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Optimization of *in vitro* fertilization with X-sorted spermatozoa in cattle

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Introduction: Dairy farming has a preferential demand for female calves to sustain milk production. Spermatozoa carrying the X chromosome, selected based on the higher content in DNA, allow to obtain almost exclusively female offspring. However, the separation procedures are stressful to the cells and induce mechanical and chemical modifications that negatively impact the survival, motility, and ultimately the fertilization potential. AIM: Considering that overall the X-sorted spermatozoa are characterized by compromised membrane integrity and premature capacitation, a series of experiments was conducted to test some optimizations of the *in vitro* fertilization (IVF) procedures to increase the embryo yield using commercially available X-sorted semen.

Methods: Three series of experiments were conducted that led us to progressively refine the IVF procedure with X-sorted spermatozoa. First different protocols for semen processing were assessed for the yield in motile spermatozoa. Then the most promising were used in an IVF setting to investigate the fertilization ability. Finally the most performant protocol was used to investigate the effect of different IVF co-incubation times on the embryo yield.

Results: By modifying some parameters of the discontinuous density gradient centrifugation such as the volume of the gradient, the proportion of PVP-coated colloidal silica solution, the centrifugation speed and the time of processing, along with the duration of the IVF culture, higher motility and fertilization and blastocyst rates were obtained ($P<0.05$).

Discussion: Overall, while studies indicate that different bulls may require specific conditions for optimal fertilization and embryo yield, the present report shows that small adjustments to semen processing can significantly improve the efficiency of the *in vitro* embryo production with X-sorted semen.

KEYWORDS

spermatozoa, IVF, cattle, embryo, density gradient, assisted reproduction, sexed semen

1 Introduction

The increased cost of materials, fuels, and labor and the fluctuating milk price can considerably thin the profit of dairy farmers, with the birth of male calves representing an additional factor that contributes to economical loss (Lucy and Pohler, 2025; Pascottini et al., 2025). Dairy breeds, that underwent intense selective pressure for the ability to transform energy intake in milk, are poor meat producers with low performance in daily weight gain, low meat market price compared to specialized beef breeds, and are generally less appreciated by the consumers. Therefore, keeping male calves on a dairy farm represents a cost with minimal returns and farmers usually sell them at a modest price to free up resources - space, food, management time, veterinary care - to invest in the female herd (Cottle et al., 2018).

It is therefore understandable that dairy farmers are willing to go the extra step to secure the birth of female calves. Indeed, since the early years of assisted reproductive technologies applied to livestock, practitioners performing Multiple Ovulation and Embryo Transfer (MOET) strived to develop methodologies to genotype the flushed embryos in order to identify the female ones through sequential biopsy and PCR (Shea, 1999; Tavares et al., 2016). However only a quota of the MOET-produced embryos is of high-enough quality to sustain this rather invasive procedure, hence most of the produced embryos remained of unknown sex. Furthermore, reduced viabilities and pregnancy rates were reported with cryopreserved sexed embryos, overall preventing this technology to broadly spread (Taneja et al., 1998; Shea, 1999; Hasler et al., 2002; Korhonen et al., 2012).

In the early 2000s, the use of flow cytometry allowed to sort the spermatozoa based on the differential DNA content of the X and Y chromosomes, not just experimentally, but as a commercial product (Seidel and Dejarnette, 2022). Hence X-sorted semen became available for routinary artificial insemination (AI) and *in vitro* fertilization (IVF) procedures, yielding offspring of the desired sex in approximately 90% of the cases (Johnson, 2000; Seidel and Garner, 2002; Seidel, 2013; Xie et al., 2020). Nevertheless spermatozoa that undergo sorting partially lose their fertilization potential, such that lower pregnancy rates (Schenk and Seidel, 2007; Dejarnette et al., 2008; Maicas et al., 2020; Steele et al., 2020; Kumar et al., 2024) and higher pregnancy loss (Crowe et al., 2025) are commonly observed. Furthermore X-sorted semen is customarily sold at lower concentrations per straw than conventional semen, likely contributing to amplify the inefficiency.

To overcome these drawbacks and improve the success rate, several studies proposed AI schemes with 2–3 inseminations at different time points, either at fixed time or upon estrous detection (Ketchum et al., 2021; Oosthuizen et al., 2021; Vanwye et al., 2024). However, repeated inseminations are impractical in some settings and economically disadvantageous, especially if a top-ranking bull is used. In this view, the application of IVF seems more amenable to scale-down the costs of a 'sexed-semen operation', reducing the number of straws needed and the animal management, since the spermatozoa coming from a single straw may be used for fertilizing oocytes of multiple heifers/cows and the oocyte retrieval does not require hormone administration.

Bovine IVF has been around for several decades and it has achieved a rather satisfactory success compared to other mammalian species. Nevertheless standardized protocols have been mainly established for unsorted semen. Here we describe a series of experiments conducted to optimize the outcomes of IVF procedures with bovine X-sorted semen.

2 Materials and methods

2.1 Collection of cumulus-enclosed oocytes and *in vitro* maturation

Bovine ovaries were recovered at a local abattoir (IT 2270M CE; Inalca S.p.A., Ospedaletto Lodigiano, LO, Italy) from Holstein cows subjected to routine veterinary inspection and according to the specific health requirements. No animals were raised, nor euthanized for conducting these analyses. Instead, ovaries were collected from animals culled for human meat consumption, in fulfillment with the 3Rs principle: Replacement, Reduction, and Refinement of animals used for experimentation established by EU Directive 2010/63/EU and subsequent amendments. The leftover tissues were disposed according to the regulation (EC) No 1069/2009 of the European Parliament and of the council of 21 October 2009.

Ovaries with more than 10 medium antral follicles visible on the surface were collected in sterile saline (NaCl, 9 g/L), supplemented with penicillin 100 U/mL and streptomycin 0.1 mg/mL (pen/strep) at 26-28° C and transported to the laboratory within 3 hrs and processed as previously described with minor modifications (Modina et al., 2007). The following collection, selection and culture procedures were conducted as previously described (Luciano et al., 2013). Briefly, medium antral follicles (diameter 2–6 mm) were punctured with a 16-gauge needle connected to an aspiration pump (COOK-IVF, Brisbane, QLD, Australia) to collect the follicular fluid containing the cumulus-enclosed oocytes (CEOs) in a 20 mL tube prefilled with TCM-199 supplemented with HEPES 20 mM, 1790 U/l heparin, and 0.4% of bovine serum albumin (BSA) (H-M199). The sediment, composed of CEOs and other follicular cells, was examined under a stereomicroscope to retrieve CEOs medium brown in color, with five or more complete layers of cumulus cells and oocytes with finely granulated, homogenous ooplasm. The selected CEOs were washed twice in H-M199 and *in vitro* matured in groups of 25–30 for 24 hrs in 500 µL of TCM-199 modified to contain 0.68 mM L-glutamine, 25 mM $NaHCO_3$, 0.4% fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 µg/mL kanamycin, and 0.1 IU/mL recombinant human FSH (Gonal-F, Merck-Serono) in humidified air under 5% CO_2 at 38.5° C. The 4-well dishes containing the IVM medium were pre-equilibrated in the incubator for at least 4 hrs before starting the culture.

At the end of the IVM, the CEOs were moved to the *in vitro* fertilization (IVF) dish.

2.2 Spermatozoa separation by discontinuous density gradient centrifugation and *in vitro* fertilization

X-sorted semen was processed either following the previously described protocol for unsorted semen in use in our laboratory (Lodde et al., 2021), or modified as detailed below.

Briefly, starting from 1–2 hrs before the end of IVM, X-sorted, commercially available, cryopreserved semen of a bull of proven fertility, previously used in AI schemes (Frau et al., 2024), was thawed by submerging the straw(s) in a 35 °C water bath for 1 min and the content transferred in sterile 2 mL tubes. Upon thawing, a 10 µL drop was visually assessed for progressive motility, expressed as the percentage of spermatozoa showing progressive and straight motion on the total spermatozoa visible in a given microscope field (Srivastava et al., 2013). The content of the straw was then gently layered on discontinuous density gradients (DDGs) prepared by mixing Percoll, a commercial PVP-coated colloidal silica solution (Merck Life Science S.r.l., Milano, Italy), and Ca^{2+}-free, HEPES-buffered Tyrode Albumin Lactate Pyruvate (Ca^{2+}-free TALP) in different proportions and centrifuged, as detailed in Table 1. When different protocols for semen preparation were tested, the content of multiple straws was pulled after the initial motility check, gently mixed, and equally divided into aliquots that underwent parallel downstream procedures.

Upon DDG centrifugation, the separated fraction of spermatozoa was recovered in a fixed volume of 100 µL - corresponding to the pellet - and was washed twice by centrifuging in Ca^{2+}-free TALP. Wash volume, centrifugation speed, and time were adjusted according to the size of the DDG, as detailed in Table 1. A 10 µL drop of spermatozoa suspension was used to visually assess the progressive motility, as described above, by 3 operators, 2 of whom were blinded. Spermatozoa concentration was calculated using a Neubauer chamber after diluting 1:4 in HCl 1M and adjusted to 220.000 spermatozoa/mL. The appropriate volume of spermatozoa suspension was then added in 100 µL drops of TALP supplemented with 0.6% (w/v) BSA fatty acid free, 10 µg/mL heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine (TALP IVF), containing the CEOs. Spermatozoa and CEOs were co-incubated at 38.5°C under 5% CO_2 in humidified air for 8–18 hrs, according to the experimental design. TALP IVF drops, covered with paraffin oil (Vitrolife, Göteborg, Sweden), were pre-equilibrated in the incubator starting from the night before the beginning of IVF. At the end of the co-incubation, the presumptive zygotes were either fixed to assess the fertilization rate or cultured for 8 days as detailed below.

2.3 *In vitro* embryo culture

At the end of the IVF culture, residual cumulus cells and spermatozoa were removed by vortexing for 1–2 min at 35 Htz in 500 µL of synthetic oviduct fluid (SOF) buffered with 10 mM of HEPES and 5 mM of $NaHCO_3$ (SOF wash), in a 5 mL round bottom tube. The inner wall of the tube was washed with an additional 500 µL SOF wash and the presumptive zygotes were allowed to sink for 30–60 sec. The medium was recovered from the bottom of the tube and examined under a stereomicroscope to retrieve the presumptive zygotes that, after 2 washes in SOF wash, were transferred in SOF buffered with 25 mM of $NaHCO_3$ and supplemented with MEM essential and nonessential amino acids, 0.72 mM sodium pyruvate, 2.74 mM myo-inositol, 0.34 mM sodium citrate, and 5% calf serum (SOF-IVC). Incubation was performed at 38.5 °C with a humidified gas mixture composed of 5% CO_2, 5% O_2, and 90% N_2 for 8 days. The 4-well dishes, containing 500 µL/well of SOF-IVC and 2 mL of water in the space between the wells, were pre-equilibrated in the incubator for at least 8 hrs before the start of IVC.

At the end of the culture period, the formed blastocysts were counted using a stereomicroscope and the blastocyst rate was calculated on the total number of initial structures. Furthermore, the blastocysts were classified as early, expanded, and hatching/hatched based on the observation of an initial or well-developed blastocoel (early or expanded morphology, respectively), and emergence - initial or complete - from the zona pellucida (hatching/hatched morphology).

2.4 Fluorescence microscopy

All the structures obtained at the end of the culture were fixed in a mixture of 60% methanol and 40% Dulbecco's phosphate buffered saline at 4°C, stained with 0.5 mg/mL propidium iodide or 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) and examined under a fluorescence microscope to confirm the morphological observations.

Some embryos were fixed, stained, and imaged at the end of the IVF culture to monitor the formation of pronuclei.

TABLE 1 Percentage, volume, and centrifugation settings of 3 discontinuous density gradients (DDGs) and subsequent washes used for processing bovine X-sorted semen.

	Percoll (%)	Volume (mL)	Separation		Wash	
			Speed (g)	Time (min)	Speed (g)	Time (min)
DDG-A	45/90	4.0	707	30	397	10
DDG-B	45/90	1.0	918	5	194	3
DDG-C	67.5/78.7	0.8	2029	5	151	3

2.5 Statistical analysis

Statistical analyses were conducted using GraphPad Prism version 8.4.3 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Continuous data were first assessed for normal distribution by the Shapiro-Wilk test and then analyzed either by t-test if normally distributed or using a non-parametric test (Wilcoxon test) if the distribution was not normal. Categorical data are presented as percentages and analyzed by Fisher's exact test. $P<0.05$ was considered statistically significant.

2.6 Experimental plan

Three series of experiments were conducted that led us to progressively refine the IVF procedure with X-sorted spermatozoa. All the experiments were performed at least 3 times. A graphical representation of the experimental plan is provided in Figure 1.

In Experiment 1 (Figure 1A), three DDGs (namely DDG-A, DDG-B, and DDG-C) were compared to test the efficiency of retrieval of live spermatozoa. The biological endpoints of this set of experiments were spermatozoa motility and concentration. Statistical significance was analyzed by two-tailed paired t-test (motility) or two-tailed Wilcoxon matched-pairs signed rank test (concentration), according to the results of the normality test.

In Experiment 2 (Figure 1B), DDG-A and DDG-C were compared to test the fertilization ability. The biological endpoints of this set of experiment were PN formation and data were analyzed by two-tailed Fisher's exact test.

In Experiment 3 (Figure 1C), different co-incubation times of DDG-C processed spermatozoa and COCs were tested to assess the

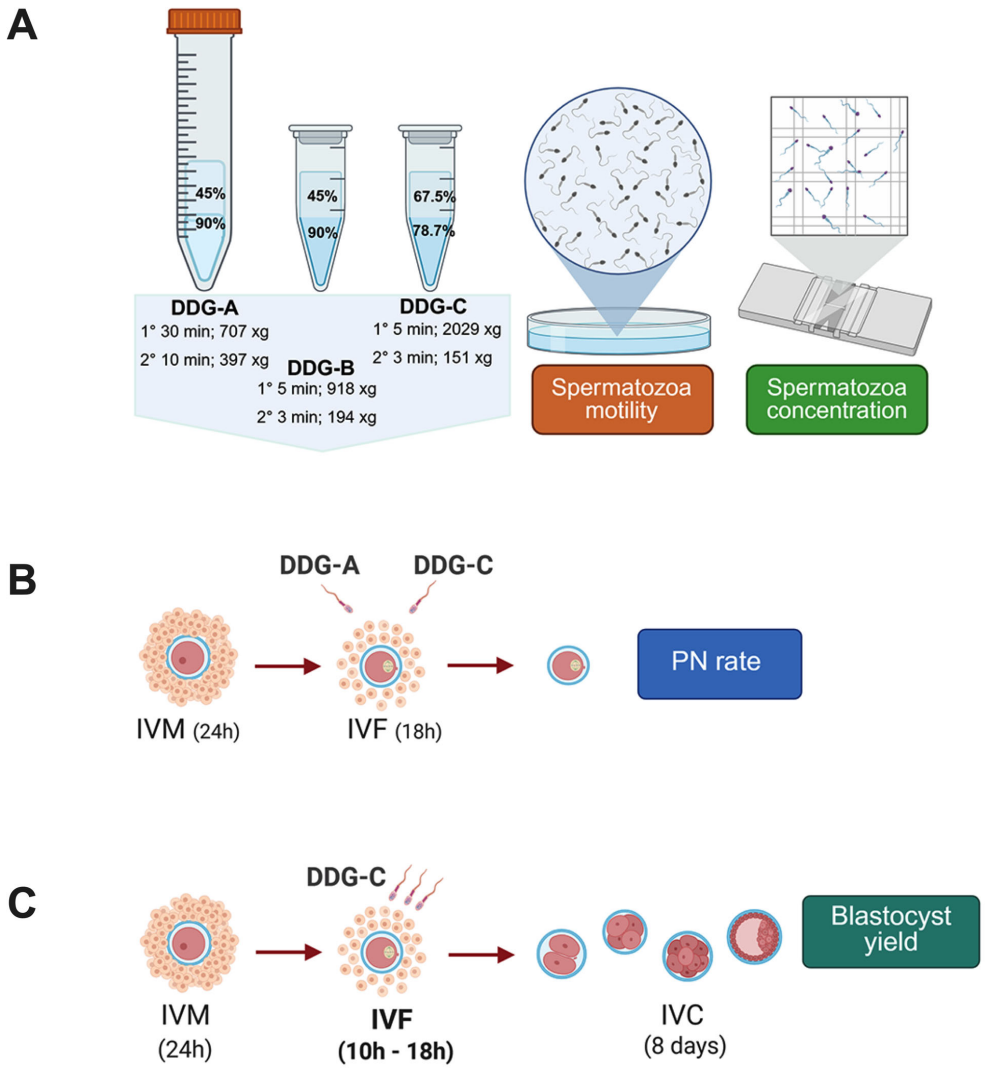

FIGURE 1
Experimental plan. Graphical representation of the experimental procedures conducted to assess the motility and concentration of X-sorted spermatozoa using differential density gradients (DDGs) A, B, and C **(A)**, the fertilization rate of X-sorted spermatozoa processed using DDG-A and B **(B)**, and the ideal IVF time of co-incubation for one selected DDG **(C)**. The colored boxes report the biological endpoints for each experimental phase. Created with BioRender.

effect on developmental competence. The biological endpoints of this set of experiment were blastocyst rate and morphology and data were analyzed by two-tailed Fisher's exact test.

3 Results

3.1 Motility and concentration of X-sorted spermatozoa processed using different DDGs

In the first series of experiments we tested if decreasing the time required for the procedures and modifying the DDG composition might affect the retrieval of viable spermatozoa. This hypothesis was tested by comparing the motility and the concentration of X-sorted spermatozoa obtained using 3 different DDG protocols. Specifically, DDG-A, representing the standard for unsorted semen in our lab, was composed of 2 mL of 45% Percoll layered on top of 2 mL of 90% Percoll. With DDG-B, the volume was reduced to a quarter of DDG-A and the time of processing was decreased, but the composition (45/90) remained unchanged. DDG-C was also small and required a shorter processing time, but compared with the previous two it was modified to have a higher density on top, intermediate between 45 and 90% (67.5%), and a lower density at the bottom, intermediate between 67.5 and 90% (78.75%).

After centrifugation and wash, the obtained sperm suspension with small-volume DDGs (B and C) had a cloudy appearance, likely due to persistent Percoll residues. The opacity made the counting difficult in the Neubauer chamber and posed a risk of Percoll contamination of the IVF drop. An additional wash was therefore included to obtain a clear sperm suspension. The additional wash was performed also with DDG-A to avoid the introduction of confounding factors.

Since DDG-B underperformed in preliminary trials, giving a remarkably low motility (10%) and a low spermatozoa concentration (950.000 sperm/mL), it was immediately discarded and not carried over in the following experiments. When comparing the remaining two gradients, spermatozoa concentration was not significantly changed (P = 0.375, Wilcoxon test, matched pairs, N = 4), but the motility was superior when using DDG-C compared to DDG-A (P = 0.0462, two-tailed paired t-test, N = 4, Figure 2).

3.2 Fertilization rate of X-sorted spermatozoa processed using different DDGs

The fertilization rate of X-sorted spermatozoa processed with DDG-A and DDG-C was investigated on 65 presumptive zygotes fixed at the end of the IVF culture. Notably, 87% of the oocytes co-incubated with spermatozoa prepared with DDG-C showed 2 pronuclei (2PN) and were therefore considered successfully fertilized. The remaining 13% were either metaphase I (MI) and therefore considered not matured oocytes, metaphase II (MII) and therefore considered unfertilized oocytes, or polyspermic zygotes (Figures 3A, B). The pronuclei rate with DDG-A was instead significantly lower (P<0.0001, Fisher's exact test, Two-tailed), only 26%, with the majority of the oocytes failing to be fertilized and being at the MII stage (Figures 2A, B).

3.3 Time of co-incubation

Despite a considerably high 2PN rate obtained with DDG-C, the blastocyst rate was rather low. Since premature capacitation has

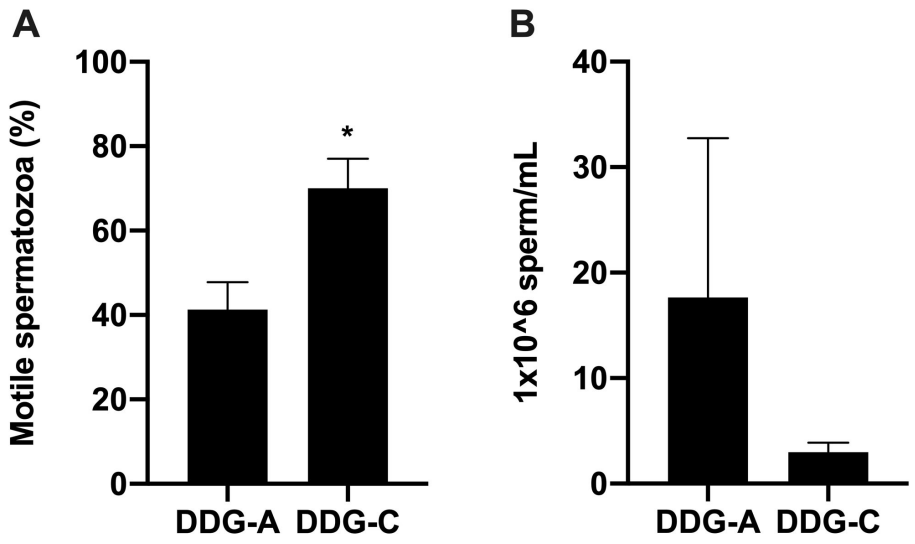

FIGURE 2
Spermatozoa motility and concentration after discontinuous density gradient (DDG) separation. The bar graphs represent the mean ± SEM of the percentage of motile spermatozoa **(A)** and the spermatozoa concentration **(B)** following separation with two different protocols, named DDG-A and DDG-C. N = 4. *P≤ 0.05, two-tailed paired t-test **(A)**. Two-tailed Wilcoxon matched- pairs signed rank test **(B)**.

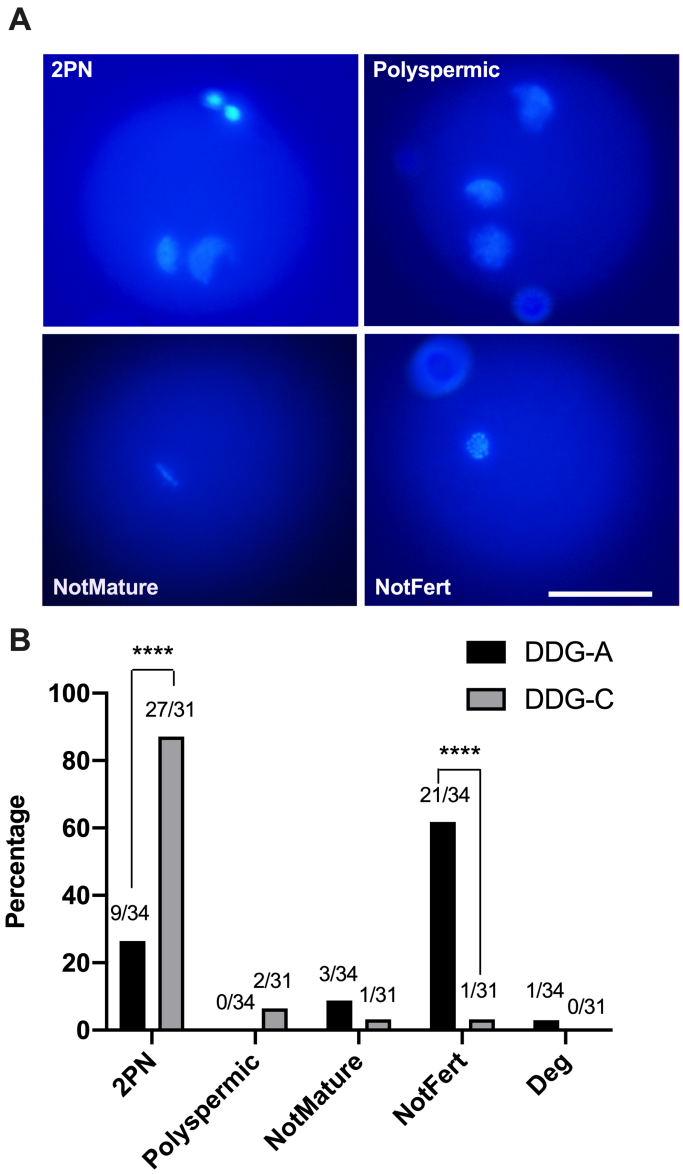

FIGURE 3
Fertilization rate upon IVF with spermatozoa separated using different discontinuous density gradients (DDGs). Representative fluorescence microscopy images **(A)** and distribution **(B)** of zygotes displaying 2 pronuclei (2PN), zygotes with multiple pronuclei (Polyspermic), not mature oocytes (NotMature), unfertilized oocytes (NotFert) and degenerated oocytes. Blue: DNA. Bar 50 µm. The bar graph represents the percentage of structures formed upon IVF with spermatozoa prepared using DDG-A (n=34) or DDG-C (n=31). ****$P \leq 0.0001$, two-tailed Fisher's exact test.

been reported for X-sorted semen (Maxwell and Johnson, 1999; Umezu et al., 2017), we reasoned that 18 hrs co-incubation might be too long and that formed zygotes might benefit of an earlier switch to embryo culture conditions. This hypothesis was tested by comparing the blastocyst rate obtained by switching the presumptive zygotes from IVF to IVC conditions after 10 or 18 hrs and further culture for 8 days. We observed that reducing the co-incubation time to 10 hrs significantly improved the overall blastocyst rate (Figure 4A). The morphological observation did not indicate differences in the quality of the blastocyst, as the rate of early, expanded, and hatched blastocyst was not different (Figure 4B).

For practical reasons, a pilot experiment was conducted to further reduce the co-incubation time to 8 hrs and the IVM time to 22 hrs. However, an additional beneficial effect was not observed (not shown).

4 Discussion

Given the interest of dairy farming for the selection on the maternal lineage, we conducted a series of experiments aimed at improving the outcome of IVF procedures with commercially available X-sorted semen. Our findings indicate that the

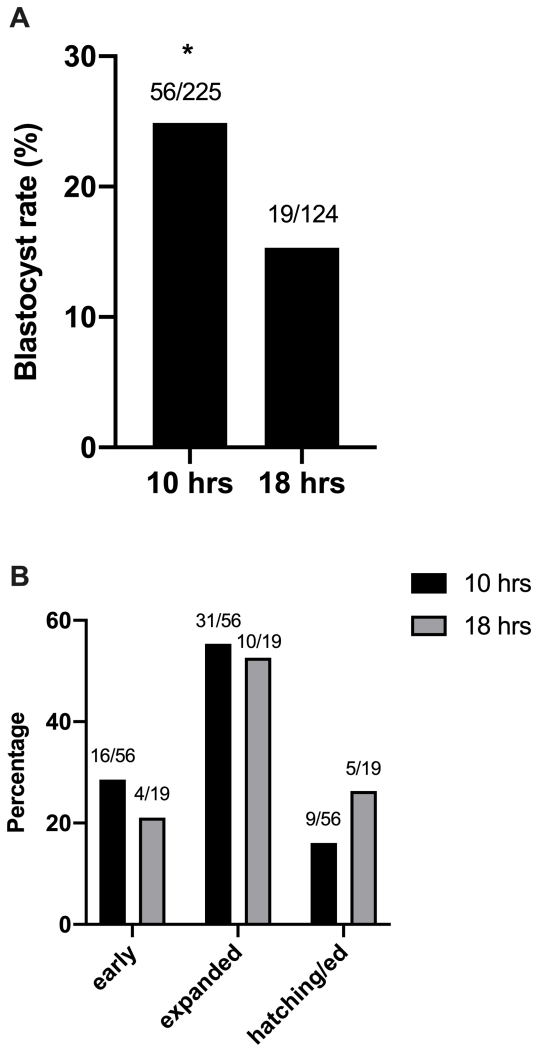

FIGURE 4
Blastocyst rate and morphology upon co-incubation of CEOs with X-sorted spermatozoa for 10 or 18 hrs before embryo culture. The bar graphs represent the distribution of blastocyst formation **(A)** and blastocyst morphology **(B)** after 8 days of culture following co-incubation of the CEOs with X-sorted spermatozoa for 10 or 18 hrs, n=225 and n=124, respectively. *P≤ 0.05, two-tailed Fisher's exact test.

discontinuous density gradient (DDG) and the timing of gametes co-incubation are critical points to consider when optimizing IVF protocols with X-sorted spermatozoa. Indeed by adjusting these parameters we obtained a significant improvement in the motility and fertilizing ability of the spermatozoa and an increased developmental competence of the zygotes, providing an optimized tool for genetic selection for dairy breeders.

Without the pretense of being exhaustive and acknowledging that other parameters might also be involved, the main elements considered when devising optimization to the spermatozoa separation and IVF protocol were: 1) the customary lower concentration and higher price of sorted semen straws, 2) the pre-capacitation typical of sorted spermatozoa, and 3) the burst of reactive oxygen species (ROS) occurring at fertilization time.

The low amount and increased costs directed us to develop a procedure based on colloidal silica solution density gradients rather than swim-up to remove cryoprotectants and damaged cells, since the former is known to provide higher absolute quantitative improvement in spermatozoa retrieval in bulls (Arias et al., 2017) as well as in other species (Prakash et al., 1998; Chatdarong et al., 2010; Santiago-Moreno et al., 2014; Galarza et al., 2022). Additionally, we reasoned that reducing the distance that the cells must travel through the density gradient and in the subsequent washes may facilitate the recovery rate and decrease the processing time, as suggested by (Missio et al., 2018). Quite surprisingly we observed that a smaller volume of the gradient per se did not necessarily improve the retrieval rate, as evidenced by the inefficiency of DDG-B (a small volume, 45/90% Percoll gradient), highlighting that changes in the overall composition of the gradient and centrifugation parameters are critical.

Indeed DDG separation occurs according to the density of the cell which is associated to the integrity of the plasmalemma and of the spermatozoa acrosomal membranes (Sharma and Agarwal, 1996; Somfai et al., 2002). Efficient separation in the gradient should work as a replacement of the *in vivo* selection of motile spermatozoa progressing through the female reproductive tract, while damaged spermatozoa and cellular debris are left behind (Holt, 2009). Since the process of sperm sorting is known to perturb the integrity of the plasmalemma (Maxwell et al., 1996; Suh et al., 2005), it is conceivable that refinements in the density of the gradient play a major effect in the separation of sexed spermatozoa, that are scarce and damaged compared to the unsorted ones. Our results confirm this hypothesis and specifically they seem to point out that a gradient with a higher density at the top (67.5% Percol of DDG-C, compared to 45% of DDG-A and B) more efficiently blocked the initial entrance of heavily compromised cells, while the progression to a less stringent density (78.5% Percoll of DDG-C compared to 90% of DDG-A and B) allowed for the retainment of a bigger portion of intact spermatozoa, thus maximizing the enrichment of the motile fraction without decreasing the fertilization potential.

Once accurately calibrated, the shorter processing time required by a small volume of the gradient may have represented an additional advantage, reducing the exposure of viable spermatozoa to dead, immotile, and abnormal cells that produce reactive oxygen species (ROS), previously shown to decrease the fertilization potential (Aitken and Clarkson, 1988). Accordingly, the combination of these factors allowed us to obtain a higher rate of normally fertilized zygotes, as demonstrated by the high 2 PN rate achieved with DDG-C. These findings are also in agreement with a study from Zhang and collaborators (Zhang et al., 2020) showing that treatment with antioxidants improves the oxidative stress of sorted spermatozoa and increase the IVF outcome.

Considerations on ROS bursts during IVF also indicated that a possible factor to ponder was the exposure of the formed zygotes to a ROS-enriched environment. Indeed, spermatozoa physiologically produce high levels of ROS during the final stages of capacitation, when they are released from the oviductal epithelium, just before their final ascent to the fertilization site (Aitken and Nixon, 2013).

In this context, each sperm's exposure to ROS is brief and helps complete their preparation for fertilization. However, if a spermatozoon does not achieve fertilization, the continued ROS-induced peroxidation determines the formation of lipid aldehydes which will trigger further free radical production in the mitochondria, prompting the cells to initiate apoptosis (reviewed by (Aitken, 2017)).

In bovine IVF, oocytes are co-incubated with conventionally-treated, unsorted spermatozoa in a confined space, i.e. the culture drop, for 10–20 hours. In these conditions, despite the fact that fertilization has already occurred at 10 hours, increasing the IVF time up to 18–20 hours, mainly for logistical reasons, does not affect blastocyst formation (Ward et al., 2002). Instead when the sorting process has compromised the integrity of the cells, inducing alterations in the membranes, ROS production, and speeding up the capacitation and acrosome reaction (Seidel, 2003; Moce et al., 2006; Reese et al., 2021; Guo et al., 2023), the prolonged IVF timing may be useless or even contribute to the low developmental competence commonly observed with X-sorted spermatozoa (Magata et al., 2021; Leme et al., 2024; Oliveira et al., 2024).

In agreement with this hypothesis, shortening the oocyte-spermatozoa co-incubation time had a positive outcome in our experimental settings. Furthermore, our observation matches the conclusions of a study conducted on ovine *in vitro* embryo production that recommends a short gamete co-incubation time during IVF with sex-sorted frozen-thawed spermatozoa (Morton et al., 2005). In summary, we observed that adjusting the gradient density and reducing the co-incubation time of gametes positively impacted the outcome of IVF with X-sorted spermatozoa, enhancing sperm motility and embryo production rates. While studies indicate that different bulls may require specific conditions and settings for optimal fertilization and embryo yield (Inaba et al., 2016; Travnickova et al., 2021), this study provides evidence that small adjustments to spermatozoa processing, tailored to the characteristics of sorted sperm, could significantly improve IVP efficiency.

Notably, improvements in the efficiency of gender selected semen has been listed among the 'Innovative approaches to enhance fertility and reproductive success in cattle over the future 20 years', due to the potential for herd management and sustainability that this technology bears (Lucy and Pohler, 2025). Nevertheless lowering the costs and increasing the conception rate are steps required to unleash its full potential. In this view several procedures still need to be optimized, starting from the sorting strategies, that should become more efficient and less damaging, to the development of *in vitro* culture approaches tailored to the metabolic needs of sorted spermatozoa and gender-selected embryos. While this work provides a proof of concept that by using an optimized IVF scheme more embryos can be produced, suggesting that a higher developmental competence can be achieved, future experiments will be needed to test the pregnancy rate upon transfer in recipient heifers.

Besides improving the efficiency of selection for the female lineage with an expected positive effect on the genetic gain (Kumar et al., 2024; Ngcobo et al., 2024), this optimization might prove useful in experimental conditions where dimorphic patterns of development between female and male embryos are investigated (Kochhar et al., 2003; Lechniak et al., 2003; Sidrat et al., 2019; Kalo et al., 2025), including differences in epigenetic reprogramming (Dobbs et al., 2013), gene expression (Richardson et al., 2023), placental formation (Legault et al., 2024).

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because no animals were raised, nor euthanized for conducting these analyses. Instead, ovaries were collected from animals culled for human meat consumption, in fulfillment with the 3Rs principle: Replacement, Reduction, and Refinement of animals used for experimentation established by EU Directive 2010/63/EU and subsequent amendments.

Author contributions

FFF: Conceptualization, Investigation, Methodology, Writing – review & editing. GM: Investigation, Validation, Writing – review & editing. EA: Investigation, Writing – review & editing. RP: Methodology, Writing – review & editing. VL: Supervision, Visualization, Writing – review & editing. FF: Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Visualization, Writing – original draft.

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Conflict of interest

Author RP was employed by company Progest Biotecnologia Ltda.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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