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Effect of different mini-volume colloid centrifugation configurations on flow cytometrically sorted sperm recovery efficiency and quality using a computer-assisted semen analyzer

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Contents

Straws of sex-sorted sperm are usually packaged at a low concentration (e.g., ~2.1 × 10^6 sperm/ml) and cost significantly more than unsorted conventional semen from the same sire. In order to maximize the efficiency of using sex-sorted sperm under in vitro fertilization conditions, the selection of an appropriate sperm separation technique is essential. In this study, the effect of using different silane-coated silica colloid dilutions and layering configurations during centrifugation of sex-sorted sperm was examined over an extended period of incubation time. Sperm recovery and viability after centrifugation using the colloid separation technique were measured along with several sperm motility parameters using CASA. For this purpose, frozen and thawed sex-sorted sperm samples were centrifuged using mini-volume single-layer (40%, 60% and 80%) and mini-volume two-layer (45%/90%, 40%/80% and 30%/60%) separation configurations using PureSperm®. A single layer of 40% PureSperm® recovered significantly more sex-sorted sperm (78.07% ± 2.28%) followed by a single layer of 80% PureSperm® (68.43% ± 2.33%). The lowest sperm recovery was obtained using a two-layer PureSperm® dilution of 45%/90% (47.57% ± 2.33%). Single-layer centrifugation recovered more sorted sperm (68.67% ± 1.74%) than two layer (53.74% ± 1.74%) ($p < .0001$). A single layer of 80% PureSperm® exhibited the highest sorted sperm viability (72.01% ± 2.90%) after centrifugation ($p < .05$). The mini-volume single layer of 80% PureSperm® was determined to be an effective alternative to a two-layer centrifugation configuration for sex-sorted sperm selection. In addition, single-layer colloid dilution of 80% performed either as well as or significantly outperformed the other treatments, as well as the control, with regard to motility (MOT) for all time periods of analysis.

1 | INTRODUCTION

Semen from many species of mammals has been found to contain a certain proportion of sperm that does not meet the minimal requirements for fertilization. The reasons for these detrimental sperm alterations can be considered at two different levels: (i) primary sperm alterations produced during spermatogenesis and spermiogenesis and (ii) secondary sperm alterations produced after sperm semen manipulation ex vivo (i.e., iatrogenic damage) (Kjelland et al., 2011).

Different sperm selection methods have been developed to separate the "normal" (or desirable) motile sperm population from the non-motile sperm, seminal plasma, semen extender media and debris with the aim of increasing gamete performance and overall in vitro fertilization (IVF) success (Henkel & Schill, 2003; Morrell, 2006). Several

sperm isolation methods coexist today such as a simple dilution and washing (Brackett & Oliphant, 1975; Mortimer, 1994b), filtration method (Anzar & Graham, 1995), density gradient centrifugation using silane-coated silica particles (Avery & Greve, 1995; Lessley & Garner, 1983) and self-migration techniques (i.e., swim-up, swim-down methods) (Mortimer, 2000a; Rodriguez-Martinez, Larsson, & Pertoft, 1997).

Importantly, the ideal sperm isolation system should offer some preferred attributes according to the following criteria: (i) does not cause sperm damage or compromise/modify the sperm fertilization capacity, (ii) provides a high recovery rate of normal and highly motile sperm, (iii) produces a pellet that is clean and free of dead spermatozoa and bacteria to avoid contamination or generation of reactive oxygen species (ROS) and (iv) minimizes transfer of endotoxin substances to the fertilization drop (Björndahl et al., 2010; Henkel & Schill, 2003; Mortimer, 2000a). Unfortunately, none of the currently available techniques meet all of the aforementioned requirements. However, the best recovery rate of desirable spermatozoa in terms of motility, morphology, membrane and chromatin integrity is found in colloid centrifugation systems, in comparison with other separation techniques (Morrell & Rodriguez-Martinez, 2016).

The mini- (Ord, Patrizio, Marello, Balmaceda, & Asch, 1990) and continuous-density centrifugation (Shalika, Dugan, Pelesh, & Padilla, 1995) methods were originally proposed for poor-quality sperm samples or samples with a low sperm count. Under those circumstances, the mini-density gradient (Ng, Liu, & Baker, 1992; Sakkas et al., 1993) and single-layer centrifugation (Abraham, Johannisson, & Morrell, 2016; Gloria et al., 2016; Shalika et al., 1995) procedures recovered significantly more motile, morphologically normal, hypoosmotic swelling test-resistant spermatozoa in comparison with other sperm separation methods. Therefore, the result of the aforementioned procedures allowed more oocytes to be fertilized per frozen-thawed straw of semen. These considerations are extremely important when sex-sorted sperm are used in IVF and when the highest sperm recovery efficiency is essential.

Density centrifugation can either be continuous or discontinuous (Morrell, 2006). Two-layer colloid configurations with centrifugation are commonly used in the sperm preparation protocol for in vitro fertilization. The dilution of 100% stock colloid to 40% and 90% is commonly used for two-layer centrifugation of sperm in the bovine IVF field (Avery & Greve, 1995; Gordon, 2003; Parrish, Krogenaes, & Susko-Parrish, 1995).

The aim of this study was to compare different silane-coated silica particle dilutions, that is percentages of the 100% stock colloid, by examining sex-sorted live and motile and dead or non-motile sperm separation from frozen-thawed samples using single-layer versus two-layer centrifugation configurations. Sperm recovery yield, viability and motility parameters were evaluated over an extended time period (i.e., up to 18 hr).

2 | MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The Institutional Animal Care and Use Committee (IACUC) ethical approval process was not required for this study as animals were not directly involved, and straws of frozen semen were procured from a commercial source, that is CRI-Genex. CRI-Genex is CSS (Certified Semen Services)-approved and follows all health, ethical and animal welfare according to the NAAB (National Association of Animal Breeders). The research was conducted under the institutional guidelines of the Department of Animal Science at UC Davis.

2.1 | Sex-sorted sperm samples

Cryopreserved sex-sorted bovine semen samples from three sires were obtained from a commercial bovine semen supplier (Genex Cooperative, Inc., Shawano, WI). The straws of semen from each sire (n = 5) originated from the same batch.

2.2 | Sperm thawing and processing

Straws of sex-sorted sperm were thawed at 37°C for 45 s. The straws were dried individually, the sealed end was removed, and the contents were then expelled into pre-labelled 1.5-ml tubes warmed to 37°C. Straw contents were well-mixed, and the volume was measured. Two samples were used to determine total sperm concentration and sperm viability using the NucleoCounter® SP-100™ Sperm Cell Counter (ChemoMetec, Allerod, Denmark) and following the manufacturer's instructions (Ravn, 2006). For sperm concentration, an aliquot (5 µl) from each sire was diluted with 500 µl reagent S100 and, after mixing, was loaded into a cassette containing propidium iodide (Abraham et al., 2016). The cassette was inserted into the fluorescence detector, and the total number of cells (T, $\times 10^6$ cells/ml) in the sample was reported.

The sperm recovery (%) was calculated using the following equation (1):

$$\text{Sperm recovery}(\%) = \frac{\text{Final concentration} \times \text{Final volume}}{\text{Initial concentration} \times \text{Initial volume}} \times 100 \quad (1)$$

For determining sperm viability, a further 5-µl aliquot from each sire was diluted with phosphate-buffered saline (500 µl), pH 7.1, supplied by the same manufacturer (Chemometec, Denmark), before loading into another cassette and inserting into the fluorescence detector to determine membrane integrity and report the number of non-viable cells (N, $\times 10^6$ cells/ml). The viability count was determined by subtracting the non-viable cells from the total number of cells (T-N) and expressing the result as a percentage. Another sample was placed into a computer-assisted sperm analysis (CASA) system (Hamilton-Thorne IVOS, Beverly, MA, USA). The remaining sample was placed on a density gradient column (PureSperm®, Spectrum Technologies, Healdsburg, CA, USA) for centrifugation (700 × g for 15 min) at room temperature. A second centrifugation (300 × g for 5 min) was performed after discarding the supernatant and re-suspending the spermatozoa pellet in TALP-Sperm (pH = 7.4, 295 mOsm) (Parrish et al., 1986). Next, volume was measured along with total sperm concentration and sperm viability

determined in order to calculate recovery and viability rate. The final pellet was then re-suspended gently by manual pipetting in 100 μl of a modified synthetic oviductal fluid-SOF-FERT (Tervit, Whittingham, & Rowson, 1972) for incubation at 38.5°C in humidified atmosphere of 5% CO_2 in air. The final formulation of SOF-FERT consisted of the following: 107.7 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.49 mM, $MgCl_2$, 1.17 mM $CaCl_2$, 5.3 mM sodium lactate, 25.07 mM $NaHCO_3$, 0.20 mM sodium pyruvate, 0.5 mM fructose, 5 μg/ml gentamicin, 20 μg/ml heparin, penicillamine (3 μg/ml), hypotaurine (11 μg/ml) and 6 mg/ml essentially fatty acid free of bovine serum albumin.

2.3 | Computer-assisted sperm analysis (CASA)

The evaluation of sperm motility and related parameters using a CASA system allows for an objective assessment of different cell characteristics (Amann & Waberski, 2014; Lu, Huang, & Lu, 2014; Mortimer, 2000b; Verstegen, Iguer-Ouada, & Onclin, 2002). Sperm samples in the present study were analysed using a CASA system according to the settings in Lenz, Kjelland, Vonderhaar, Swannack, and Moreno (2011). The CASA motility variables measured were percentage of total motile sperm (MOT), percentage of progressively motile sperm (PMOT), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL, μm/s), average lateral head displacement (ALH, μm), the number of times the sperm head crosses the mean path/s (BCF, Hz), straight-line sperm motility (STR, %) and linear sperm motility (LIN, %). The temperature setting of the stage warmer was set at 37°C.

A pre-warmed four-cell-chambered Leja slide (IMV International Corp., Maple Grove, MN, USA) was loaded according to manufacturer recommendations. Given that the sperm had previously been stained with Hoechst 33342 from the sex-sorting process, the CASA was set up under the IDENT fluorescent optics option. The number of sperm analysed per treatment averaged 361 ± 121 sperm.

2.4 | Experimental design

Aliquots of sperm from each sire were made available for performing sperm concentration and viability measurements using the NucleoCounter. Total sperm concentration and sperm viability analyses were performed before and after using the different colloid centrifugation configurations (i.e., single and two layer).

The different colloid dilutions, as percentages of 100% stock colloid, and the number of layers used for centrifugation were as follows: single layer (40%, 60% and 80%) and two layer (45%/90%, 40%/80% and 30%/60%). The different colloid centrifugation configurations were made using PureSperm® 100% and PureSperm® Buffer (PureSperm®, Spectrum Technologies, Healdsburg, CA, USA). A sample consisting of 2.57 ± 0.37 × 10^6 sex-sorted sperm cells/ml was carefully placed over each colloid centrifugation layout. For the two-layer colloid centrifugation layout, 500 μl of each respective stock colloid dilution was placed carefully in a 1.5-ml microtube. For the single-layer respective stock colloid dilution, 1 ml was added to a 1.5-ml microtube. All microtubes were equilibrated for at least 15 min (<30 min) on a warm plate at 37°C before loading the semen. After the second centrifugation, a sample was analysed by CASA at 0, 2, 4, 6, 8 and 18 hr. A sample of thawed sex-sorted semen from each bull was not centrifuged, but maintained and evaluated in the same manner as the centrifuged sperm.

2.5 | Statistical analysis

Statistical analyses were performed using InfoStat software version 2015 (Di Rienzo et al., 2015) together with R version 3.2.3 packages (R Core Team 2015). To consider the complete experimental design and technical constraints, independent linear mixed-effects models were estimated for each output variable. The corresponding model assumptions were verified using residual versus predicted values and normal quantile–quantile plots. The a posteriori analyses included Fisher's least significant difference (LSD) using Bonferroni-adjusted *p*-values at each time period or main effect. All statistical tests, except when noted otherwise, were two-sided with a cut-off at .05 for statistical significance.

3 | RESULTS

The centrifugation configuration clearly affected sperm recovery (Figure 1), viability (Figure 2) and its kinetic characteristics, that is CASA motility variables. A statistical model, that is equation (2), was developed, and it is worth mentioning that both output variables did not depend on time effect or its interactions as depicted in equation (3) (see Supporting Information). Average sex-sorted sperm recovered (×10^6 cell/ml) was 2.00 ± 0.30, 1.57 ± 0.30, 1.73 ± 0.18, 1.51 ± 0.20, 1.36 ± 0.27 and 1.27 ± 0.32 for 40%, 60%, 80%, 30%/60%, 40%/80% and 45%/90%, respectively. Sex-sorted sperm recovery rate box plot results for the different treatments showed a clear descending pattern as the silane-coated silica colloid density increases (Figure 1a). Interestingly, the two-layer 45%/90% colloid dilution configuration had the highest range (41%), standard deviation (10%) and lowest median recovery rate (52%). In addition, single-layer colloid configurations outperformed two-layer colloid configurations as shown in Figure 1b and formally ratified by model results in Figure 1c. More specifically, LSD groups A, B and C had greater or equal recovery rates compared to C, CD and D, as depicted in Figure 1d, with regard to the number of colloid layers used. The best ($p < .05$) sorted sperm recovery was achieved using the 40% layer (78.07% ± 2.28%) followed by the 80% layer (68.43% ± 2.33%), Figure 1c. The poorest sperm recovery was obtained with the 45%/90% (47.57% ± 2.33%) colloid layer configuration.

Overall, single-layer colloid configurations recovered more sorted sperm (68.67% ± 1,74%) than two-layer colloid configurations ($p < .0001$) (Figure 1d). On the other hand, the membrane integrity of sorted sperm did not show a clear pattern in either mean or variance (Figure 2a and b). The non-centrifuged semen, the single-layer colloid dilutions of 40% and 60% and the two-layer colloid dilutions of 45%/90% showed higher variability than the other configurations.

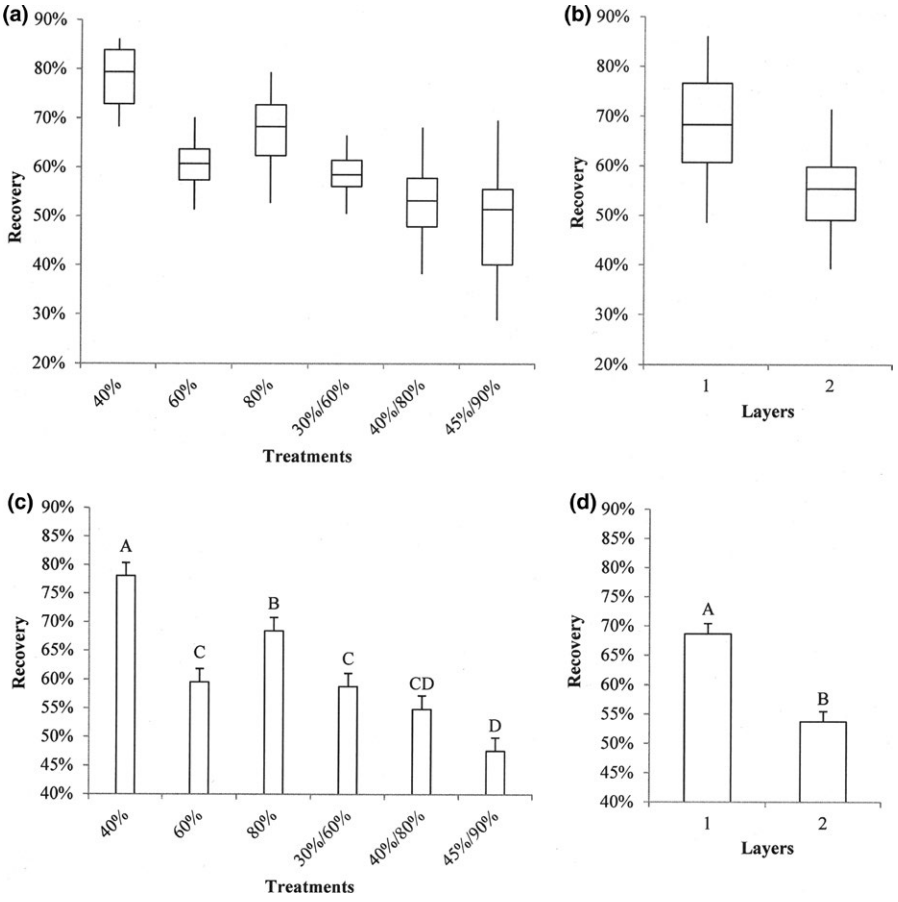

FIGURE 1 Recovery rates for sex-sorted sperm following centrifugation through one or two layers of PureSperm® of different dilutions. Recovery rate box plot data are presented for the six centrifugation treatments tested and also grouped by the number of colloid layers in panels (a and b), respectively. Model-adjusted mean ± *SE* estimation results are presented in panels (c and d) for the corresponding centrifugation configurations of panels (a and b), respectively. Fisher's least significant difference (LSD) test results are represented in letters (Bonferroni-adjusted $p < .05$)

The single-layer colloid dilution of 80% showed the highest viability rate (72.01% ± 2.90%); meanwhile, 40%, 60% and 30%/60% colloid configurations produced the lowest viability results (Figure 2c). There was not a significant difference in sperm viability between single-layer and two-layer configurations ($p = .59$, Figure 2d). The CASA results for the assessed motility parameters of equations (4–5) are summarized in Tables S1–S4. The data in Table S4 elucidate that the single-layer colloid dilution of 80% performed either as well as or significantly outperformed the other treatments with regard to motility (MOT) for all time periods of analysis.

4 | DISCUSSION

Straws of sex-sorted sperm are usually packaged at a low concentration (e.g., ~2.1×10^6 sperm/ml) and cost significantly more than unsorted conventional semen from the same sire. To maximize the efficiency of using sex-sorted sperm under in vitro fertilization conditions, the selection of an appropriate sperm separation technique is essential. Centrifugation of sperm using a single or two layer of colloid isolates a specific subpopulation fraction, enriched with high motile sperm, from seminal plasma, cryoprotective agents, extender media, background materials, debris, non-motile cells and bacteria which are retained in the upper fractions (Avery & Greve, 1995; Henkel & Schill, 2003; Parrish et al., 1995). The present study compared different colloid dilutions and configurations (single layer and two layer) based on their effect on the sperm recovery rate, sperm viability and multiple sperm functional characteristics, that is evaluated CASA motility variables. Two-layer 45%/90% (Parrish et al., 1995) and three-layer 30%/60%/90% (Cesari et al., 2006) Percoll® stock dilutions have been widely used to prepare bull sperm for in vitro fertilization (Henkel & Schill, 2003; Mendes, Burns, De La Torre-Sanchez, & Seidel, 2003; Mortimer & Mortimer, 2013; Samardzija, Karadjole, Getz, et al., 2006). Single-layer centrifugation has arisen as an alternative method to enrich ejaculates with highly motile sperm before cryopreservation or cooling (Nongbua, Johannisson, Edman, & Morrell, 2017), thereby enabling the processing of whole/large ejaculates (i.e., boar and stallion) and improving cryopreserved bovine straws of semen from poor-quality ejaculates (Gloria et al., 2016), and as a result facilitating its commercial application for animal breeding (Morrell, van Wienen, & Wallgren, 2011; Morrell & Wallgren, 2011).

The average sex-sorted sperm recovery rate obtained with all PureSperm® dilutions and layering configurations combined in the present study (i.e., 54% ± 2%) is similar to that of other studies (Rodriguez et al., 2012). Remarkably, our results showed that the most popular colloid dilutions and layering configuration (i.e., 45%/90%) present the lowest recovery rate (48% ± 3%), although other studies mentioned even lower recovery rates (30%–45%) with Percoll® and BoviPure® (Lessley & Garner, 1983; Parrish et al., 1995; Samardzija, Karadjole, Getz, et al., 2006; Samardzija, Karadjole, Matkovic, et al.,

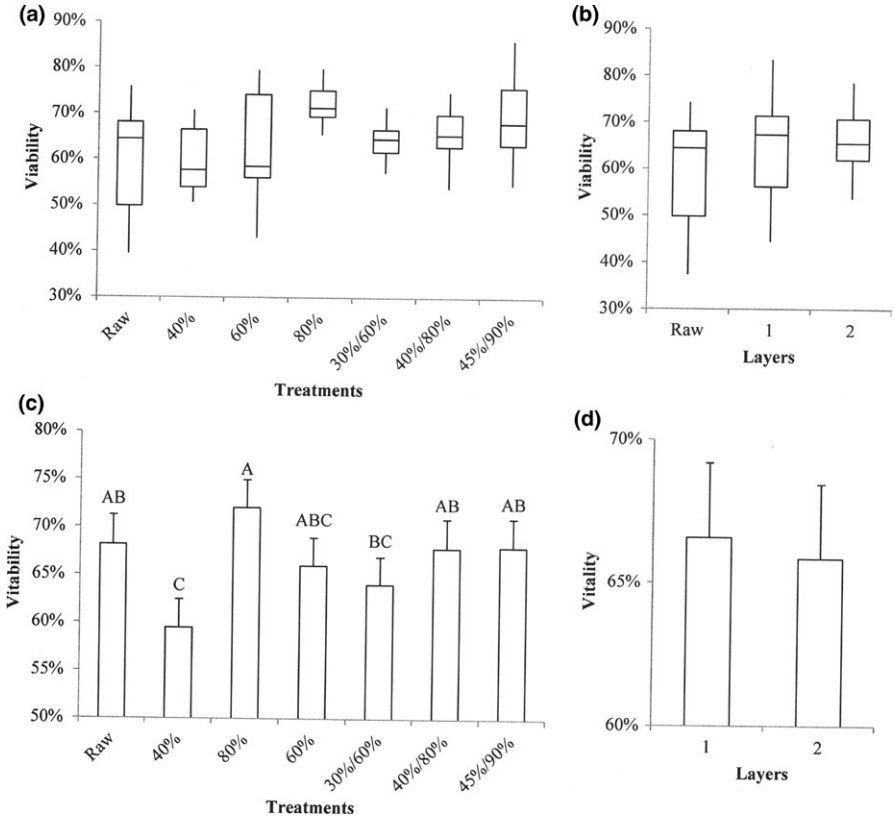

FIGURE 2 Viability rates for sex-sorted sperm without centrifugation ("Raw") and following centrifugation through one or two layers of PureSperm® of different dilutions. Viability rate box plot data are presented for the non-centrifuged sample and the six centrifugation treatments tested and also grouped by the number of colloid layers in panels (a and b), respectively. Model-adjusted mean ± *SE* estimation results are presented in panels (c and d) for the corresponding centrifugation configurations of panels (a and b), respectively. Fisher's least significant difference test results are represented in letters (Bonferroni-adjusted $p < .05$)

2006; Samardžija et al., 2006). Surprisingly, low sperm recovery rates (e.g., <20%) after density gradient centrifugation can also be found in the scientific literature (Machado et al., 2009) and this situation is not only restricted to cattle, but has also been reported in humans (Claassens, Menkveld, & Harrison, 1998). Differences in the number of colloid layers, colloid stock dilutions, layering procedure, equilibration conditions, centrifugal force and duration, among others, may have contributed to the variations between published reports (Beydola, Sharma, Lee, & Agarwal, 2013; Henkel & Schill, 2003; Mortimer, 2000a). The higher recovery rate found in single-layer centrifugation sperm selection in comparison with two-layer centrifugation could be explained by its easier and shorter preparation procedure (Morrell, Garcia, Pena, & Johannisson, 2011; Morrell, Rodriguez-Martinez, & Johannisson, 2010; Thys et al., 2009). Extra care and caution is required when layering two or more densities of colloid in the 1.5-ml tube to preserve the interface between the two layers. Consequently, any degree of layer mixing could compromise the efficiency of the sperm selection procedure.

In this study, the single-layer 40% PureSperm® configuration showed the highest recovery rate, although it also displayed the lowest viability indicating that many dead sperm passed through the colloid column. In contrast, the single-layer 80% PureSperm® configuration demonstrated a good combination of recovery rate and viability for sex-sorted sperm. This finding is important given that IVF blastocyst developmental rates with sex-sorted sperm have been reported as either reduced (Bermejo-Alvarez, Lonergan, Rath, Gutierrez-Adan, & Rizos, 2010; Blondin et al., 2009; Lu, Cran, & Seidel, 1999; Lu & Seidel, 2004; Rodriguez et al., 2012; Trigal et al., 2012; Wilson et al., 2006) or similar (Peippo et al., 2010; Xu et al., 2006; Zhang, Lu, & Seidel, 2003) when compared to those using non-sorted semen from the same sire. Even though other authors found no significant differences between single-layer and two-layer colloid configurations, they showed that single-layer recovery (total sperm, motility, and progressive motility) and in vitro fertilization yield (cleavage, blastocyst formation, and total embryo cells) performed as well as the discontinuous density gradient (Thys et al., 2009) or swim-up controls (Abraham et al., 2016). Future research should focus on validating/correlating this finding, that is single-layer 80% PureSperm® configuration, under an in vitro embryo production system to maximize straw utilization and improve blastocyst rates with sex-sorted semen.

Because sex-sorted sperm may have motion pattern characteristics somewhat different than that of conventional semen (in some instances), for example increased velocity or lower straight-line motility values, the use of standard density gradient centrifugation protocols for sperm selection, which are normally used for conventional semen, may not be entirely adequate for sex-sorted sperm. The combination of the low sperm concentration loaded in each commercial straw and the altered sperm movement pattern results in inefficient sperm sedimentation after density gradient centrifugation (Dell'Aqua et al., 2006). Consequently, the number of sex-sorted sperm recovered is low which can result in a challenge to fertilize at the appropriate sperm concentration, therefore limiting the potential number of fertilized oocytes. The inferior cleavage and blastocyst formation rates reported (Barcelo-Fimbres, Campos-Chillon, & Seidel, 2011; Liu et al., 2015; Lu et al., 1999; Merton, Haring, Stap, Hoebe, & Aten, 1997; Palma, Olivier, Neumuller, & Sinowatz, 2008; Wilson et al., 2006; Zhang et al., 2003) may be a result of the low sperm concentration obtained after selection

by density gradient. Therefore, a minimal volume density colloid sperm selection method and high centrifuge rotation speed could contribute to ameliorate sex-sorted sperm performance (Dell'Aqua et al., 2006).

The sperm motility pattern is considered one of the principal traits associated with overall semen quality analysis and fertilizing capacity (Graham & Mocé, 2005; Li, Kalo, Zeron, & Roth, 2016; Mocé & Graham, 2008; Petrunkina, Waberski, Gunzel-Apel, & Topfer-Petersen, 2007; Rodriguez-Martinez, 2007; Sellem et al., 2015). Several reviews and articles report that sperm motility and progressive motility are improved after colloid density centrifugation (Henkel & Schill, 2003; Mortimer, 1994a, 2000a; Rodriguez-Martinez et al., 1997; Samardzija, Karadjole, Getz, et al., 2006; Veznik, Svecova, Zajicova, Reckova, & Rubes, 2007). The results of the present study support these previous findings, with motility improving especially with the 80% single-layer centrifugation in comparison with the non-centrifuged sample (see Table S4). On the other hand, progressive motility did not differ between the best treatment (i.e., 80% single-layer centrifugation) and the non-centrifugation counterpart. It should be noted that CASA motility variables reported here (MOT, PMOT, VAP, VSL, VCL, ALH, BCF, STR, and LIN) are similar to previously published articles (Blondin et al., 2009; Carvalho, Sartori, Machado, Mourao, & Dode, 2010; Lenz et al., 2011), although none of these publications performed CASA analysis over a prolonged period of time.

5 | CONCLUSIONS

The results of the present study elucidate an optimal sex-sorted sperm separation protocol that may allow for more oocytes to be fertilized per frozen-thawed straw of semen. The single-layer 80% PureSperm® centrifugation layout was the more effective method in terms of sperm recovery and viability. Furthermore, major motility characteristics were increased or remained high during the evaluated time period after the 80% PureSperm® single-layer centrifugation. Mini-volume single-layer 80% PureSperm® centrifugation configuration would appear to be a very desirable alternative to two-layer and large-volume colloid preparations for sex-sorted sperm.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

LBF carried out the experiment and participated in the data collection. JLC and PJR contributed in the experimental design, experiment logistics and execution. PJR collaborated with acquisition of funding. CF helped with statistical analysis. LBF, JLC, CF, HHO, MEK and PJR participated with data analysis and drafted the manuscript with critical revision of the whole text content.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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