ Harvester as described in Experiment 1. Thereafter, the sperm suspension (700 μL) was transferred to a 15 mL centrifugation tube and 3.3 mL WashTALP were added. A centrifugation step for 3 min at 300×*g* (room temperature) followed and the supernatant was removed down to the 100 μL mark of the tube. This extra washing step had to be included to remove remaining egg yolk particles that would impair the MitoTracker® staining process. Next, WashTALP was added to yield a total volume of 1 mL.

Subsample 2 was processed with BoviPure™ gradients as described in Experiment 1. Processed sperm were split into three aliquots. All three tubes were filled to a total amount of 1 mL with WashTALP. They were kept for about 15 min at 38.5 °C in a humidified atmosphere with 5% CO_2-in-air, until sperm processing with the Harvester was finished.

2.5.4. Staining with MitoTracker® dyes

After processing sperm with the Harvester and BoviPure™ gradient, four tubes with 1 mL were available. One tube was from the Harvester preparation and three tubes were from the BoviPure™ preparation. Spermatozoa were fluorescently labelled with either MitoTracker® Red FM or Green FM by adding 1 mL of dye solution (800 nM MitoTracker® in WashTALP) to 1 mL sperm suspension (final concentration for MitoTracker®: 400 nM). For example, sperm from the Harvester were stained with MitoTracker® Green FM and sperm from the BoviPure™ gradient were stained with MitoTracker® Red FM. Control samples for any effect of the MitoTracker® dyes were only run for BoviPure™-processed samples. Control samples comprised a basic control (1 mL WashTALP) and a solvent control (1 mL WashTALP with 0.08% DMSO added). All treatments were incubated for 15 min at 38.5 °C in a humidified atmosphere with 5% CO_2-in-air and then centrifuged at 300×*g* for 3 min (room temperature). The supernatant was removed and 3 mL WashTALP were added to each of the four tubes. Another centrifugation step at 300×*g* for 3 min followed. Supernatant was removed again down to the 100 μL mark and a 10 μL sample from each tube was taken to evaluate sperm motility via IVOS II. The basic control was only used to verify that neither DMSO nor the processing itself had a negative impact on sperm motility. For the other three samples, sperm concentrations were determined using a Neubauer hemocytometer and each sample was adjusted to 2×10^6 motile sperm cells/mL in FertTALP (TALP basic medium supplemented with 23.8 mM $NaHCO_3$, 6 mg/mL BSA and 0.1 I.U./mL heparin, 10 μM hypotaurine and 1 μM epinephrine; 305 to 315 mOsmol). Equal numbers of motile BoviPure™ sperm and Harvester sperm were mixed.

To exclude a systematic effect of one of the MitoTracker® dyes on the spermatozoa's fertilizing ability, two independent *in vitro* fertilization sessions were performed for each bull. MitoTracker® dyes were swapped between the sessions for sperm processed by BoviPure™ or Harvester.

2.5.5. In vitro fertilization

Presumptive matured cumulus-oocyte-complexes were washed once in FertTALP and then placed in groups of 50–70 COCs in 250 μL FertTALP in a plastic four-well dish. For fertilization, 250 μL sperm suspension (2×10^6 motile sperm cells/mL) were added to each well (final sperm concentration: 1×10^6 motile sperm cells/mL). One oocyte group was fertilized with spermatozoa from the solvent control, another oocyte group with the mix of spermatozoa from the two semen preparations (Harvester/BoviPure™). Sperm cells and COCs were co-incubated for 18 h at 38.5 °C in a humidified atmosphere with 5% CO_2. The IVF for the bulls was performed on different days.

2.5.6. Fluorescence microscopy

All presumptive zygotes were decumulated by vortexing and stained using Hoechst 33342 (5 μg/mL) for 10 min at room temperature. After washing in phosphate-buffered saline (AppliChem, A0964; 9.55 g/L) supplemented with 0.1% polyvinyl alcohol, control zygotes and zygotes fertilized with the BoviPure™/Harvester-sperm-mix were mounted separately in groups of 10 zygotes in a drop of 1,4-diazabicyclo[2.2.2]octane (DABCO; 25 mg DABCO® (Carl Roth, Karlsruhe, Germany; oNo. 0718) in 1 mL PBS and 9 ml Glycerol, pH 8.6) on microscopy slides. On the same day, zygotes were evaluated by fluorescence microscopy (Zeiss Axioscope 5 with Colibri 5 LED light and Axiocam 202 mono). A multi band pass filter (FS 90 HE LED; Carl Zeiss GmbH, Oberkochen, Germany) and excitation at 469/38 nm BP and 385/30 nm BP was used to visualize MitoTracker® Green FM and Hoechst 33342. Filter set 45 (Carl Zeiss GmbH) and excitation at 555/30 nm BP was used for visualizing MitoTracker® Red FM. All zygotes were screened for the occurrence of (at least) two pronuclei to confirm fertilization. Polyspermic fertilized zygotes were classified as such by the occurrence of a minimum of three pronuclei. Fertilization rates of zygotes from the control group and from zygotes fertilized with BoviPure™/Harvester-prepared sperm were compared to exclude a potential negative affect of the MitoTracker® dyes on fertilizing ability of the spermatozoa. Additionally, the origin of the fertilizing sperm cell(s) was evaluated for zygotes fertilized with BoviPure™/Harvester-prepared, i.e. MitoTracker®-labelled, sperm (Fig. 1).

2.6. Statistical analysis

Data were analyzed using Excel® and GraphPad Prism® (Version 9.3.1). Student's t-test for paired samples with Bonferroni-

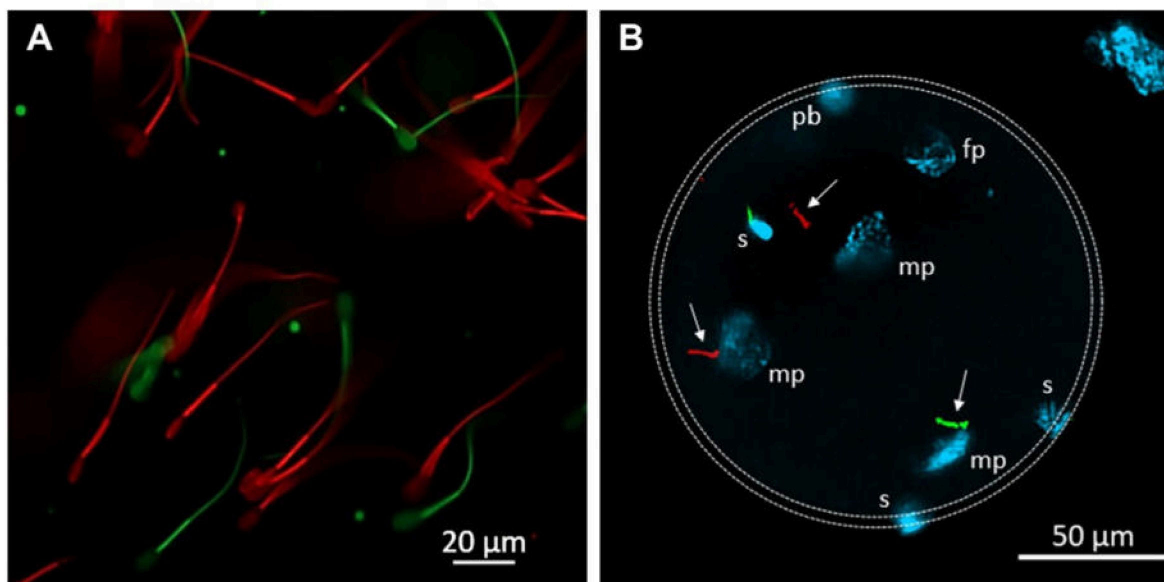

Fig. 1. Evaluation of the origin of the fertilizing sperm cell.
A) Representative image of a mix of spermatozoa labelled with MitoTracker® Red FM or Green FM prior to insemination. B) Overlay image of a zygote penetrated by three spermatozoa at 18 h post fertilization. Spermatozoa were labelled with MitoTracker® Green FM and Red FM prior to insemination. DNA (blue) is stained with Hoechst 33342. Please note that sperm attached to the zona pellucida may have lost their tail due to the mechanical denudation process. Dotted line: zona pellucida; FP: female pronucleus; MP: male pronucleus; S: zona attached sperm cell; P: polar body; →: midpiece of penetrated spermatozoa with decondensed DNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Holm correction was used to analyze the data on sperm quality and sperm recovery.

In the fertilization assay, the total number of monospermic fertilized zygotes with sperm after BoviPure™ or Harvester treatment was compared with a Chi square (χ^2) test. Likewise, the number of penetrated sperm cells for polyspermic fertilized zygotes was compared.

Data are presented as mean ± standard deviation and were considered to be significantly different when $P < 0.05$.

3. Results

3.1. Experiment 1: sperm quality parameters

3.1.1. Sperm motility parameters

To focus on the practical relevance, we compared the data of 5 min incubated BoviPure™ treated sperm and unprocessed sperm with the data of the Harvester treated semen. Nevertheless, data after 30 min incubation can be found in the supplemental files (Table S2).

CASA analysis revealed that BoviPure™ gradient centrifugation and VetCount™ Harvester both doubled the proportion of motile sperm cells (86.3 ± 14.6% and 89.4 ± 8.3%) compared to the untreated semen (42.5 ± 19.4%; $P < 0.05$; Table 1). Similarly, both treatments significantly increased the proportion of progressively motile spermatozoa (Fig. 2).

3.1.2. Flow cytometry analyses

Both treatments nearly doubled the proportion of viable, acrosome intact (VAI) spermatozoa (Fig. 2). The VAI sperm fraction was significantly higher for the Harvester treatment (88.8 ± 5.3%) than for the BoviPure™ gradient (86.5 ± 4.7%) (Fig. 2). Following both purification methods, the sperm population consisted of a significantly greater percentage of VAI spermatozoa with a low free intracellular Ca^{2+} concentration and low membrane fluidity compared to the untreated semen (78.6 ± 6.0% and 76.5 ± 4.4% vs. 37.1 ± 13.2%; Fig. 2). After normalizing the free intracellular calcium concentration $[Ca^{2+}]_i$ of VAI spermatozoa (unprocessed sperm: $[Ca^{2+}]_i = 1$) we noted that the Harvester-selected spermatozoa had an even lower $[Ca^{2+}]_i$ (0.67 ± 0.1; $P < 0.05$) than the BoviPure™ gradient-selected sperm (0.84 ± 0.14; Table 3). Determination of the DNA fragmentation index (DFI) showed that only BoviPure™ gradient centrifugation significantly reduced the %DFI of the selected sperm population. However, the %DFI of the non-treated semen was already low (1.0 ± 0.2%; Table 3).

3.1.3. Sperm morphology

Following both sperm preparation methods, the obtained sperm population consisted of a significantly higher percentage of spermatozoa with normal morphology compared to unprocessed

Table 1
Sperm motility parameters of untreated (5 min), BoviPure™ gradient-treated (5 min) and Harvester-treated bovine semen samples (n = 6 bulls). All values are mean ± SD. Small uppercase letters (a, b) indicate a significant difference in one column between samples ($P < 0.05$).

Treatments	Total Motility (%)	Progressive Motility (%)	VCL (µm/s)	LIN (%)	ALH (µm)	BCF (Hz)
Untreated	42.53 ± 19.35^a	41.85 ± 18.79^a	229.49 ± 31.14^a	49.92 ± 3.78^a	9.44 ± 1.12^a	31.44 ± 4.42^a
BoviPure™ gradient	86.33 ± 14.60^b	84.43 ± 14.10^b	260.57 ± 32.33^b	46.47 ± 5.95^{a,b}	11.34 ± 1.03^b	27.50 ± 2.38^b
VetCount™ Harvester	89.36 ± 8.30^b	85.13 ± 7.75^b	224.76 ± 13.49^a	41.87 ± 2.88^b	10.51 ± 0.67^a	28.41 ± 2.65^b

VCL: curvilinear velocity; LIN: linearity; ALH: average amplitude of lateral head displacement; BCF: beat cross frequency.

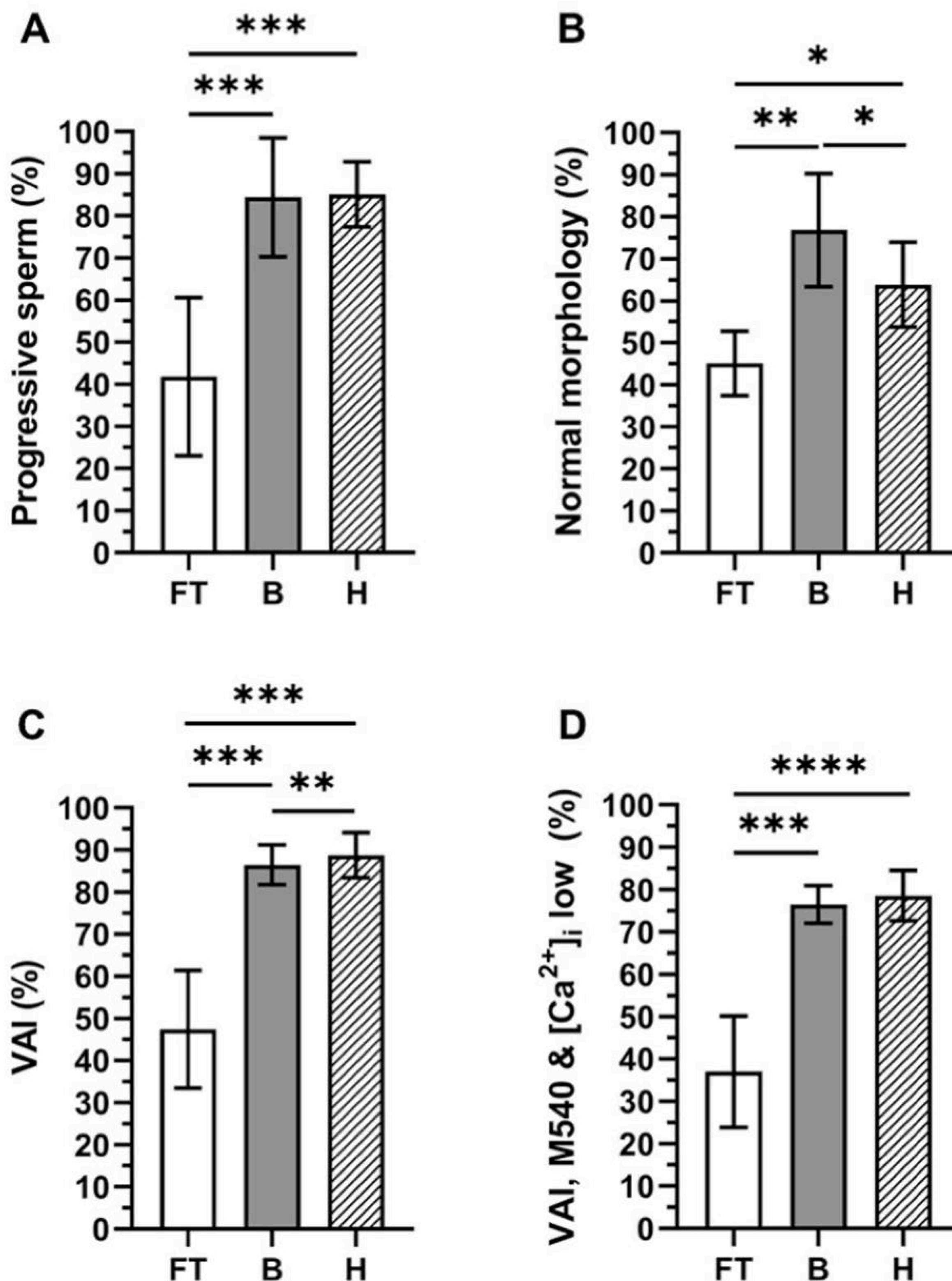

Fig. 2. Sperm quality parameters for only frozen-thawed (*FT*), BoviPure™ purified (*B*) and VetCount™ Harvester-treated sperm (*H*). Proportions of A) progressively motile sperm cells, B) spermatozoa with normal morphology, C) viable, acrosome intact (VAI) spermatozoa and D) VAI sperm cells with low membrane fluidity (M540 low) and low free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ are shown. Results are presented as mean ± SD, n = 6 bulls. Stars indicate a significant difference between samples: ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05.

Table 2
Flow cytometry analyses of untreated, BoviPure™ gradient-treated and Harvester-treated bovine semen samples (n = 6 bulls). All values are mean ± SD. Small letters (a, b, c) indicate a significant difference in one column between samples (P < 0.05).

Treatment	VAI (%)	VAI, M540 low & $[Ca^{2+}]_i$ low (%)	Normalized $[Ca^{2+}]_i$	%DFI
Untreated	47.4 ± 13.9^a	37.1 ± 13.2^a	1^a	1.04 ± 0.21^b
BoviPure™ gradient	86.5 ± 4.7^b	76.5 ± 4.4^b	0.84 ± 0.14^b	0.12 ± 0.07^a
VetCount™ Harvester	88.8 ± 5.3^c	78.6 ± 6.0^b	0.67 ± 0.10^c	0.91 ± 0.20^b

VAI: viable, acrosome intact; $[Ca^{2+}]_i$: free intracellular calcium concentration; %DFI: % spermatozoa with moderate to high DNA fragmentation index.

semen (Fig. 2). The improvement was even higher after the BoviPure™ gradient centrifugation (76.8 ± 13.4%; P < 0.05) than for Harvester treated semen (63.8 ± 10.2%) (Fig. 2). Semen purified via BoviPure™ gradient centrifugation or VetCount™ Harvester treatment had only half of the proportion of spermatozoa with a defect acrosome (16.6 ± 11.5% and 20.3 ± 14.2%) compared to unprocessed semen (39.8 ± 9.4%; P < 0.05; Table 2). Interestingly, we observed significantly less spermatozoa with a curled end piece in the BoviPure™-treated semen than in the Harvester-treated samples (Table 2).

3.1.4. *Sperm recovery*

Sperm recovery was not significantly different after VetCount™ Harvester treatment of 1 mL frozen-thawed semen, 200 μL frozen-thawed semen, or BoviPure™ gradient centrifugation (12.4 ± 3.6%, 10.8 ± 2.7% and 14.4 ± 5.1%, respectively). Noteworthy, Harvester-treated samples were less concentrated compared to samples prepared by BoviPure™ gradient centrifugation due to the large fluid volume which was retrieved from the collection chamber (Table 4).

3.2. *Experiment 2: competitive fertilization assay*

3.2.1. *Fertilization rates*

In this experiment a total of 735 presumptive zygotes (123 ± 11 per bull) fertilized with the BoviPure™/Harvester-sperm-mix and 434 zygotes (72 ± 33 per bull) from the control group (DMSO only) were screened for pronuclei. The control group reached a fertilization rate of 73.6 ± 13.0%, the BoviPure™/Harvester group 83.7 ± 21.9%. The overall polyspermy rate for the control group was 19.5 ± 9.8% and 28.3 ± 12.7% for the BoviPure™/Harvester group. The ratio of not fertilized, monospermic, or polyspermic fertilized zygotes from the BoviPure™/Harvester group is given in Fig. 3 for each bull.

3.2.2. *Fertilizing ability*

The evaluation focused primarily on the number of monospermic fertilized zygotes after competitive *in vitro* fertilization with sperm from a BoviPure™ and VetCount™ Harvester preparation. In three bulls, the fertilization rates did not differ for sperm from both preparations (Fig. 4A; Fig. S4A). However, in two bulls, sperm cells selected by the VetCount™ Harvester fertilized significantly more oocytes than BoviPure™ treated spermatozoa (Fig. 4A; S4A). But, in one bull the opposite result was observed, i.e. more oocytes were fertilized by spermatzoa after BoviPure™ gradient centrifugation (Fig. 4A; S4A). The labeling with either MitoTracker® Red FM or MitoTracker® Green FM had no significant impact on the fertilizing ability of the spermatozoa.

In zygotes that showed polyspermy the total number of penetrated sperm cells was counted and assigned to either VetCount™ Harvester or BoviPure™ gradient treatment. Only in one bull significantly more Harvester than BoviPure™-treated spermatozoa penetrated oocytes (Fig. 4B; S4B). For the other bulls no significant difference between the number of penetrated sperm from each preparation method was observed (Fig. 4B; Fig. S4B).

4. Discussion

The aim of this study was to evaluate the VetCount™ Harvester as a new method for purifying frozen-thawed bull semen for *in vitro* fertilization. Sperm quality parameters and fertilizing ability of Harvester-selected spermatozoa were assessed and compared to sperm cells separated by BoviPure™ gradient centrifugation.

VetCount™ Harvester treatment and BoviPure™ gradient centrifugation both yielded a high-quality sperm fraction. Either method significantly improved normal morphology, motility, and viability of the bovine semen samples and selected predominantly viable, acrosome intact sperm cells with low membrane fluidity and low intracellular calcium concentration ($[Ca^{2+}]_i$). Changes in membrane fluidity and intracellular calcium concentration are indicators for sperm stability and/or sperm activation, i.e. capacitation. It mainly depends on the context of a study or the experimental settings how the parameters can be interpreted. In the early stage of capacitation, phospholipids in the plasma membrane start to rearrange in viable sperm cells and membrane fluidity increases [21,22]. Later on, in the capacitation process, there is a controlled influx of calcium ions from Ca^{2+} storing cell organelles and from the extracellular space into the sperm cell's cytoplasm. This influx is crucial for various cell signaling pathways, it is e.g. involved in hyperactivated motility and acrosome reaction [23]. Therefore, free $[Ca^{2+}]_i$ is tightly regulated and maintained at low levels in spermatozoa. Harvester selected spermatozoa showed the lowest $[Ca^{2+}]_i$, indicating that these sperm cells were more capable of regulating their calcium homeostasis during sperm preparation than BoviPure™ gradient treated spermatozoa. To evaluate if this finding points towards a superior fertilization competence of Harvester-selected sperm over BoviPure™-selected sperm or vice versa, the competitive fertilization assay was performed. However, no consistent pattern in favor of either technique was evident.

Using the VetCount™ Harvester for preparing bovine frozen-thawed semen for IVF has some advantages over the BoviPure™ gradient centrifugation method. Minimal training is required for

Table 3
Selected morphological parameters of untreated, BoviPure™ gradient-treated and Harvester-treated bovine semen samples (n = 6 bulls). All values are mean ± SD. Small letters (a, b, c) indicate a significant difference in one column between samples (P < 0.05).

Treatments	Normal morphology (%)	Defect acrosome (%)	Curled end-piece (%)
Untreated	45.1 ± 7.6^a	39.8 ± 9.4^a	7.2 ± 5.8^{a,b}
BoviPure™ gradient	76.8 ± 13.4^b	16.6 ± 11.5^b	2.1 ± 2.4^a
VetCount™ Harvester	63.8 ± 10.2^c	20.3 ± 14.2^b	13.2 ± 6.9^b

Table 4
Sperm recovery rate, sperm concentration and volume of samples following BoviPure™ gradient centrifugation (300 μL semen purified per gradient) or VetCount™ Harvester treatment (1 mL or 200 μL semen) are shown for each bull. Sperm concentration calculated for BoviPure™ gradient is biased due to the addition of medium (200 μL per gradient) to the obtained sperm suspension before sampling. SD = standard deviation.

	BoviPure™ gradient (300 μL x 2)			VetCount™ Harvester (1 mL)			VetCount™ Harvester (200 μL)		
	Sperm conc. [sperm/μL]	Volume [μL]	Recovery rate [%]	Sperm conc. [sperm/μL]	Volume [μL]	Recovery rate [%]	Sperm conc. [sperm/μL]	Volume [μL]	Recovery rate [%]
Bull 1	12,172	600	16.57	15,094	700	15.14	2258	700	12.19
Bull 2	10,656	600	11.48	14,438	600	10.37	2656	700	12.90
Bull 3	15,984	600	13.60	21,750	750	13.88	4008	700	11,00
Bull 4	8516	600	5.99	8617	800	6.46	1867	700	5.39
Bull 5	17,789	600	19.17	16,313	700	12.30	2555	700	11.06
Bull 6	10,008	600	19.35	12,188	700	16.50	2422	700	11.99
Mean	**12,521**		**14.36**	**14,733**		**12.44**	**2628**		**10.75**
SD	3625		5.14	4378		3.63	731		2.72

handling the Harvester device and the hands-on time is very small. Semen and medium can be easily applied into the chambers according to manufacturer's instructions, and sperm separation occurs within 30 min of incubation. The basic principle of the Harvester is the active movement of motile sperm cells from the lower chamber into the upper chamber by passing a microporous membrane. No significant manipulation steps that could possibly harm the sperm are involved. Though a higher percentage of spermatozoa with curled end pieces in fixed samples from the Harvester treated group was observed, there was no apparent negative effect on sperm motility, function or fertilizing competence.

The migration behavior of sperm has already been exploited for decades in a sperm separation technique so called ‘Swim-up’. For this method, a tube is filled with 1 ml of TALP based medium under which frozen-thawed semen is carefully layered. While incubating for about 1 h, motile sperm cells move to the medium's top and can be aspirated [24]. Because of the similarity in the underlaying separation principle, the classical ‘Swim-up” would have been a more comparable technique to the VetCount™ Harvester. Nevertheless, in this study we decided to compare Harvester treatment with BoviPure™ gradient centrifugation since this procedure is used as a standard in many bovine IVF laboratories.

When comparing the purified sperm suspension between both methods it was obvious that BoviPure™ treated sperm suspensions were much cleaner than Harvester-treated samples. Harvester-treated suspensions contained a considerable amount of egg yolk particles originating from the semen extender. These particles were probably sucked through the pores into the upper chamber when the medium was aspirated. To prevent particles from crossing the membrane, a very simple solution can be to aspirate a smaller volume of purified sperm. However, sperm concentration is already relatively low following Harvester treatment and a reduction of the aspirated volume would likely result in a critically low number of sperm available for IVF, even when purifying 1 mL pure frozen-thawed semen.

Harvester and BoviPure™ treatment yielded a sperm recovery rate between 10% and 15%. In literature, very heterogenous data about sperm recovery rates for different gradient centrifugation protocols can be found. Depending on animal species, kind of gradient, and centrifugation protocol a lot of studies show higher recovery rates (30–60%) [8,25–27] compared to our findings. Others describe similar sperm recovery rates for BoviPure™ gradient treatment of bull semen to what we found [28,29]. Although recovery rates for VetCount™ Harvester and BoviPure™ gradient were not significantly different in our study, Harvester filtrated sperm was less concentrated due to the larger obtained fluid volume. When applying 1 ml pure, frozen-thawed semen to the Harvester, purified sperm is concentrated enough for direct use in IVF. However, when applying a smaller volume of semen, such as a single 0.25 ml straw, the concentration of purified sperm can be too low for direct use in IVF. This should be taken in account since in most bovine IVP laboratories the sperm content of one to two straws of frozen semen normally is sufficient enough for IVF, and

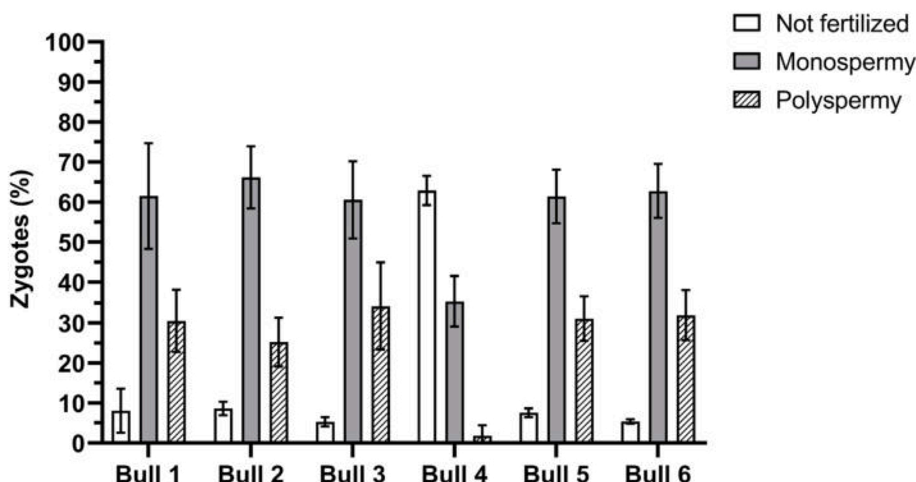

Fig. 3. General distribution of non-fertilized, monospermic and polyspermic fertilized zygotes after *in vitro* fertilization with a mix of sperm prepared by BoviPure™ gradient or VetCount™ Harvester. Data are shown for each of the six bulls as mean ± SD of two independent experiments per bull.

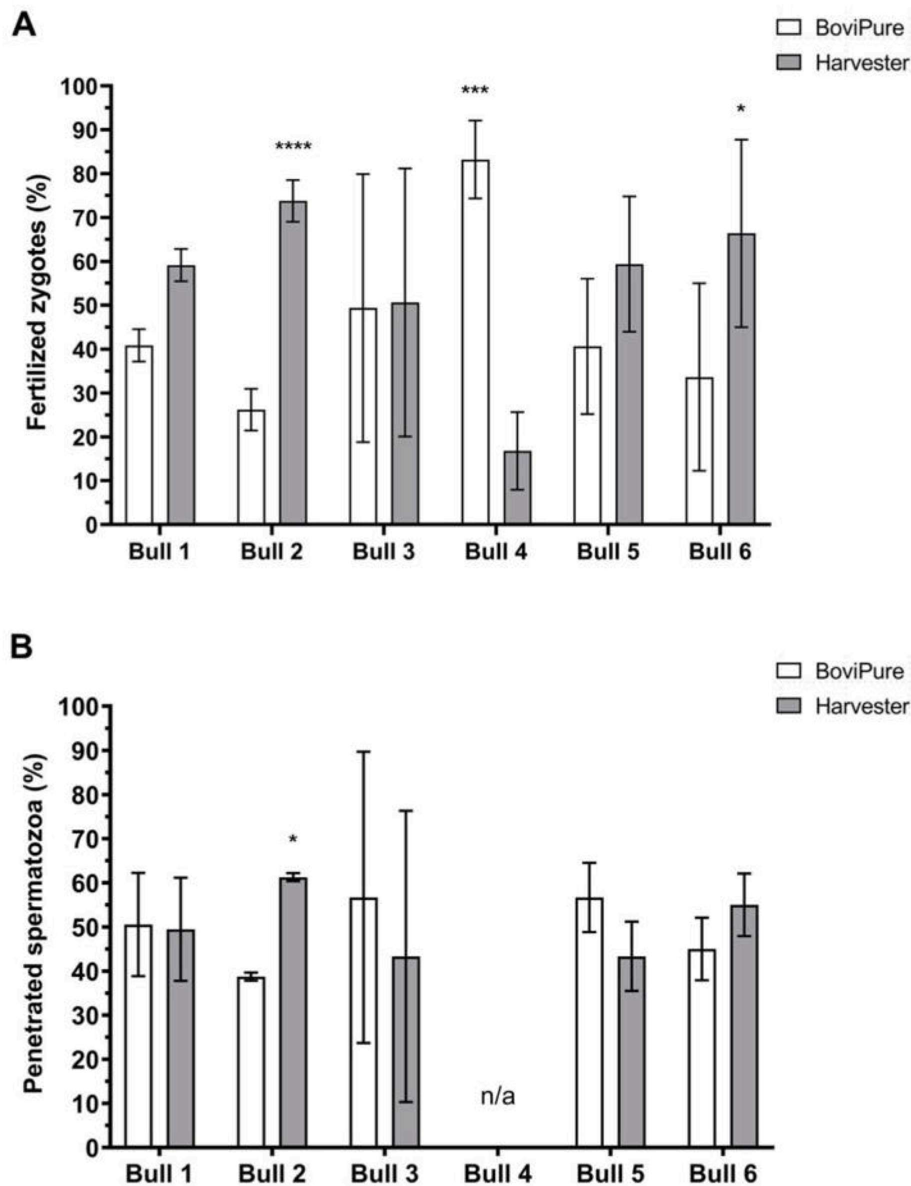

Fig. 4. Evaluation of the origin of the fertilizing spermatozoon.
A) Monospermic fertilized zygotes fertilized by a spermatozoon either after Harvester-treatment or BoviPure™ gradient centrifugation. B) Percentage of spermatozoa after Harvester-treatment or BoviPure™ gradient centrifugation that penetrated zygotes showing polyspermy. Bull 4 was excluded from analysis for Figure B due to only a very small number of polyspermic zygotes. The raw data are available in Fig. S4. Results are represented as mean ± SD of two independent experiments per bull. Stars indicate a significant difference between treatment groups: ****$P < 0.0001$, ***$P < 0.001$, *$P < 0.05$.

using 1 ml pure frozen-thawed semen would be wasteful. Therefore, this is one of the main limitations of the device for its application in commercial bovine IVF companies. To yield higher concentrated, purified sperm with the Harvester technique some adaptions to the system are possible. One option would be to add a chemoattractant, such as progesterone, to the collection medium to increase the number of spermatozoa that migrate into the upper chamber of the Harvester. It is well known (e.g. for bovine, human and rabbits) that progesterone is naturally produced and secreted by the oocyte surrounding cumulus cells and that capacitated sperm cells are chemotactically attracted by it [30–32]. Some microchips have been developed where sperm separation is based on chemotactically behavior of spermatozoa towards a progesterone gradient [10,33,34], and conventional methods like Percoll® gradient were improved by the addition of progesterone [35]. Also, Acetylcholine, another chemoattractant, was successfully used in microfluidic devices to separate good quality spermatozoa [9,36]. A second option to promote sperm migration is to include a temperature gradient into the device to use the thermotactic behavior of sperm, e.g. by inserting micro heaters (described by Yan (2021) [37]) into the Harvester's top. Nevertheless, especially with regard to the last-mentioned option, costs and benefits of any adaption have to be considered.

Even though there are possible adaptions that may further improve the Harvester for preparing frozen-thawed semen for bovine IVF, we see its current application limited to research facilities. For institutions that work with research students, the Vet-Count™ Harvester could be a good alternative for sperm purification to minimize the training period, human error and variability between users.

5. Conclusion

The VetCount™ Harvester selects high-quality, fertile sperm cells from frozen-thawed bull semen similar to sperm obtained by BoviPure™ gradient centrifugation. In a competitive situation with BoviPure™ selected spermatozoa, the fertilization capacity of Harvester selected sperm varies depending on the bull. In contrast to BoviPure™-treated semen, a Harvester-treated sperm suspension is less clean and concentrated.

Declaration of competing interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Rebecca Herbicht: Methodology, Investigation, Formal analysis, Writing – original draft, Visualization, Project administration. **Gregor Neufeld:** Investigation, Formal analysis, Writing – review & editing. **Claudia Klein:** Conceptualization, Writing – review & editing. **Heiko Henning:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Supervision, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.06.019.

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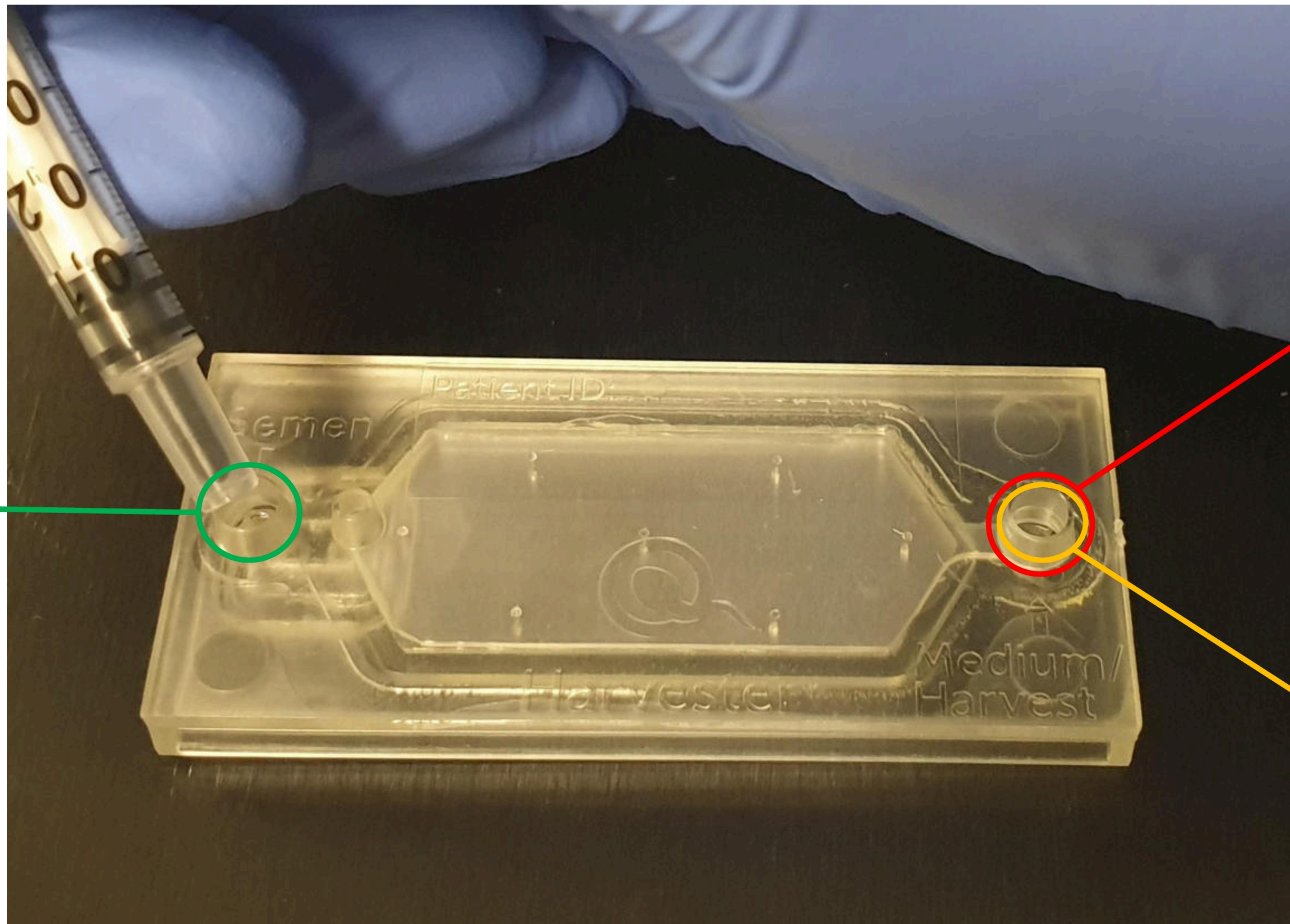


Figure S1. Photograph of the VetCount^{TM} Harvester illustrated with descriptions of the access ports.

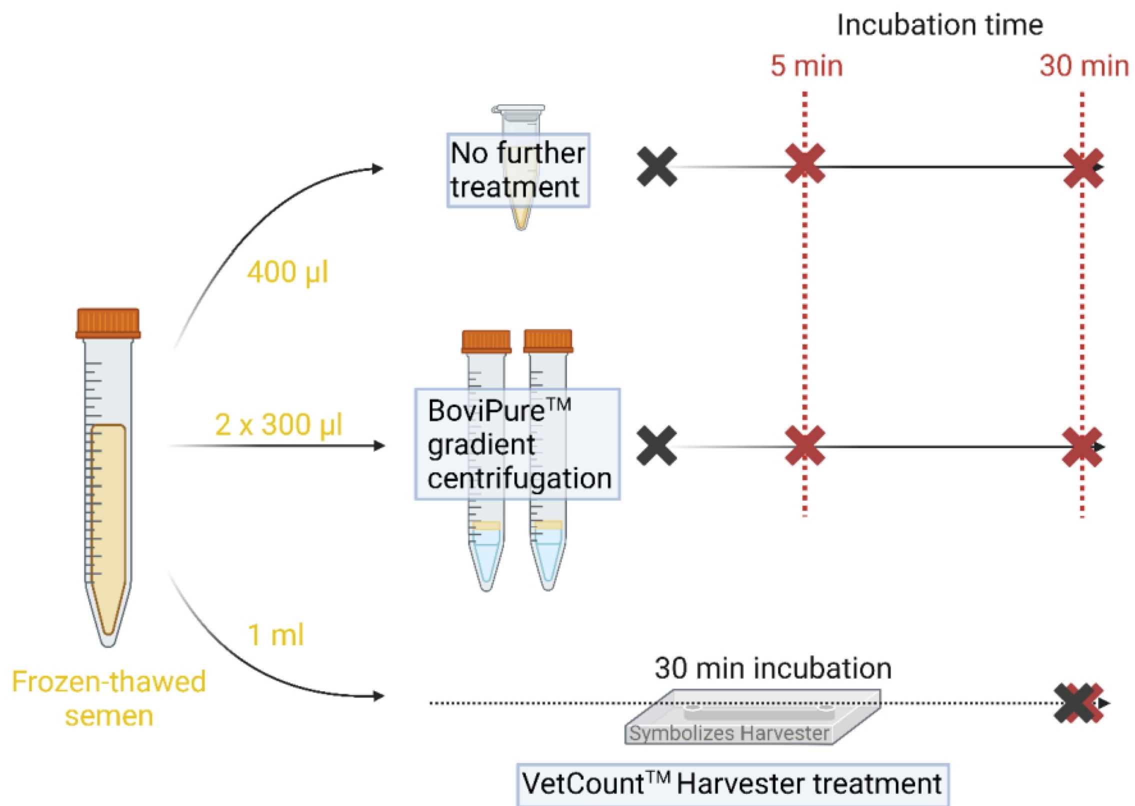


Figure S2. Workflow of sperm preparation for Experiment 1.
X: Sample for sperm motility assessment was taken; **X**: Samples for assessment of sperm morphology, DNA fragmentation and other flow cytometry analyses were taken. Figure was created with BioRender.com.

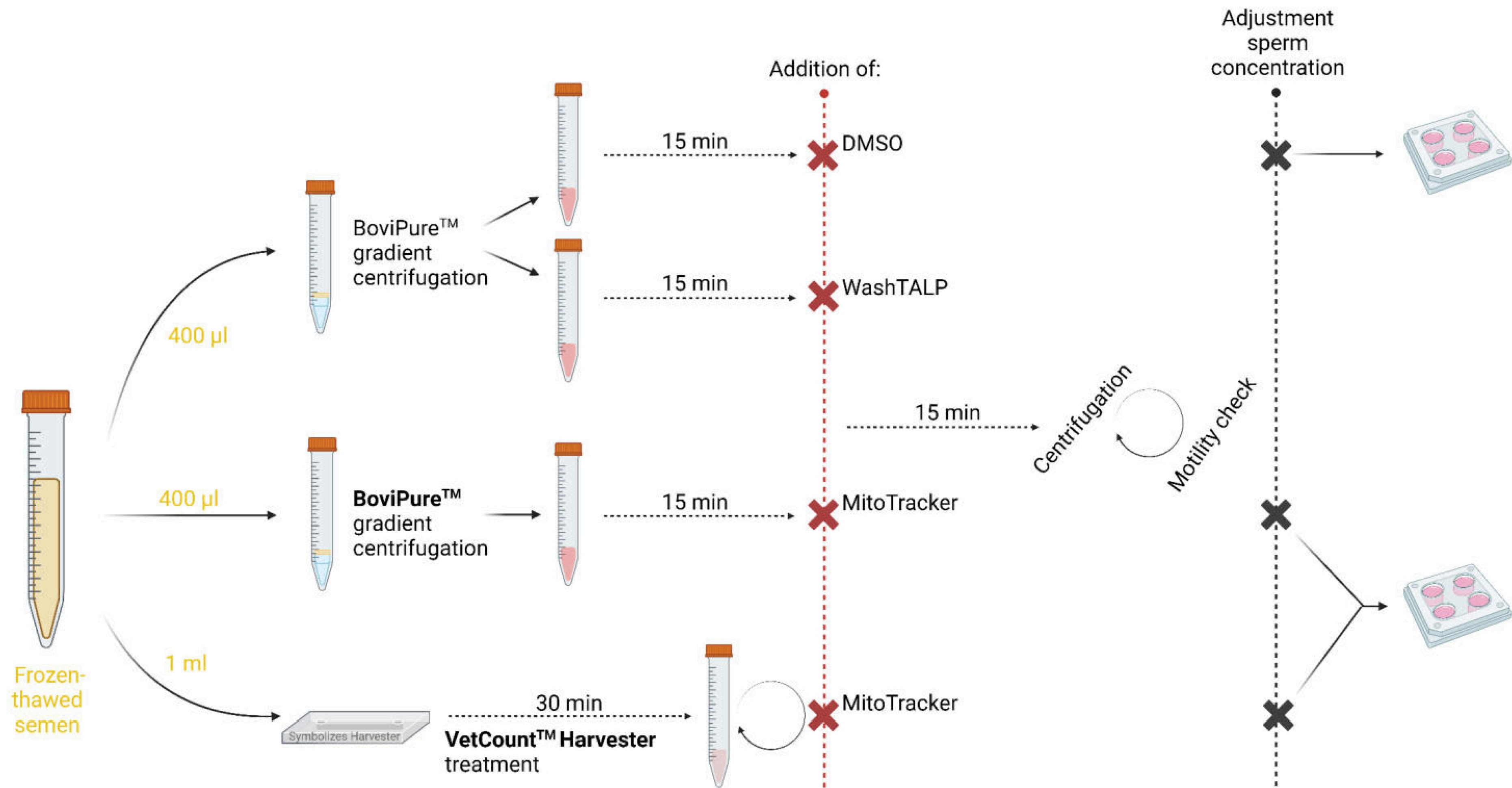
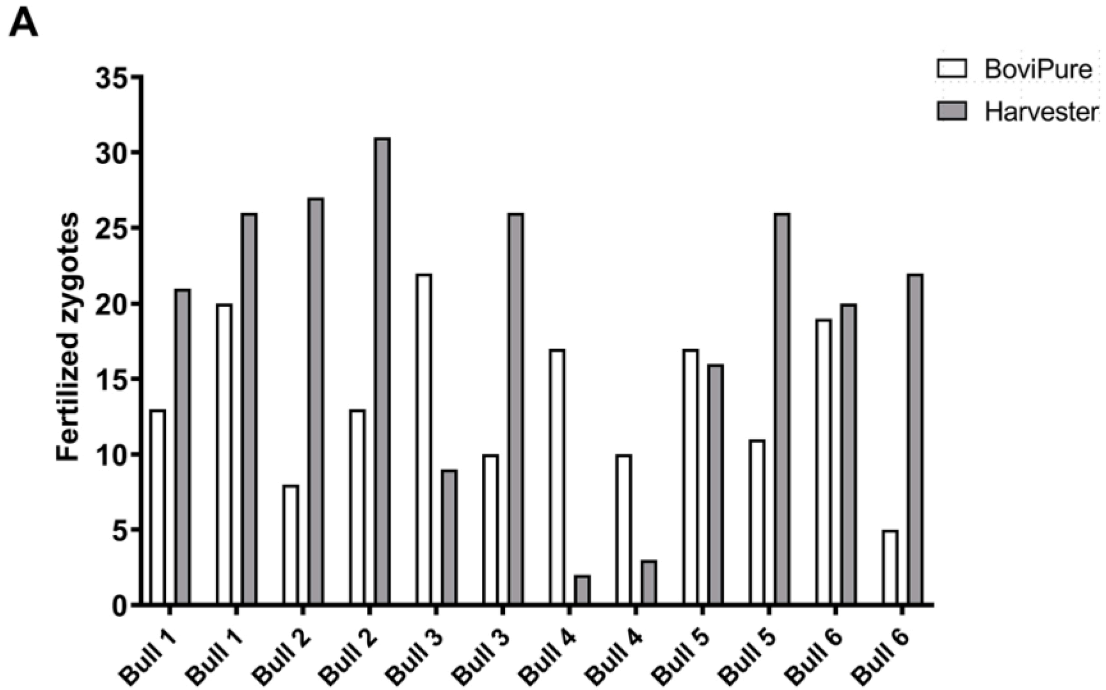


Figure S3. Workflow of sperm preparation for Experiment 2.

Times refer to incubation periods. Each circular arrow symbols a washing step (3 min, 300 X *g*, room temperature). Figure was created with BioRender.com.



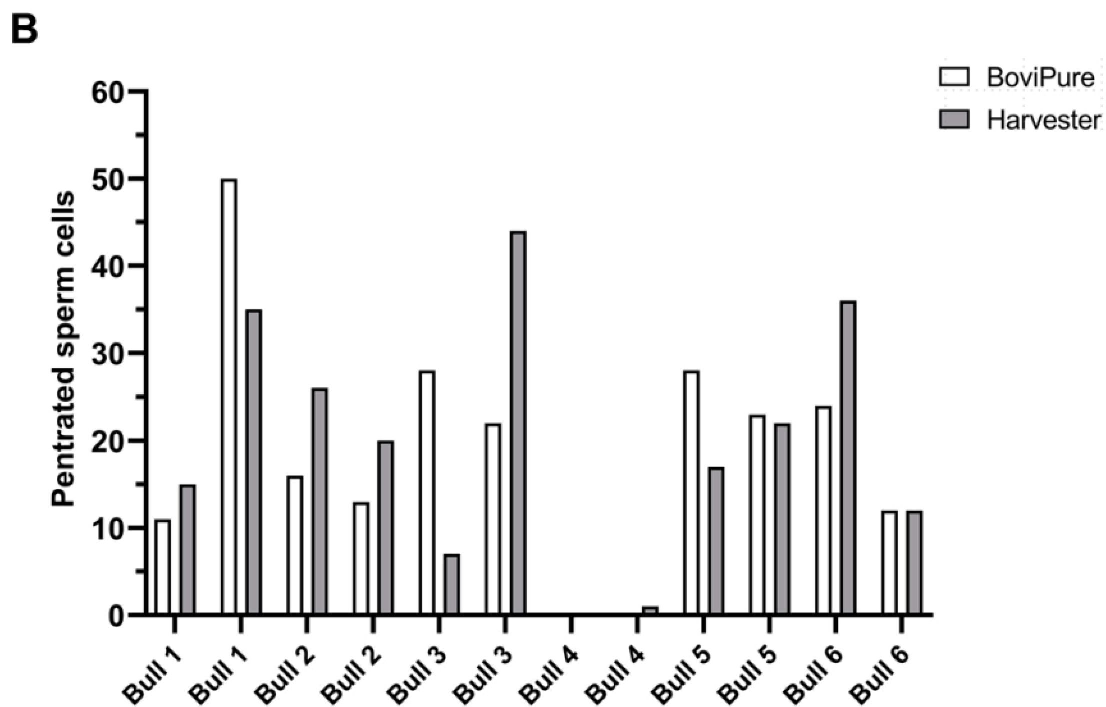


Figure S4. Evaluation of the origin of the fertilizing spermatozoon.
(**A**) Total number of monospermic fertilized zygotes that were fertilized by a spermatozoon either after Harvester-treatment or BoviPure^{TM} gradient centrifugation.
(**B**) Total number of spermatozoa either after Harvester-treatment or BoviPure^{TM} gradient centrifugation that penetrated zygotes showing polyspermy. Data shown separately for each bull and both experimental replicates per bull.

Table S1

Parameter Settings CASA IVOS II (Animal Breeder, Boar and Equine Breeder Version 1.10; Hamilton Thorne Bioscience, Beverly, USA).

Analysis Setup	Setting
Objective	Zeiss 10x NH IVOS-II 160 mm
Objective Magnification	1.21
Frame Count	30
Frame Capture Speed	60 Hz
Camera Exposure	16 ms
Camera Gain	300
Integrate Time	500 ms
Illumination Primary	LED
Photometer	60 - 70
Stage Temperature	37 °C
Chamber Depth	20 µm
Capillary Correction	1.3
Head brightness Min	149
Head Size	6 - 70 $µm^2$
Cell Travel Max	15 µm
Progressive STR	30 %
Progressive VAP	15 µm/s
Static VAP	5 µm/s
Static VSL	3 µm/s

Table S2

Sperm motility parameters of untreated (30 min), BoviPure™ gradient treated (30 min) and Harvester treated bovine semen samples. All values are mean ± SD. Small uppercase letters (a, b, c) indicate a significant difference in one column between samples ($P < 0.05$).

Treatment	Total Motility (%)	Progressive Motility (%)	VCL (µm/s)	LIN (%)	ALH (µm)	BCF (Hz)
Untreated	44.6 ± 16.0[a]	42.9 ± 15.0[a]	286.4 ± 30.0[a]	38.5 ± 2.6[a]	12.9 ± 1.2[a]	26.5 ± 1.6[a]
BoviPure™ gradient	73.2 ± 13.6[b]	71.7 ± 13.5[b]	218.4 ± 19.5[b]	50.2 ± 3.1[b]	10.0 ± 0.6[b]	25.7 ± 2.7[a]
VetCount™ Harvester	89.4 ± 8.3[c]	85.1 ± 7.8[c]	224.8 ± 13.5[b]	41.9 ± 2.9[c]	10.5 ± 0.7[b]	28.4 ± 2.7[a]

LIN: linearity; ALH: average amplitude of lateral head displacement; VCL: curvilinear velocity; BCF: beat cross frequency