Animal Reproduction Science 202 (2019) 1–9

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Use of microfluidics to sort stallion sperm for intracytoplasmic sperm injection

Raul A. Gonzalez-Castro, Elaine M. Carnevale*

Department of Biomedical Sciences, Colorado State University, Equine Reproduction Laboratory, 3101 Rampart Road, Fort Collins, CO, 80521, USA

ARTICLE INFO

Keywords:
Stallion
Sperm
ICSI
Equine
Microfluidics
Sorting
Swim-up

ABSTRACT

We determined if microfluidic sorting (MF) of frozen-thawed stallion sperm improves sperm population characteristics and results in embryo development after intracytoplasmic sperm injection (ICSI). The efficiency and efficacy of MF sperm separation was evaluated by comparing pre- and post-separation sperm population variables. Procedural comparisons were performed after sorting with MF, single-layer colloidal centrifugation (SLC) or swim-up (SU), and cleavage and embryo development were evaluated after ICSI using MF-sorted sperm. In Experiment 1, when compared to the original sperm sample, MF sorting resulted in a sperm subpopulation with greater motility, morphology, viability, and membrane as well as DNA integrity. After sorting by MF, SLC and SU in Experiment 2, motility, viability, and membrane integrity were similar for sperm sorted using MF and SLC; however, morphology and DNA integrity were greater in sperm sorted using MF when compared with SLC. Swim-up was the least effective sorting method. In Experiment 3, sperm were processed using MF and SLC prior to ICSI. Motility, morphology and DNA integrity were similar for sperm subpopulations sorted using either method; but viability was greater for sperm sorted using MF than SLC. Sorting did not improve sperm membrane integrity. Sorting with MF prior to ICSI resulted in similar cleavage and blastocyst development rates as SLC. We concluded that MF separation of stallion sperm resulted in a subpopulation with improved sperm population parameters, comparable or better than SLC and SU. Embryos were produced after ICSI using MF sperm sorting.

1. Introduction

Intracytoplasmic sperm injection (ICSI) is the only repeatable and effective method to produce horse embryos *in vitro* (Carnevale, 2008). The selection of individual sperm for ICSI is dependent on embryologists' experience and primarily based on sperm motility and morphology (Sessions-Bresnahan et al., 2014). When sperm are selected using morphological markers and motility in men with male factor infertility, sperm with DNA damage are not excluded (Celik-Ozenci et al., 2004; Avendaño et al., 2009), which can negatively affect embryo quality and pregnancy (Barroso et al., 2009).

Fresh, frozen and refrozen sperm are used for ICSI in horses and can result in cleavage and embryo development, although sperm processing can affect success rates (Choi et al., 2002, 2006). Optimal methods to evaluate and sort stallion sperm for ICSI have not been identified. Potentially, the use of sperm from most stallions could result in production of embryos after use of ICSI. Differences in cleavage and embryo development rates, however, are observed after use of semen from different stallions when there are experimental and clinical conditions (Lazzari et al., 2002; Colleoni et al., 2012; Herrera et al., 2012; Hinrichs, 2013; Choi et al., 2016).

* Corresponding author.
E-mail address: elaine.carnevale@colostate.edu (E.M. Carnevale).

https://doi.org/10.1016/j.anireprosci.2018.12.012
Received 10 August 2018; Received in revised form 11 December 2018; Accepted 31 December 2018
Available online 02 January 2019
0378-4320/

Stallions with relatively lesser *in vivo* fertility also have less *in vitro*-fertilizing potential (Colleoni et al., 2012). Because the individual stallion and sperm quality affect ICSI success, there is a need for selecting high quality sperm to improve pregnancy outcomes (Carnevale, 2008). Sperm samples, therefore, are usually sorted prior to ICSI to obtain a sperm subpopulation of greater quality, increasing the probability of selecting sperm that when used will result in optimal fertility.

Different methods have been used to sort stallion sperm prior to ICSI, with the most common being a swim-up (SU) procedure or density gradient centrifugation (Colleoni et al., 2011; Choi et al., 2016). For men and stallions, both methods provide a sperm subpopulation with greater percentages of desirable end points for measurements of motility, viability, morphology, mitochondrial membrane potential, and membrane integrity and DNA intactness (Sakkas et al., 2000; Macpherson et al., 2002; Sieme et al., 2003; Morrell et al., 2009, 2010; Colleoni et al., 2011; Kim et al., 2015; Luppi et al., 2015). The microfluidic sorting (MF) technology can be used for sperm separation as an alternative method to obtain a subpopulation with relatively greater motility, viability, morphology and DNA integrity (Matsuura et al., 2013; Asghar et al., 2014; Nosrati et al., 2014; Shirota et al., 2016). Because centrifugation is not used, the MF can be used to reduce the content of reactive oxygen species (ROS) of sperm and the potential for DNA damage (Aitken and Clarkson, 1988; Barroso et al., 2000). There is no information available regarding use of MF methods combined with ICSI for embryo production in horses.

We hypothesized that use of MF for separating stallion sperm would result in improved values for sperm quality variables when compared to use of unsorted sperm and sperm where other separation methods were imposed. Aims of the studies were to: 1) determine the extent that use of MF would improve values for sperm quality variables; 2) compare values for sperm quality variables of frozen-thawed sperm after sorting using MF, single-layer colloidal centrifugation (SLC), and the SU procedure; and 3) determine if there would be cleavage and embryo production with use of sperm that were sorted using the MF technologies and ICSI.

2. Materials and methods

2.1. Chemicals, reagents and animal care

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Animal care and semen collection procedures were performed in accordance with the Animal Care and Use Committee at Colorado State University.

2.2. Experiment 1: sorting of stallion sperm using MF

2.2.1. Sperm samples and MF

Frozen-thawed sperm samples were obtained from 20 stallions. Ten of the samples were obtained from research stallions and were cryopreserved in-house as previously described (Kirk et al., 2005). A single ejaculate was collected from ten stallions, using an artificial vagina (CSU Model™; Animal Reproduction Systems, Chino, CA, USA) equipped with an in-line nylon micromesh filter (Animal Reproduction Systems) to remove the gel fraction. Volume, sperm concentration, and motility were evaluated using a graduate cylinder, 590B Equine Densimeter (Animal Reproduction Systems) and computer-assisted sperm analysis (CASA; Sperm Vision ® *Therio, MOFA Global, Verona, WI, USA), respectively*. Filtered semen was extended in pre-warmed E–Z Mixin® "BF" (Animal Reproduction Systems) at 50×10^6 sperm/mL, before the sperm suspension was centrifuged at 400 *g* for 15 min. The supernatant was discarded, and the pellet was re-suspended to 200×10^6 progressively motile sperm/mL in E–Z Freezin™ -"LE" Equine Semen Extender (Animal Reproduction System) and packed in 0.5-mL straws (IMV Technologies, Maple Grove, MN, USA). Straws were positioned in a floating straw rack (Animal Reproduction Systems) and cooled at 4 °C for 30 min. Straws were placed 4 cm above liquid nitrogen for 15 min and then plunged into liquid nitrogen for storage.

Ten samples of frozen sperm were obtained from other facilities and contained sperm of variable quality from commercial stallions. The samples were frozen in various extenders, containing approximately 200×10^6 motile sperm/mL before freezing. These samples were representative of sperm that is obtained for clinical ICSI.

For thawing, a small section of each straw (approximately a tenth of the length) was cut under liquid nitrogen to mimic clinical procedures. The cut sections were thawed at 37 °C in G-BSA [G-MOPS™ (Vitrolife Sweden AB, Frölunda, Sweden) plus 0.4% BSA]. Frozen-thawed sperm were processed using MF with the use of a commercial device (FERTILE PLUS™ Sperm Sorting Chip, DxNow Inc., MA, USA) previously used for human sperm. The manufacturer's guidelines were followed with modifications for stallion sperm as noted. Thawed sperm were suspended in ≤800 µL G-BSA and placed into the loading chamber. The collection chamber had 500 µL of G-BSA added, and the device was incubated in room air at 37 °C for 20 min, allowing sperm to swim up and through a porous membrane into the collection chamber. From the collection chamber, 300 µL of sorted sperm were removed for evaluation.

2.2.2. Sperm analysis

Frozen-thawed sperm were analyzed before and after MF sorting for the following variables (percentage per total sperm) of: 1) total motile sperm, MOT+, 2) normal morphology, MORPH+, 3) live sperm, as a measurement of viability and sperm head membrane integrity, LIVE+, 4) positive hypoosmotic swelling, indicative of membrane integrity in the principal piece of the tail, HOS+, and 5) DNA fragmentation, DNA–.

For MOT+, visual assessment by a single observer was performed, using phase-contrast microscopy at X 200 magnification. For MORPH + and LIVE+, an aliquot (20 µL) of the sperm sample was mixed on a slide with 20 µL of an eosin-nigrosin stain (Hancock Stain®, Animal Reproduction Systems Inc.). The mixture was smeared on a slide and dried on a warming plate at 37 °C to avoid hypotonic artifacts. Sperm ($n = 100$ per sample) were examined under X 1000 magnification using bright field microscopy.

Morphological abnormalities included defects of the head (altered head size and shape head, acrosome defects, nuclear vacuoles and deformities), midpiece (proximal or distal cytoplasmic droplets, swollen or rough midpiece, and fractured axonemal fibers), and principal piece (cytoplasmic retention, bending and coiling). For LIVE+, sperm with an intact post-acrosomal plasma membrane that did not uptake eosin in the head region were considered to be live, and sperm with any detectable eosin in the head region were counted as dead (Brito, 2007; Mocé and Graham, 2008). LIVE + sperm were characterized on each slide as the percentage of unstained sperm per 200 total sperm (Brito, 2007).

For HOS+, 10 µL of the sperm sample was mixed with 100 µL of hypoosmotic sucrose solution (100 mOsm/Kg) in a 1.5 mL microtube. The mixture was incubated at 37 °C for 30 min. Sperm (n = 300 per sample) were examined under X 400 magnification with contrast field microscopy. Sperm with unaltered tail morphology were considered HOS–, and sperm with a distinctive curling of the tail were considered HOS+ (Jeyendran et al., 1984). Results are expressed as percentages of HOS + sperm per total sperm.

Sperm chromatin dispersion test was used to determine DNA–. Briefly, 50 µL of 1% low-melting point agarose in 1.5 mL microtube was placed in a water bath at 90 °C for 3 min and then moved to a slide warmer at 37 °C for 5 min. An aliquot (10 µL) of the sperm sample was mixed with the melted agarose. The mixture was pipetted onto a 0.65% agarose-precoated slide, covered with cover glass, and cooled at 4 °C for 5 min. After removing the cover glass, the slide was incubated in the dark with an acid denaturation solution (0.08 N HCl) for 7 min at room temperature. The slide was then immersed in Lysing Solution I (0.4 M 2-Amino-2-(hydroxymethyl)-1,3-propanediol, 0.4 M 2-mercaptophenol, 1% sodium dodecyl sulfate, and 50 mM ethylenediaminetetraacetic acid, pH 7.5) for 10 min and Lysing Solution II (0.4 M 2-Amino-2-(hydroxymethyl)-1,3-propanediol, 2 M NaCl, and 1% sodium dodecyl sulfate, pH 7.5) for 5 min. After washing with distilled water for 5 min, the slide was consecutively immersed for 2 min in 70%, 90% and 100% ethanol. The slide was stained with Dip Quick Stain (Jorgensen Laboratories Inc., Loveland, CO, USA), and 300 sperm were counted under X 1000 magnification using bright field microscopy. Sperm with a large or medium halo of DNA dispersion were considered to have intact DNA, and sperm with small or no halo of DNA dispersion were considered to have fragmented DNA (DNA–). DNA– was determined for each slide as the percentage of sperm with fragmented DNA per total sperm (Fernandez et al., 2003, 2005; López-Fernández et al., 2007; Olaciregui et al., 2016)

2.3. Experiment 2: Comparison of sperm population sorting using MF, SLC, and the SU procedure

2.3.1. Sperm samples and sorting

Sperm from 19 stallions were frozen in-house (n = 10) or obtained from other facilities (n = 9) as described in Experiment 1. A single straw from each stallion was thawed in a water bath at 37 °C for 30 s before being divided into three aliquots for sorting using MF, SLC and SU. For MF, thawed sperm samples were processed as described for Experiment 1. For SLC, thawed samples (100 µL, ~40×10^6 sperm) were suspended in ≤ 200 µL of G-BSA and layered onto 500 µL of Equipure™ (Nidacon, International AB, Gothenburg, Sweden) diluted with G-BSA (75% v/v) in a 1.5-mL microtube and centrifuged at 200 g for 8 min. Supernatant was discarded, and the remaining sediment (50 µL) was washed in 300 µL G-BSA at 400 g for 3 min for analysis. For SU, thawed sperm were overlaid by 1 mL of G-IVF (Vitrolife, plus 0.4% BSA) at 38.2 °C. The tube was positioned at ~45° angle and incubated in 6% CO_2 and air at 38.2 °C for 15 min. Approximately, 0.5 mL of supernatant was removed and centrifuged at 300 g for 3 min. The supernatant was discarded and the remaining sediment (100 µL) was used for evaluation.

2.3.2. Sperm analysis

Frozen-thawed sperm were analyzed before and after sorting for MOT+, MORPH+, LIVE+, HOS + and DNA–. Assessments of MORPH+, LIVE + and HOS + were performed as described for Experiment 1. For MOT + and DNA–, sufficient numbers of sperm were available to evaluate larger numbers of sperm using different methods. For MOT + evaluation, thawed sperm were diluted in prewarmed E–Z Mixin® "BF" (Animal Reproduction System) to a final sperm concentration of 50×10^6 sperm/mL. Total sperm motility was analyzed using a computer-assisted sperm analyzer (Sperm Vision® *Therio)*. *For* DNA–, samples were evaluated following the protocol for Sperm Chromatin Structure Assay (Evenson and Melamed, 1983). Briefly, an aliquot of (100 µL) frozen-thawed sperm was layered onto 500 µL of Equipure™ in a 1.5 mL microtube and centrifuged for 10 min at 200 g to remove extender. For samples sorted by SLC, this step was not needed, and the SLC samples were not exposed to another centrifugation step with Equipure™ prior to the DNA assay. The supernatant was removed, and the pellet was re-suspended in TNE-Tris (1.0 mM 2-Amino-2-(hydroxymethyl)-1,3-propanediol, 15.0 mM NaCl and 1.0 mM ethylenediaminetetraacetic acid, pH 7.4). Sperm concentration was adjusted to 2×10^6 sperm/mL. A 200 µL sperm-TNE-Tris suspension was mixed with 400 µL of a detergent solution (0.08 N HCl, 15 mM NaCl and 0.1% Triton X-100). Thirty seconds later, 1.2 mL of acridine orange solution (20 mM Na_2HPO_4, 10 mM citric acid, 1 mM ethylenediaminetetraacetic acid, 15 mM NaCl and 6 µg acridine orange/mL) was added. A minimum of 20,000 events per sample were analysed within 3–5 min using an Accuri ™ C6 Flow cytometer and CFlow Plus (Becton Dickinson, Franklin Lakes, NJ, USA). Percentage of DNA fragmentation (DNA–) was assessed by selecting sperm to the right of the main population on a dot plot of red (FL3, > 670 nm/long pass band) compared with green (FL1, 530/30 nm band pass) fluorescence.

2.4. Experiment 3: Use of MF for separation of sperm prior to ICSI and comparison with SLC

2.4.1. Sperm samples and sorting for oocyte injection

Sperm frozen with various extenders and methods in 0.25-, 0.5- or 5-mL straws (n = 40 analyzed samples) from 21 commercial stallions were included. Commercial sperm samples were incorporated into the clinical program without specific preconditions and rotating days for different sample processing. Individual straws were cut under liquid nitrogen to use a minimal number of sperm. The

cut sections were thawed at 37 °C in G-BSA. Sperm were sorted by MF or SLC as described in Experiments 1 and 2. In samples processed by MF (n = 22), sperm from the collection chamber were used for final selection. The SLC samples (n = 18) were processed, and sperm from the sediment were washed prior to final selection. After processing and just prior to ICSI, the final selection of sperm for oocyte injection was based on motility and morphology at X 200 magnification by the ICSI technician.

2.4.2. Sperm analysis

Samples were analyzed before and after sorting for MOT+, MORPH+, LIVE+, HOS + and DNA– as described in Experiment 1.

2.4.3. Oocyte collection and sperm microinjection

Oocyte donors were mares 3 to 25 yr (n = 20, mean ± SEM of 12.2 ± 4.7 yr). Oocytes were collected by transvaginal, ultrasonic-guided follicle aspirations during the follicular phase of the estrous cycle from dominant follicles, yielding maturing oocytes (Carnevale, 2016). Maturing oocytes completed maturation in Medium 199 with Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate (GIBCO BRL Life Technologies, Grand Island, NY, USA) with additions of 10% fetal calf serum, 0.2 mM pyruvate and 25 mg/mL gentamicin for approximately 20 h at 38 °C in 6% CO_2 and air. During the study, matured oocytes in metaphase II with extrusion of a polar body were used for ICSI. Sperm injections (Day 0 = day of ICSI) were performed by a single technician using a piezo drill into 50 oocytes (n = 29 for sperm processed by MF, n = 21 for sperm processed by SLC). After ICSI, potential zygotes were placed into 30-μL drops of culture medium (Dulbecco Modified Eagle medium/F12, GIBCO BRL Life Technologies, with 10% fetal calf serum) under oil (Ovoil™, Vitrolife, Göteborg, Sweden) at 38 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. The day after ICSI, potential zygotes were observed under X 200 magnification for cleavage; if complete cell division was not observed, but markers of imminent cleavage were noted, such as change in shape or fragmentation of the oolemma, the potential zygote was reassessed for cleavage on the following day. Cleaved embryos were evaluated on Days 5 and daily thereafter until development into a blastocyst or confirmation of embryo degeneration. Development after ICSI was defined per injected oocyte as Cleavage, ≥ 2 cells by Day 2, and Blastocyst, development of a blastocyst.

2.5. Statistical analysis

Statistical analyses were performed using R Core Team Software 3.4.2017 (R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria). Data obtained for sperm variable analyses were tested for normality and homogeneity of variances using the Shapiro-Wilk and Levene test. In Experiment 1, Wilcoxon Rank Sum test for independent samples was used to compare sperm population quality parameters between in-house and commercial samples. Because multiple sperm variables were different between in-house and commercial samples, further comparisons were performed separately by sample source. Percentages for sperm quality variables before and after MF sorting were examined by two-factor repeated measures design with sample type (in-house and commercial samples) as the between-factor and sorting (unsorted and sorted sperm) as the within-factor. A mixed model was fit to each sperm variable separately, with type of samples, sorting and type and sorting interaction were fixed effects. In Experiment 2, percentages for sperm variables before and after sorting among methods were analyzed using one-factor repeated measures design with sorting methods as the within-factor and stallion as a random effect for repeated measures. A mixed model was fit to each sperm variable separately. Tukey adjusted pairwise comparisons were considered among sorting methods. In Experiment 3, comparisons of percentages for sperm variables before and after sorting within each method and between methods were performed by Wilcoxon test for paired and independent samples, respectively. Associations among values for sperm variables after sorting were performed by Spearman's rank correlation (r_s). Percentages for sperm variables after sorting that resulted in positive (Yes) or negative (No) ICSI outcome for Cleavage and Blastocyst were compared by Wilcoxon Rank Sum test for independent samples. Logistic regressions were conducted to investigate the association between sorting methods and ICSI outcomes for Cleavage and Blastocyst. Odd ratios (OR) and 2.5% and 97.5% confidence interval (CI) were calculated. Values for sperm variables are presented as mean ± SEM. Statistical differences were considered to occur at $P < 0.05$.

3. Results

3.1. Experiment 1: sorting of stallion sperm using MF

When compared to original sperm samples, values for MOT+, MORPH+, LIVE+, and DNA– were greater ($P \leq 0.05$) after MF sorting of in-house and commercial samples (Table 1), there being no significant interaction between sample type and sorting for these variables ($P > 0.08$). In-house samples, however, had a greater HOS+ value before ($P < 0.004$) and after ($P < 0.0001$) sorting than commercial samples, with an interaction between sample type and sorting ($P > 0.015$). In commercial samples, HOS+ was not affected ($P > 0.2$) by MF sorting (Table 1).

3.2. Experiment 2: Comparison of sperm population sorting using MF, SLC, and SU procedures

When the original sperm population was compared to sorted sperm, MF was the only sorting method that when used there was improvement in MORPH+, LIVE + and DNA– (Table 2). There was a greater MOT + after MF and SLC than in the original sperm population. There was none of the sorting methods that were assessed with which there was an increase for HOS + when compared to the original samples, although the percentage of HOS + was greater after sorting with SLC than SU (Table 2). Values for sperm

Table 1
Mean ± SEM percentages (range) for sperm population quality variables for unsorted and sorted sperm populations using microfluidics from in-house ($n = 10$) and commercial stallion samples ($n = 10$); Values with different superscripts for unsorted and sorted sperm for individual sperm variables differ at $^{a,b}P < 0.05$ and $^{c,d}P = 0.05$; MOT+, percentage of total motile sperm; MORPH+, percentage of morphologically normal sperm; LIVE+, percentage of live sperm; HOS+, percentage of sperm with functional tail membrane; DNA–, percentage of sperm with fragmented DNA.

	MOT+		MORPH+		LIVE+		HOS+		DNA–	
Samples	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted
In-house	37.2 ± 4.1[a] (20-60)	62.2 ± 4.9[b] (40-90)	60.1 ± 3.9[a] (47-85)	75.5 ± 3.1[b] (62-89)	55.8 ± 5.1[a] (25-73)	73.6 ± 3.8[b] (55-98)	33.7 ± 2.3[a] (19-44)	48.9 ± 3.1[b] (33-67)	12.3 ± 1.4[a] (6-19)	5.6 ± 1.4[b] (1-13)
Commercial	22.0 ± 4.1[a] (10-50)	57.0 ± 3.7[b] (50-80)	58.4 ± 3.1[a] (42-72)	74.0 ± 3.2[b] (52-84)	55.5 ± 3.5[c] (46-80)	68.3 ± 4.5[d] (47-91)	21.2 ± 1.9 (11-34)	24.9 ± 3.6 (7-45)	22.3 ± 4.6[a] (10-52)	8.2 ± 2.6[b] (2-26)

Table 2
Mean ± SEM percentages for sperm quality variables for the original samples (Unsorted, $n = 19$) and sperm sorted using microfluidics (MF), single layer colloidal centrifugation (SLC) and swim−up procedure (SU); Values with different superscripts within a row differ at $^{a,b,c}P < 0.05$; MOT+, percentage of total motile sperm; MORPH+, percentage of morphologically normal sperm; LIVE+, percentage of live sperm; HOS+, percentage of sperm with functional tail membrane; DNA–, percentage of sperm with fragmented DNA.

	Unsorted	Sorting Methods		
		MF	SLC	SU
MOT+	43.7 ± 3.3^{a}	53.9 ± 3.5^{bc}	61.8 ± 3.9^{c}	49.2 ± 4.0^{ab}
MORPH+	57.0 ± 5.2^{a}	71.1 ± 4.1^{b}	62.2 ± 5.2^{a}	62.6 ± 4.8^{a}
LIVE+	65.8 ± 2.2^{a}	73.9 ± 2.3^{b}	67.9 ± 2.5^{ab}	63.9 ± 2.6^{a}
HOS+	33.6 ± 2.0^{ab}	30.5 ± 1.8^{ab}	35.4 ± 2.0^{b}	30.1 ± 1.9^{a}
DNA–	12.7 ± 1.5^{a}	6.0 ± 1.2^{b}	12.1 ± 1.9^{a}	10.5 ± 1.5^{a}

population variables were not different after SU when compared to values with the original samples (Table 2).

3.3. Experiment 3: Use of MF for sorting sperm prior to ICSI and comparison with SLC

Prior to sorting with MF or SLC, mean values for sperm population variables (MOT+, MORPH+, LIVE+, HOS + and DNA–) were not different from those of the original samples (Table 3). With the exception of HOS+, mean values for sperm population variables were greater after sorting with MF or SLC (Table 3). Only the mean value for LIVE + was greater for sperm sorted using MF as compared with the value when there was use of SLC. There were no differences in mean values for the other sperm variables after sorting (Table 3). Some values for sperm population variables were correlated after sorting using MF and SLC (Table 4), although correlations were not significant between sorting methods (MF and SLC).

After ICSI, 54% (27/50) of all sperm-injected oocytes cleaved, and 46% (23/50) of sperm-injected oocytes developed into a blastocyst by Day 7, however, there was no blastocyst development beyond Day 7 of culture. There were no embryo developmental associations related to sorting methods used and ICSI outcomes for Cleavage [$P > 0.3$; MF: OR = 1 (CI, 0.4–1.9) and SLC: OR = 1.74 (CI, 0.6–5.6)] or Blastocyst [$P > 0.4$; MF: OR = 1 (CI, 0.3–1.50) and SLC: OR = 1.55 (CI, 0.5–4.9)]. Mean values for sperm variables that were associated positive or negative for Cleavage or Blastocyst were not different among sorting methods; with the exception that MORPH+ was greater ($P < 0.02$) for sperm populations that did not result in blastocyst formation when sperm were separated using SLC (Table 5).

4. Discussion

Results of several studies with different species provide ample evidence that the use of MF provides a sperm subpopulation with improved characteristics for assisted reproductive technologies (Matsuura et al., 2013; Asghar et al., 2014; Nosrati et al., 2014; Shirota et al., 2016). In the present study, stallion sperm were sorted using a MF approach. The device that was used was designed with two chambers separated by a membrane filter with 8-µm micropores. Motile stallion sperm, having a head width of approximately 3 µm (2.79 to 3.26 µm) (Brito, 2007) and can transit through the membrane and into the collection chamber when using MF. Motile sperm move selectively against gravity and through the micropores, with less motile or nonmotile sperm and debris remaining behind (Asghar et al., 2014). Advantageously, the use of MF does not require centrifugation, which can cause sublethal damage to sperm and expose DNA to relatively greater concentrations of ROS that can ultimately lead to DNA fragmentation (Aitken and Clarkson, 1988; Hughes et al., 1998; Barroso et al., 2000). In the present study, sperm samples from in-house and commercial stallions differed for MOT+, HOS + and DNA–, before sorting, with in-house samples having values indicating greater ($P < 0.02$)

Table 3
Mean ± SEM percentages (range) of sperm quality variables of samples used for ICSI in Experiment 3 for Unsorted (original sample) and Sorted sperm using microfluidics (MF, $n = 22$) and single layer colloidal centrifugation (SLC, $n = 18$); Values with different superscripts for Unsorted and Sorted within a row for sperm quality variables differ at $^{a,b}P < 0.05$; Values within a column differ at $^{A,B}P < 0.05$ between MF and SLC. MOT+, percentage of total motile sperm; MORPH+, percentage of morphologically normal sperm; LIVE+, percentage of live sperm; HOS+, percentage of sperm with functional tail membrane; DNA–, percentage of sperm with fragmented DNA.

	MOT+		MORPH+		LIVE+		HOS+		DNA–	
	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted	Unsorted	Sorted
MF	23.7 ± 3.2^{a} (10–60)	55.9 ± 2.8^{b} (40–90)	54.7 ± 3.5^{a} (18–74)	73.8 ± 2.4^{b} (49–95)	51.9 ± 2.8^{a} (22–80)	67.5 ± 3.2^{bA} (39–91)	21.3 ± 1.4 (11–34)	21.5 ± 2.3 (4–45)	21.9 ± 2.9^{a} (7–52)	9.4 ± 1.6^{b} (1–26)
SLC	25.0 ± 3.2^{a} (10–50)	53.5 ± 3.9^{b} (25–80)	54.5 ± 3.6^{a} (25–76)	66.1 ± 4.0^{b} (19–88)	46.4 ± 2.9^{a} (22–68)	57.8 ± 3.8^{bB} (19–86)	21.4 ± 2.2 (12–51)	21.0 ± 2.0 (9–36)	17.3 ± 2.1^{a} (7–36)	12.4 ± 1.8^{b} (4–30)
Total	24.6 ± 2.5^{a} (10–60)	54.8 ± 2.6^{b} (25–90)	54.6 ± 2.9^{a} (18–76)	70.8 ± 3.0^{b} (19–95)	49.4 ± 2.9^{a} (22–80)	62.7 ± 3.5^{b} (19–91)	21.3 ± 1.6 (11–51)	21.2 ± 2.0 (4–45)	19.6 ± 2.4^{a} (7–52)	11.3 ± 1.8^{b} (1–30)

Table 4
Correlations among sperm quality variables after sorting using microfluidics (MF) and single layer colloidal centrifugation (SLC) (n = 40 samples); No differences in correlations were observed with use of MF and SLC; Spearman's correlation coefficients (r_s) noted with asterisk (*) were significant at $P < 0.05$. MOT+, percentage of total motile sperm; MORPH+, percentage of morphologically normal sperm; LIVE+, percentage of live sperm; HOS+, percentage of sperm with functional tail membrane; DNA–, percentage of sperm with fragmented DNA.

	MORPH+	LIVE+	HOS+	DNA–
MOT+	r_s = 0.52* P = 0.0001	r_s = 0.53* P = 0.0001	r_s = 0.07 P = 0.7	r_s = –0.56* P < 0.0001
MORPH+		r_s = 0.40* P = 0.004	r_s = –0.03 P = 0.8	r_s = –0.44* P = 0.001
LIVE+			r_s = 0.32* P = 0.04	r_s = –0.68* P < 0.0001
HOS+				r_s = 0.001 P = 0.9

Table 5
Cleavage and Blastocyst development rates per sperm injected oocyte after sorting of sperm with use of microfluidics (MF) and single layer colloidal centrifugation (SLC) and mean ± SEM percentages for sperm variables in which ICSI resulted in positive (Yes) or negative (No) outcomes; Values with different superscripts for individual sperm variables within sorting method differ at $^{a,b}P < 0.05$; MOT+, percentage of total motile sperm; MORPH+, percentage of morphologically normal sperm; LIVE+, percentage of live sperm; HOS+, percentage of sperm with functional tail membrane; DNA–, percentage of sperm with fragmented DNA.

		Rates	Outcome	MOT+	MORPH+	LIVE+	HOS+	DNA–
Cleavage	MF	48.3% (14/29)	YES	53.1 ± 3.1	73.9 ± 3.0	67.1 ± 3.9	21.3 ± 3.7	11.8 ± 2.1
			NO	57.2 ± 2.9	74.8 ± 2.7	68.2 ± 3.6	20.2 ± 8.5	8.4 ± 2.0
	SLC	61.9% (13/21)	YES	52.9 ± 4.1	62.1 ± 6.4	59.3 ± 4.6	22.4 ± 2.1	12.1 ± 1.9
			NO	53.2 ± 8.5	74.9 ± 6.1	57.2 ± 5.0	19.3 ± 1.4	11.7 ± 1.8
Blastocyst	MF	41.3% (12/29)	YES	54 .0 ± 3.8	74.1 ± 3.8	65.9 ± 4.9	21.2 ± 3.6	11.8 ± 2.2
			NO	56.1 ± 3.1	74.9 ± 2.5	68.7 ± 3.2	20.1 ± 8.6	9.4 ± 7.6
	SLC	52.3% (11/21)	YES	52.8 ± 3.9	57.2 ± 6.0^a	61.8 ± 4.6	20.9 ± 2.7	10.8 ± 2.2
			NO	54.1 ± 6.6	77.4 ± 2.1^b	54.4 ± 4.8	20.1 ± 2.1	12.7 ± 1.8

sperm quality. Consequently, analyses were performed grouping samples by origin (in-house and commercial) as sperm quality is a potential confounding factor for sperm sorting efficiency, and freezing methods used in the present study were standardized only for the in-house samples. Results of this study indicate MF can be used with stallion sperm and result in a sperm subpopulation with improved motility, morphology, viability and DNA integrity, independent of the starting point values for most individual sperm quality variables. In sperm samples with disrupted membrane integrity as measured using the HOS test, the use of MF did not result in an improvement of membrane integrity in the separated sperm populations when this technology was used. Human sperm sorted with similar MF devices as that used in the present study have a subpopulation with greater motility, viability, normal morphology, nuclear maturity, and DNA integrity (Asghar et al., 2014). In humans, a negative relationship was observed between sperm DNA fragmentation and pregnancy outcomes when there was use of IVF and ICSI technologies (Avendaño et al., 2009; Zhao et al., 2014). The use of sperm with > 30% DNA fragmentation resulted in reduced pregnancy rates after IVF, but a similar association was not detected after use of ICSI (Bungum et al., 2006; Li et al., 2006). The selection of sperm based on motility and morphology characteristics for ICSI from a population with relatively greater DNA fragmentation (> 50%) resulted in a similar pregnancy rate when compared to selection of sperm with a lesser DNA fragmentation (< 15%), however, there was an increase in miscarriages suggesting a potential negative effect on embryonic/fetal development (Dar et al., 2013). Although sperm DNA fragmentation may not result in a reduction in cleavage and early pregnancy after use of ICSI in horses (Gonzalez-Castro and Carnevale, 2018), this fragmentation may be associated with embryo loss at later stages of development as occurs in humans (Miller and Smith, 2001; Loutradi et al., 2006;. During embryo development in humans, developmental abnormalities, implantation failure or early pregnancy loss are associated with a paternal effect related to nuclear or chromatin defects in sperm such as aneuploidy, genetic anomalies and DNA fragmentation (Barroso et al., 2009). The use of MF was the only sorting method in the present study that resulted in the selection of a sperm population with less DNA fragmentation; therefore, some advantages of MF sperm sorting could become evident at development stages later than observed in the present study.

The use of current sperm sorting methods, such as density gradient centrifugation or SU procedures, can provide a means to select a more desirable population of sperm prior to use for ICSI in horses and humans (Choi et al., 2016; Galli et al., 2016; Simopoulou et al., 2016). A proteomic comparison of human sperm has been used to evaluate density gradient centrifugation results in sperm with greater capacitation potential than SU (Luppi et al., 2015); but in most studies, use of both methods result in detection of sperm of similar quality for motility, morphology, viability or DNA integrity (Sakkas et al., 2000; Zini et al., 2000; Kim et al., 2015, 2017). In the present study, SU was the least effective method that was assessed to separate small quantities for sperm with greater mean values for quality variables with the use of MF and SLC resulting in sperm subpopulations with greater normal morphology, motility, viability and DNA integrity. Consistent with the results from the present study, MF sorted sperm in a previous study had a greater

normal morphology and DNA integrity when compared to sperm sorted using density gradient centrifugation and SU in sperm samples from infertile and fertile men (Schulte et al., 2007; Shirota et al., 2016; de Martin et al., 2017). Human sperm separated by MF also have less ROS when compared with sperm sorted using basic washing or SU (Asghar et al., 2014). This finding indicates that the greater ROS generation with use of SU resulted from the centrifugation steps (Aitken and Clarkson, 1988; Hughes et al., 1998), which are avoided using MF. Fresh sperm from stallions processed using single- or double-layer colloidal centrifugation have greater motility, normal morphology, and DNA integrity when compared to unprocessed sperm (Morrell et al., 2009, 2010). The use of density gradient centrifugation results in a greater viability, but not motility or morphology, for fresh stallion sperm when compared to unsorted samples, and use of the SU results in samples have greater motility, but not normal morphology or viability. The use of density gradient centrifugation and SU result in similar viability, but use of SU results in a greater motility and lesser normal morphology in the sorted sperm subpopulation (Sieme et al., 2003). The efficiency of sperm sorting based on motility can be co-founded by the sperm quality of the originating sample (Xue et al., 2014; Simopoulou et al., 2016). Findings in the present study indicate SU is not an efficient method to sort small numbers of limited quality stallion sperm, and SLC and MF are more efficient techniques that are less sensitive to the confounding effects of quality of the original sample.

In clinical practice, the stallion effect on ICSI is confounded by mare selection and can require additional ICSI cycles if differences in samples are to be detected (Galli et al., 2016). In the present study, there were no differences between the ICSI outcomes that were related to the sorting methods when there were a relatively small sample numbers. Larger sample sizes (n = 197 for cleavage and n = 321 for blastocyst formation) would have been required to detect statistical differences at a power of 0.8. Human fertilization and pregnancy outcomes after standard *in vitro* fertilization are similar when there is use of density gradient centrifugation and SU (Kim et al., 2015). In horses, there were greater cleavage and blastocyst rates after ICSI when sperm were sorted using density gradient centrifugation followed by SU when compared to sperm processed by density gradient centrifugation alone, but the outcomes did not differ when compared to sperm processed by basic washing or SU (Choi et al., 2016). In the present study, sperm motility and morphology were used as the final criteria for sperm selection, and this could have negated some of the effects of sorting method. Selection of a sperm sorting method in a clinical program, to some extent, is going to depend on particular aspects of the original sperm sample. In the present studies, there was use of MF sorting with relatively few numbers of sperm and with sperm populations with compromised quality. In addition, MF was the only sorting method that when used there was a subpopulation of sperm with greater DNA integrity when compared to use of SLC and SU. Although, MF was the only sorting method in the present study that when used there was a subpopulation of sperm with relatively greater DNA integrity than with use of the other methods, the advantage of using MF sorting for clinical ICSI with horses is still to be determined.

Conflict of interest

The authors declare that there are no known conflicts of interest.

Acknowledgements

Vista Equine Colorado, LLC and Dr. Jacobo Rodriguez provided some frozen sperm samples, and DxNow Inc. provided FERTILE PLUS™ Sperm Sorting Chips. The research was funded by The Cecil and Irene Hylton Foundation and the Equine Assisted Reproduction Program at Colorado State University. Doctoral scholarship was granted to Raul Gonzalez-Castro by Becas Chile (CONICYT, Ministry of Education of Chile). JoAnne Stokes performed ICSI procedures.

References

Aitken, R.J., Clarkson, J., 1988. Significance and antioxidants of reactive oxygen species in defining the efficacy of sperm preparation techniques. J. Androl. 9, 367–376.

Asghar, W., Velasco, V., Kingsley, J.L., Shoukat, M.S., Shafiee, H., Anchan, R.M., Mutter, G.L., Tüzel, E., Demirci, U., 2014. Selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species. Adv. Healthc. Mater. 3, 1671–1679. https://doi.org/10.1002/adhm.201400058.

Avendaño, C., Franchi, A., Taylor, S., Morshedi, M., Bocca, S., Oehninger, S., 2009. Fragmentation of DNA in morphologically normal human spermatozoa. Fertil. Steril. 91, 1077–1084. https://doi.org/10.1016/j.fertnstert.2008.01.015.

Barroso, G., Morshedi, M., Oehninger, S., 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum. Reprod. 15, 1338–1344. https://doi.org/10.1093/humrep/15.6.1338.

Barroso, G., Valdespin, C., Vega, E., Kershenovich, R., Avila, R., Avendaño, C., Oehninger, S., 2009. Developmental sperm contributions: fertilization and beyond. Fertil. Steril. 92, 835–848. https://doi.org/10.1016/j.fertnstert.2009.06.030.

Brito, L.F.C., 2007. Evaluation of stallion sperm morphology. Clin. Tech. Equine Pract. 6, 249–264. https://doi.org/10.1053/j.ctep.2007.09.004.

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., Giwercman, A., 2006. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum. Reprod. 22, 174–179. https://doi.org/10.1093/humrep/del326.

Carnevale, E.M., 2008. Clinical considerations regarding assisted reproductive procedures in horses. J. Equine Vet. Sci. 28, 686–690. https://doi.org/10.1016/j.jevs.2008.10.005.

Carnevale, E.M., 2016. Advances in collection, transport and maturation of equine oocytes for assisted reproductive techniques. Vet. Clin. North Am. - Equine Pract. 32, 379–399. https://doi.org/10.1016/j.cveq.2016.07.002.

Celik-Ozenci, C., Jakab, A., Kovacs, T., Catalanotti, J., Demir, R., Bray-Ward, P., Ward, D., Huszar, G., 2004. Sperm selection for ICSI: shape properties do not predict the absence or presence of numerical chromosomal aberrations. Hum. Reprod. 19, 2052–2059. https://doi.org/10.1093/humrep/deh361.

Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S., Hinrichs, K., 2002. Developmental competence in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. Reproduction 123, 455–465. https://doi.org/10.1530/rep.0.1230455.

Choi, Y.H., Love, C.C., Varner, D.D., Hinrichs, K., 2006. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. Theriogenology 65, 808–819. https://doi.org/10.1016/j.theriogenology.2005.04.035.

Choi, Y.H., Velez, I.C., Macías-García, B., Riera, F.L., Ballard, C.S., Hinrichs, K., 2016. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. Theriogenology 85, 1289–1296. https://doi.org/10.1016/j.theriogenology.2015.12.015.

Colleoni, S., Lagutina, I., Lazzari, G., Rodriguez-Martinez, H., Galli, C., Morrell, J.M., 2011. New methods for selecting stallion spermatozoa for assisted reproduction. J. Equine Vet. Sci. 31, 536–541. https://doi.org/10.1016/j.jevs.2011.03.009.
Colleoni, S., Lazzari, G., Duchi, R., Baca Castex, C., Mari, G., Lagutina, I., Galli, C., 2012. Fertilization and development of oocytes after ICSI with semen of stallions with different in vivo fertility. J. Equine Vet. Sci. 32, 408–409. https://doi.org/10.1016/j.jevs.2012.05.030.
Dar, S., Grover, S.A., Moskovtsev, S.I., Swanson, S., Baratz, A., Librach, C.L., 2013. In vitro fertilization-intracytoplasmic sperm injection outcome in patients with a markedly high DNA fragmentation index (> 50%). Fertil. Steril. 100, 75–80. https://doi.org/10.1016/j.fertnstert.2013.03.011.
de Martin, H., Cocuzza, M.S., Tiseo, B.C., Wood, G.J.A., Miranda, E.P., Monteleone, P.A.A., Soares, J.M., Serafini, P.C., Srougi, M., Baracat, E.C., 2017. Positive rheotaxis extended drop: a one-step procedure to select and recover sperm with mature chromatin for intracytoplasmic sperm injection. J. Assist. Reprod. Genet. 1–10. https://doi.org/10.1007/s10815-017-1024-1.
Evenson, D.P., Melamed, M.R., 1983. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J. Histochem. Cytochem. 31, 248–253.
Fernandez, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R., Alvarez, J.G., 2003. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J. Androl. 24, 59–66. https://doi.org/10.1002/j.1939-4640.2003.tb02641.x.
Fernandez, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gos??lvez, J., Enciso, M., LaFromboise, M., De Jonge, C., 2005. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil. Steril. 84, 833–842. https://doi.org/10.1016/j.fertnstert.2004.11.089.
Galli, C., Colleoni, S., Duchi, R., Lazzari, G., 2016. Male factors affecting the success of equine in vitro embryo production by ovum pickup-intracytoplasmic sperm injection in a clinical setting. J. Equine Vet. Sci. 43, S6–S10. https://doi.org/10.1016/j.jevs.2016.05.014.
Gonzalez-Castro, R.A., Carnevale, E.M., 2018. Association of equine sperm population parameters with outcome of intracytoplasmic sperm injections. Theriogenology 119, 114–120. https://doi.org/10.1016/j.theriogenology.2018.06.027.
Herrera, C., Dufourq, P., Freije, M., Morikawa, I., Centeno, J.E., Aristi, V., Menghini, L., Sporleder, C., 2012. Selection of stallions for in vitro embryo production by ICSI in a commercial program. J. Equine Vet. Sci. 32, 409. https://doi.org/10.1016/j.jevs.2012.05.031.
Hinrichs, K., 2013. Assisted reproduction techniques in the horse. Reprod. Fertil. Dev. 25, 80–93. https://doi.org/10.1071/RD12263.
Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J., Thompson, W., 1998. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Hum. Reprod. 13, 1240–1247. https://doi.org/10.1093/humrep/13.5.1240.
Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70, 219–228. https://doi.org/10.1530/jrf.0.0700219.
Kim, E.K., Kim, E.H., Kim, E.A., Lee, K.A., Shin, J.E., Kwon, H., 2015. Comparison of the effect of different media on the clinical outcomes of the density-gradient centrifugation/swim-up and swim-up methods. Clin. Exp. Reprod. Med. 42, 22–29. https://doi.org/10.5653/cerm.2015.42.1.22.
Kim, S.W., Jee, B.C., Kim, S.K., Kim, S.H., 2017. Sperm DNA fragmentation and sex chromosome aneuploidy after swim-up versus density gradient centrifugation. Clin. Exp. Reprod. Med. 44, 201–206. https://doi.org/10.5653/cerm.2017.44.4.201.
Kirk, E.S., Squires, E.L., Graham, J.K., 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenology 64, 1422–1439. https://doi.org/10.1016/j.theriogenology.2005.03.006.
Lazzari, G., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Barbacini, S., Galli, C., 2002. Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. Theriogenology 58, 709–712. https://doi.org/10.1016/S0093-691X(02)00777-X.
Li, Z., Wang, L., Cai, J., Huang, H., 2006. Correlation of sperm DNA damage with IVF and ICSI outcomes: a systematic review and meta-analysis. J. Assist. Reprod. Genet. 23, 367–376. https://doi.org/10.1007/s10815-006-9066-9.
Loutradi, K.E., Tarlatzis, B.C., Goulis, D.G., Zepiridis, L., Pagou, T., Chatziioannou, E., Grimbizis, G.F., Papadimas, I., Bontis, I., 2006. The effects of sperm quality on embryo development after intracytoplasmic sperm injection. J. Assist. Reprod. Genet. 23, 69–74. https://doi.org/10.1007/s10815-006-9022-8.
López-Fernández, C., Crespo, F., Arroyo, F., Fernández, J.L., Arana, P., Johnston, S.D., Gosálvez, J., 2007. Dynamics of sperm DNA fragmentation in domestic animals. II. The stallion. Theriogenology 68, 1240–1250. https://doi.org/10.1016/j.theriogenology.2007.08.029.
Luppi, S., Martinelli, M., Giacomini, E., Giolo, E., Zito, G., Garcia, R.C., Ricci, G., 2015. Comparative proteomic analysis of spermatozoa isolated by swim-up or density gradient centrifugation. Reprod. Biol. Endocrinol. 13, 1–7. https://doi.org/10.1186/s12958-015-0027-y.
Macpherson, M.L., Blanchard, T.L., Love, C.C., Brinsko, S.P., Varner, D.D., 2002. Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. Theriogenology 58, 317–320. https://doi.org/10.1016/S0093-691X(02)00736-7.
Matsuura, K., Uozumi, T., Furuichi, T., Sugimoto, I., Kodama, M., Funahashi, H., 2013. A microfluidic device to reduce treatment time of intracytoplasmic sperm injection. Fertil. Steril. 99, 400–407. https://doi.org/10.1016/j.fertnstert.2012.10.022.
Miller, J.E., Smith, T.T., 2001. The effect of intracytoplasmic sperm injection and semen parameters on blastocyst development in vitro. Hum. Reprod. 16, 918–924. https://doi.org/10.1093/humrep/16.5.918.
Mocé, E., Graham, J.K., 2008. In vitro evaluation of sperm quality. Anim. Reprod. Sci. 105, 104–118. https://doi.org/10.1016/j.anireprosci.2007.11.016.
Morrell, J., Johannisson, A., Dalin, A.M., Rodriguez-Martinez, H., 2009. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reprod. Domest. Anim. 44, 512–517. https://doi.org/10.1111/j.1439-0531.2008.01265.x.
Morrell, J.M., Rodriguez-Martinez, H., Johannisson, A., 2010. Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. Equine Vet. J. 42, 579–585. https://doi.org/10.1111/j.2042-3306.2010.00101.x.
Nosrati, R., Vollmer, M., Eamer, L., San Gabriel, M.C., Zeidan, K., Zini, A., Sinton, D., 2014. Rapid selection of sperm with high DNA integrity. Lab Chip 14, 1142. https://doi.org/10.1039/c3lc51254a.
Olaciregui, M., Luño, V., Martí, J.I., Aramayona, J., Gil, L., 2016. Freeze-dried stallion spermatozoa: evaluation of two chelating agents and comparative analysis of three sperm DNA damage assays. Andrologia 48, 988–994. https://doi.org/10.1111/and.12530.
Sakkas, D., Manicardi, G.C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P.G., Bianchi, U., 2000. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum. Reprod. 15, 1112–1116. https://doi.org/10.1093/humrep/15.5.1112.
Schulte, R., Chung, Y., Ohl, S., Takayama, S., Smith, G.D., 2007. Microfluidic sperm sorting device provides a novel method for selecting motile sperm with higher DNA integrity. Fertil. Steril. 88, S76.
Sessions-Bresnahan, D.R., Graham, J.K., Carnevale, E.M., 2014. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using invitro fertilization, perivitelline, and intracytoplasmic sperm injections. Theriogenology 82, 274–282. https://doi.org/10.1016/j.theriogenology.2014.04.002.
Shirota, K., Yotsumoto, F., Itoh, H., Obama, H., Hidaka, N., Nakajima, K., Miyamoto, S., 2016. Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. Fertil. Steril. 105, 315–321. https://doi.org/10.1016/j.fertnstert.2015.10.023.
Sieme, H., Martinsson, G., Rauterberg, H., Walter, K., Aurich, C., Petzoldt, R., Klug, E., 2003. Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. Reprod. Domest. Anim. 38, 134–140. https://doi.org/10.1046/j.1439-0531.2003.00416.x.
Simopoulou, M., Gkoles, L., Bakas, P., Giannelou, P., Kalampokas, T., Pantos, K., Koutsilieris, M., 2016. Improving ICSI: a review from the spermatozoon perspective. Syst. Biol. Reprod. Med. 62, 359–371. https://doi.org/10.1080/19396368.2016.1229365.
Xue, X., Wang, W.S., Shi, J.Z., Zhang, S.L., Zhao, W.Q., Shi, W.H., Guo, B.Z., Qin, Z., 2014. Efficacy of swim-up versus density gradient centrifugation in improving sperm deformity rate and DNA fragmentation index in semen samples from teratozoospermic patients. J. Assist. Reprod. Genet. 31, 1161–1166. https://doi.org/10.1007/s10815-014-0287-z.
Zhao, J., Zhang, Q., Wang, Y., Li, Y., 2014. Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Fertil. Steril. 102, 998–1005. https://doi.org/10.1016/j.fertnstert.2014.06.033. e8.
Zini, A., Finelli, a, Phang, D., Jarvi, K., 2000. Influence of semen processing technique on human sperm DNA integrity. Urology 56, 1081–1084. https://doi.org/10.1016/S0090-4295(00)00770-6.