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Characterization of sperm cell membrane charge and selection of high-quality sperm using microfluidics in stallions

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A B S T R A C T

Intracytoplasmic sperm injection (ICSI) is the only method for *in vitro* embryo production (IVP) in horses. Besides oocyte developmental competence, the outcome of IVP is also highly dependent on sperm quality. Therefore, it is not only essential to employ superior methods of selecting high quality sperm, but also to be able to characterize which quantifiable properties of sperm quality are most indicative of its fertility. In men, a net negative surface charge, estimated by zeta potential (ZP) is highly correlated with sperm quality and *in vitro* embryo developmental outcomes. However, there is no information available about approximate charges or ZP in equine sperm. Therefore, in this study we aimed to characterize equine sperm ZP and identify its associations with known measures of sperm quality. Additionally, we aimed to complete a comprehensive comparison of conventional sperm selection techniques as compared to the novel method of microfluidic sorting. Ejaculates (n = 22) were partitioned into fresh (~23 °C, 0 h; n = 12) and cooled (~4 °C, 24 h; n = 10) groups, and processed by swim up (SU), density gradient centrifugation (DGC), density gradient-swim up combination (DG-SU), and microfluidic chip (MF) sorting. Motility, progressive motility, cell viability, normal morphology, and ZP were evaluated for both unprocessed fractions and post-selected fractions. The ZP of both fresh and cooled samples was net negative and also correlated with motility and progressive motility for both fresh and cooled samples ($P < 0.05$). The ZP of cooled samples was also correlated with viability ($P < 0.05$). Among the compared methods of sperm selection, MF was highly effective in selecting high quality sperm as determined by the measured parameters. Percent motility, progressive motility, normal morphology, and viability of MF selected sperm were of higher quality than sperm selected by SU, and of similar to DG-SU and DGC without the use of potentially harmful centrifugation steps. Correlations between ZP, motility, and viability parameters may indicate a role of external charge on the motility and survival of sperm within the female reproductive tract. In conclusion, we identified an average net negative ZP on equine sperm and correlations between ZP and other measures of sperm quality, as well as having identified MF as a novel effective method of equine sperm selection for IVP.

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1. Introduction

Assisted reproductive technology (ART) is an integral part of the equine breeding industry. Reasons for the implementation of ART may include physical distance between a selected mare and stallion, providing increased safety to both animals and the handler, the use of cryopreserved gametes of deceased animals, or the inability of a valuable horse to be bred by live cover, indicative of subfertility. Particularly in regard to many cases of subfertility, methods of ART could be the only option available for horse owners to breed these individuals and propagate the genetic line.

Commonly used ART includes artificial insemination (AI), embryo transfer (ET), gamete cryopreservation, and more advanced techniques such as the *in vitro* production of embryos (IVP). In horses, IVP is limited to a technique called intracytoplasmic sperm

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injection (ICSI), as traditional IVF is ineffective [1]. Optimization of ICSI often focuses on obtaining and monitoring the oocyte, in part because the number of available oocytes at any given time is significantly less than the number of available sperm cells. However, sperm viability and quality should not be overlooked as indicators of fertilization and development potential, because there may be a wide range of sperm quality within an ejaculate that may influence optimal embryo production. Therefore, researchers of male fertility often prioritize investigation into sperm physiology and its relation to both *in vivo* and *in vitro* fertility.

Zeta Potential (ZP), or electrophoretic mobility, is an estimation of cellular surface charge [2]. ZP is the electrostatic potential at the slipping plane of a cell (the radial distance extending from the cell surface at which surrounding fluid particles possess fluid mobility rather than being bound [Fig. 1A]), that is proportional to cell surface-charge density [2–5]. It has been shown that a net negative surface charge, estimated by ZP, is present in mature human sperm and is primarily based on the addition of proteins terminating in negatively charged sialic acid groups (sialoglycoproteins) to the outer plasma membrane either by the sperm or male somatic cells during spermatogenesis and epididymal maturation, respectively (Fig. 1A) [2,6–9]. Glycoconjugates adsorbed to the outer sperm plasma membrane extend outwards from the cell and form a dense layer of oligosaccharides and proteins known as the sperm glycocalyx (Fig. 1B). It has been shown that the glycocalyx plays an integral role in sperm immune evasion, transport within the female tract, preservation of sperm protein integrity, and sperm-zona pellucida binding [6,7,9–13]. The acquisition of the negative charge on sperm throughout maturation has also been described in rodent species, mongoose, and bulls, and is therefore thought to be conserved among mammals [14–16].

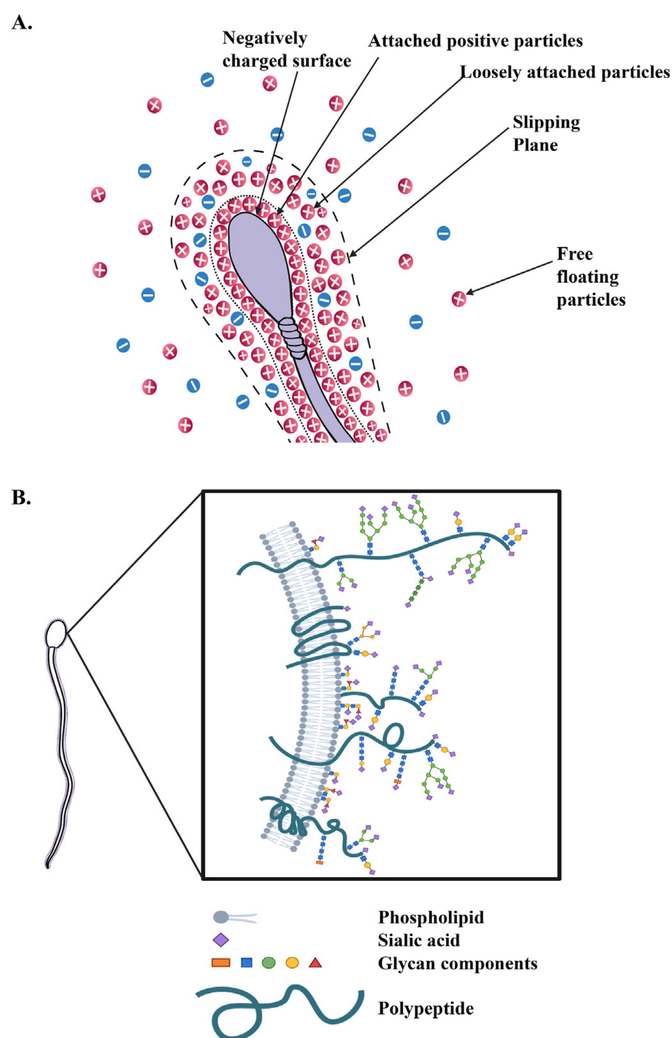


Fig. 1. Schematic diagram of Zeta Potential (ZP). **A.** Diagram of the slipping plane of a sperm cell, the point at which fluid particles are not bound, but free-floating around the cell, and where cell surface charge can be estimated as ZP. Positively charged particles are bound to the negatively charged cell surface, and subsequently surrounded by a loosely bound layer of mixed positive and negatively charged particles. The point at which charged particles are no longer bound is called the slipping plane. **B.** Glycoconjugates insert and extend away from the sperm surface, terminating in negatively charged sialic acid groups that aid in sperm function and survival.

In humans, a greater net negative ZP, largely attributed to the composition of the glycocalyx, has been correlated with increased percentages of morphologically normal sperm as well as those possessing high DNA integrity [2,6,17]. Additionally, the selection of sperm by conventional methods (selecting for motility and viability) also selected for a more negative population [18]. Based on these observations, multiple techniques to select sperm based upon ZP have been developed and implemented in human clinics [4,6,19–22]. However, no studies on equine sperm ZP or its potential applications to sperm selection have been published to date.

In addition to furthering our understanding of sperm physiology, it is necessary to implement findings in a clinical setting; particularly to improve and develop new methods of equine sperm selection for the generation of *in vitro* produced embryos. Standard methods of sperm selection that are used clinically with horses, as well as humans and livestock, are Swim Up (SU), Density Gradient Centrifugation (DGC), and Density Gradient Swim Up Combination (DG-SU). These methods select sperm based upon active motility and sperm density. However, a thorough evaluation of sperm selection methods has not been performed in horses and novel techniques used in human fertility clinics have not been well translated to the equine breeding industry.

One novel selection technique used in humans that is based on *in vivo* mechanisms of sperm selection is the microfluidic (MF) chip. The MF chip is a microfluidics-based method, which selects highly motile sperm using innate rheo-, chemo-, or thermotactic behaviors of sperm [23–26]. The use of MF chips in human and bovine clinical settings generally results in an overall enrichment of sperm with good motility, viability, and DNA integrity [22,27–29]. Multiple designs of MF devices exist, including an easy-to-use chip that selects sperm by filtering out inferior sperm as the highly viable population swims upwards through a porous membrane [27,28,30]. One of these devices was utilized in an equine ICSI study, in which it was observed that the selected population was enriched for normal morphology, motility, viability, and DNA integrity [30]. In this study, MF was also compared to single layer centrifugation and SU techniques using motility, morphology, and DNA integrity outcomes; where MF selected for a subpopulation of sperm with better DNA integrity than sperm selected by SU or single layer centrifugation. Oocytes injected with MF-selected sperm had similar cleavage and blastocyst rates to those injected with sperm selected by single layer centrifugation, and better development rates than those selected by SU, indicating that MF is an effective method of sperm selection for equine ICSI [30].

Despite this initial assessment of MF in the stallion, a full comparison to all common sperm selection techniques used in stallions has not been completed. Therefore, in this study we aimed to determine the efficacy of SU, DGC, DG-SU, and MF in selecting high quality sperm. Quality of the selected populations will be quantified by measuring parameters known to be correlated with stallion fertility (motility, progressive motility, viability, and morphology). In addition, we aim to measure the ZP of the selected

sperm populations and use them as a “high quality” grouping in comparison to the ZP of unselected, or “low quality” groups. We hypothesized, based on previous studies performed in other species, that high quality equine sperm will possess a more negative surface charge, or ZP, than low quality samples, and that MF will be an effective method of selecting sperm for equine IVP.

2. Materials and methods

2.1. Chemicals and media

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Biggers-Whitten-Whittingham (BWW) media was made from an initial salt base consisting of 89.84 mmol/L NaCl, 4.78 mmol/L KCl, 1.19 mmol/L $KH2PO_4$, 22.94 mmol/L HEPES buffer (fw: 260.3), 4.00 mmol/L $NaHCO_3$, 1.70 mmol/L $CaCl_2 + 2H_2O$, and $MgSO_4 + 7H_2O$. Salts were supplemented with 5.55 mmol/L D-glucose, 0.25 mmol/L sodium pyruvate, 11% (v/v) DL-lactic acid syrup, and 1% penicillin/streptomycin and 0.1% PVA. The completed media containing all of the components is referred to as BWW. The final media product was pH. balanced to 7.4 using HCl or NaOH and had an osmolality of 300 ± 5 mOsm/kg.

2.2. Animals and animal handling

Horses used in this study (n = 5) included both UC Davis resident and Veterinary Medicine Teaching Hospital (VMTH) Equine Reproduction Service client stallions. Samples from client stallions of VMTH were used anonymously after receiving a signed Owner Informed Consent Form. All animal and experimental protocols were approved by and performed in accordance with the Institutional Animal Care and Use Committee of the University of California Davis.

Ejaculates (n = 13) were collected using a phantom mount and a Missouri artificial vagina, as previously described [31]. Gel fractions were removed via a mesh filter (Disposable Nylon Mesh Semen Gel Filters, ARS Inc, Ontario, CA, USA), and approximately half of the remaining gel free fraction was extended 1:1 with a commercial equine semen extender (INRA96®, IMV Technologies, L'Aigle, France) warmed to 37 °C for immediate analysis and processing. This fraction will be referred to as fresh sperm. The second half of the ejaculate was extended 1:3 with the same extender and was cooled to 4 °C for a period of 24 h prior to analysis and processing [32]. This fraction will be referred to as cooled sperm.

2.3. Semen quality characterization

Sperm quality parameters were measured for both fresh and cooled samples both before and after selection protocols were performed (see section 2.4, Sperm Selection), and included assessments of motility, progressive motility, viability, and morphology.

Concentration and viability were assessed using a NucleoCounter SP-100 (ChemoMetec Allerød, Denmark), as previously described [33]. A small fraction of the sample was diluted into a lysis reagent (Reagent S100 Lysis Buffer, ChemoMetec) to measure total concentration and into DPBS (500 mL, ChemoMetec) to measure the population of non-viable cells, and then loaded into a cassette (SP-1 Casette, ChemoMetec) containing propidium iodide for automated analysis to determine the percentage of viable, or membrane-intact, sperm.

Motility and morphology were analyzed using Computer Assisted Sperm Analysis (CASA, HTM CEROS, Version 12.2 g; Hamilton Thorne Biosciences, Beverly, MA, USA) and Sperm Vision® SAR software (Minitübe, Tiefenbach, Germany) [34]. For CASA motility analysis, 3 μL of semen, diluted to approximately 20–50 million sperm/mL as per manufacturer recommendations, was loaded into a single chamber of a 4 chambered glass slide (Leja, Spectrum Technologies, Healdsburg, CA, USA). Seven randomized microscopic fields of the chamber were analyzed for each sample and sperm motility endpoints that were recorded included the sample's averaged total motility (M, %) and progressive motility (PM, %). The preset values for the CASA system were: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 70; minimum cell size, 4 pixels; mini-mum static contrast, 30; straightness threshold for progressive motility, 75%; average path velocity (VAP) threshold for progressive motility, 50; VAP threshold for static cells, 20; cell intensity, 106; and light emitting diode illumination intensity.

For morphological analysis, sperm were fixed with 10% formalin and pipetted on a glass slide for oil immersion phase-contrast microscopy under 100× magnification (Zeiss A×10 Lab A1, Zeiss, Jena, Germany). The percent of sperm with normal morphology was calculated based on a visual morphological assessment of a minimum of 100 sperm within Sperm Vision® software (Minitübe). Sperm possessing a distal droplet, but no other morphological abnormalities were considered normal [35,36].

Lastly, zeta potential (ZP) was measured as described by Ionov et al. [18] before and after processing in both fresh and cooled samples. ZP was measured with a commercially available zeta potential analyzer (Zetasizer Nano-ZS, Malvern Instruments, UK) in 1X DPBS (pH 7.0–7.3). Each individual sample was diluted to 1.5 million sperm/mL in 1X DPBS (ChemoMetec) and run through the zeta potential analyzer, and the total run time included a 2-min equilibration period and 8 successional ZP measures. The 8 repeated ZP measurements were averaged to achieve an overall ZP value, recorded in mV, for that sample.

2.4. Sperm selection

A fraction from each fresh (diluted 1:1 in INRA96) or cooled (diluted 1:3 in INRA96) ejaculate was utilized for one of the following selection methods: Swim Up (SU) [37], Density Gradient Centrifugation (DGC) [38], Density Gradient-Swim Up Combination DG-SU [39], or Microfluidics (MF) [30] and evaluated as described previously. All selection protocols for fresh and cooled samples were performed at room temperature.

The concentration of the initial extended sample used for processing was dependent on the concentration of the collected ejaculate and fell between 32 and 175 million sperm/mL, with an average of 142.45 million sperm/mL. Initial sperm concentrations were not adjusted prior to sperm processing by DGC, DG-SU, SU, and MF in order to represent natural variations seen in clinical practice. Aliquots of sperm used in quantitative measures both before and after selection were adjusted for concentration as described in relevant sections.

Wash: Semen diluted in extender was centrifuged for 8 min at 300×*g*. Supernatant was removed and the remaining sperm pellet was resuspended in BWW culture medium as a control.

SU: Sperm were deposited into the bottom of a round-bottom tube (14 mL Polypropylene Round-Bottom Tube, Falcon, Corning Science, Reynosa, Tamaulipas, Mexico) and overlaid with BWW culture medium. The tubes were then incubated at a 45° angle for 30 min at room temperature. The top ¼ fraction of media was aspirated using a pipet for subsequent analysis.

DGC: An 84% Percoll® (Sigma, St. Louis, MO, USA) solution, diluted in BWW, was layered into a centrifuge tube (15 mL Polypropylene Conical Tube, Falcon, Corning Science, Reynosa, Tamaulipas, Mexico) and overlaid with a 42% Percoll® solution. Percoll® solutions were balanced for osmolality. Unprocessed semen was then carefully deposited above the upper gradient. The

tube was centrifuged for 30 min at 300×g. The resulting pellet was removed using a fine-tip aspiration pipet, resuspended 1:1 in BWW, and homogenized prior to analysis.

DG-SU: DGC was performed as described, however the pellet was removed and immediately resuspended in BWW in a fresh tube. Sperm were then re-pelleted by centrifugation for 5 min at 300×g. Supernatant was removed and the secondary pellet was overlaid with additional culture media. Sperm were then allowed to swim up as described above and the top fraction of media was gently removed for analysis.

MF: Sperm were loaded into the inlet of the microfluidic device (ZyMōt™ Multi, 850 μL, Sperm Separation Device™, DxNow, Gaithersburg, MD, USA) through the inlet channel per manufacturer's instructions and the porous microfilter was overlaid with BWW. The device was allowed to sit at room temperature for 30 min before sperm that had passed through the microfilter were aspirated through the outlet of the collection chamber for analysis.

2.5. Statistical analysis

Data description, descriptive statistics, plots, and statistical tests were performed in JMP® Pro 14 (JMP Statistical Discovery, Cary, North Carolina). Normality of the data distribution was tested using Shapiro-Wilk test. Non-parametric methods were used for subsequent analyses since the data were not normally distributed. Spearman's correlations were used to identify potential associations between zeta potential and sample quality parameters. To identify differences in quality of samples from different selection methods, Kruskal-Wallis and follow up *post hoc* analysis were used to compare the degree of improvement in sperm quality parameters after each of the tested selection methods as compared to each other and to the initial, unprocessed sperm.

3. Results

3.1. Identification of a net negative ZP of equine sperm

In order to confirm that sperm membrane charges are conserved in equine sperm, we measured zeta potential in fresh and cooled, and quality-selected and unselected samples. We were able to confirm that equine sperm possess a net negative charge similar to that reported for human sperm. The combined average ZP of all fresh samples was −11.91 ± 2.13 (mean ± s.d.; −16.81 to −7.65 mV, min-max) and the combined average of cooled samples was −12.20 ± 1.73 (mean ± s.d.; −15.34 to −8.23 mV, min-max) (Fig. 2A).

3.2. ZP correlations with sperm quality

The associations between ZP measurements and other known measures of quality (motility, progressive motility, viability, and normal morphology) for both unprocessed samples and those processed by wash-only, SU, DGC, DG-SU, and MF of both fresh and cooled sperm were assessed. Data were not normally distributed, and thus Kruskal-Wallis and follow up *post hoc* analysis were used. Correlation tests revealed that ZP of fresh sperm was negatively correlated with both motility, and progressive motility (Fig. 2B). ZP of fresh sperm was not correlated with normal morphology or viability (P > 0.05).

In cooled sperm, there was a significant, negative correlation between ZP and motility, progressive motility, and viability (Fig. 2B). ZP of cooled samples was not correlated with normal morphology (Fig. 2B).

3.3. Variation in quality of sperm selected by different methods

Each ejaculate was divided and evaluated as one of 6 treatment groups. Quality parameters, excluding ZP, were compared among unprocessed, wash-only, and DGC, SU, DG-SU, and MF selection methods as either fresh or cooled treatments (Fig. 3).

When comparing selection efficacy of fresh sperm by total motility, unprocessed, washed, and SU groups possessed significantly less motile populations than DGC (P < 0.005; P < 0.005; P < 0.05), MF (P < 0.001; P < 0.005; P < 0.005), and DG-SU (P < 0.005; P < 0.05; P < 0.01). Numerically, the average motility of sperm selected by DGC (mean $\text{motility}_{\text{DGC fresh}}$ = 88.0%) was greatest, followed by MF (mean $\text{motility}_{\text{MF fresh}}$ = 84.5%), and DG-SU (mean $\text{motility}_{\text{DG-SU fresh}}$ = 78.7%).

Analysis of the progressive motility of fresh sperm showed that the unprocessed, washed, and SU groups demonstrated significantly lower percentages of progressively motile populations than DGC (P < 0.0005; P < 0.0005; P < 0.005), MF (P < 0.001; P < 0.005; P < 0.05), and DG-SU (P < 0.005; P < 0.05; P < 0.05). Numerically, DGC (mean progressive $\text{motility}_{\text{DGC fresh}}$ = 83.5%) selected the highest average progressive motility, followed by MF (mean progressive $\text{motility}_{\text{MF fresh}}$ = 88.3%) and DG-SU (mean progressive $\text{motility}_{\text{DG-SU fresh}}$ = 75.1%).

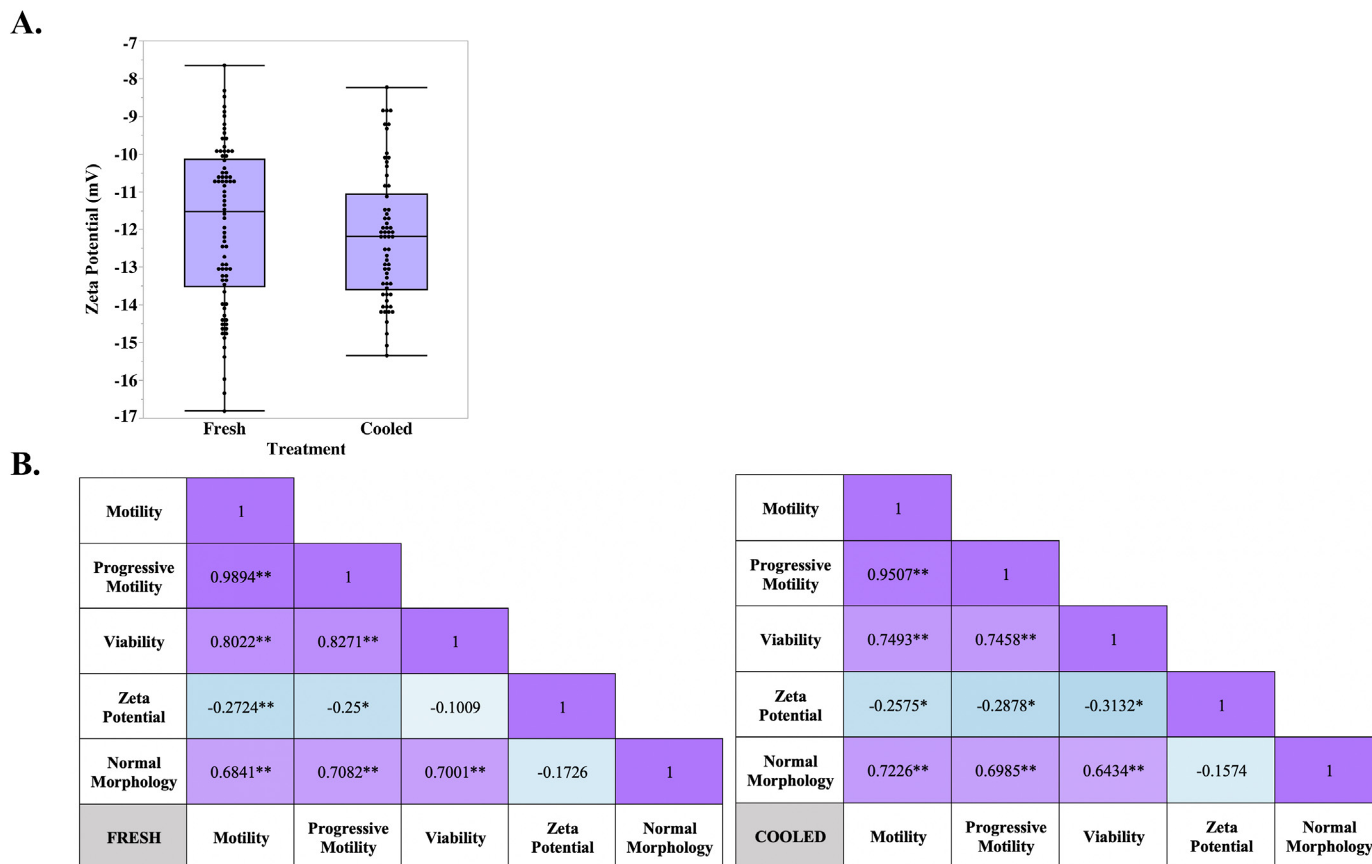
Viability analysis also showed that fresh unprocessed, washed, and SU groups had significantly lower population viability than MF (P < 0.0001; P < 0.0001; P < 0.0001), DG-SU (P < 0.001; P < 0.005; P < 0.005), and DGC groups (P < 0.01; P < 0.005; P < 0.05). The average viability of the MF (mean $\text{viability}_{\text{MF fresh}}$ = 97.8%) group was the highest, followed by DG-SU (mean $\text{viability}_{\text{DG-SU fresh}}$ = 94.6%) and DGC (mean $\text{viability}_{\text{DGC fresh}}$ = 89.4%).

In selecting for morphologically normal sperm from fresh ejaculates, unprocessed and washed groups selected for a significantly lower percentage of morphologically normal sperm than DG-SU (P < 0.005; P < 0.005), MF (P < 0.01; P < 0.01), and DGC (P < 0.05; P < 0.05). DG-SU also selected for significantly more morphologically normal sperm than SU (P < 0.05). Numerically, DG-SU selected for the highest percentage of morphologically normal sperm (mean normal $\text{morphology}_{\text{DG-SU fresh}}$ = 73.6%), followed by MF (mean normal $\text{morphology}_{\text{MF fresh}}$ = 69.5%) and DGC (mean normal $\text{morphology}_{\text{DGC fresh}}$ = 68.5%).

Results were similar for measured parameters of cooled sperm to results from fresh samples processed by the same methods. Unprocessed, washed, and SU groups possessed significantly less motile populations than DGC (P < 0.05; P < 0.05; P < 0.05), MF (P < 0.001; P < 0.001; P < 0.001), and DG-SU (P < 0.005; P < 0.005; P < 0.005). The average motility of sperm selected by MF (mean $\text{motility}_{\text{MF cooled}}$ = 80.9%) was greatest, followed by DG-SU (mean $\text{motility}_{\text{DG-SU cooled}}$ = 78.0%), and DGC (mean $\text{motility}_{\text{DGC cooled}}$ = 67.8%).

Progressive motility of cooled sperm was significantly lower in unprocessed, washed, and SU groups demonstrated significantly lower percentages of progressively motile populations than MF (P < 0.005; P < 0.005; P < 0.005), DG-SU (P < 0.05; P < 0.05; P < 0.01), and DGC (P < 0.05; P < 0.05; P < 0.05). Numerically, MF (mean progressive $\text{motility}_{\text{MF cooled}}$ = 72.7%) selected for the highest average progressive motility, then DG-SU (mean progressive $\text{motility}_{\text{DG-SU cooled}}$ = 67.3%) and DGC (mean progressive $\text{motility}_{\text{DGC cooled}}$ = 60.9%).

Viability analysis also showed that cooled unprocessed, washed, and SU groups had significantly lower population viability than DG-SU (P < 0.005; P < 0.005; P < 0.01), MF (P < 0.01; P < 0.05; P < 0.05), and DGC groups (P < 0.05; P < 0.05; P < 0.05). The average viability of the DG-SU group (mean $\text{viability}_{\text{DG-SU cooled}}$ = 93.1%) was the highest, followed by MF (mean $\text{viability}_{\text{MF cooled}}$ = 87.5%) and DGC (mean $\text{viability}_{\text{DGC cooled}}$ = 82.6%).



FRESH	Motility	Progressive Motility	Viability	Zeta Potential	Normal Morphology
Motility	1				
Progressive Motility	0.9894**	1			
Viability	0.8022**	0.8271**	1		
Zeta Potential	-0.2724**	-0.25*	-0.1009	1	
Normal Morphology	0.6841**	0.7082**	0.7001**	-0.1726	1

COOLED	Motility	Progressive Motility	Viability	Zeta Potential	Normal Morphology
Motility	1				
Progressive Motility	0.9507**	1			
Viability	0.7493**	0.7458**	1		
Zeta Potential	-0.2575*	-0.2878*	-0.3132*	1	
Normal Morphology	0.7226**	0.6985**	0.6434**	-0.1574	1

Fig. 2. Distributions and correlations of zeta potential measurements. **A.** Measured zeta potential distribution of fresh and cooled sperm, in millivolts (mV). The box plot displays median, first and third quartiles, minimum, and maximum values, and points represent individual measurements. **B.** Spearman's ρ Correlation Table. Nonparametric correlations are displayed and color coded based on direction of correlation and intensity, purple being positively correlated and blue being negatively correlated. (*) signifies $P < 0.05$, and (**) signifies $P < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In selecting for morphologically normal sperm from cooled ejaculates, unprocessed and washed groups selected for a significantly lower percentage of morphologically normal sperm than DG-SU ($P < 0.0005$; $P < 0.0005$), MF ($P < 0.05$; $P < 0.05$), and DGC ($P < 0.05$; $P < 0.05$). DG-SU also selected for significantly more morphologically normal sperm than SU ($P < 0.005$). Numerically, DG-SU selected for the highest percentage of morphologically normal sperm (mean normal morphology$_{\text{DG-SU cooled}}$ = 78.8%), followed by MF (mean normal morphology$_{\text{MF cooled}}$ = 69.1%) and DGC (mean normal morphology$_{\text{DGC cooled}}$ = 65.5%).

4. Discussion

In the present study we have successfully shown for the first time that equine sperm possess a net negative ZP, representative of a net negative surface charge, similar to that demonstrated in previous human and animal studies [2,18,40]. Results further show that there are significant correlations between ZP and sperm quality; specifically, motility and progressive motility of both fresh and cooled sperm, and viability of cooled sperm.

Our data supports recent evidence from human clinical experiments, in which samples with more optimal cell viability, total motility, and morphology were shown to possess significantly lower (more negative) zeta potentials [18]. In the aforementioned human study, ZP of fresh sperm was most correlated with motility ($P < 0.01$), which is also reflected in our data with equine sperm [18]. The authors also showed that ZP was correlated with viability ($P < 0.05$) and morphology ($P < 0.05$) [18], unlike our data in which viability was only correlated with ZP in cooled sperm and no correlation was observed between morphology and ZP. Some differences may be attributed to species differences, whilst others may be due to differences in experimental design such as the media and any unknown influences of selection techniques on ZP. For example, although ZP was correlated with quality parameters, no comparison of ZP among selection techniques is presented, as the Percoll® particles used in DGC separation are known to possess a low surface charge and may bias DGC and DG-SU results [43]. As mentioned previously, ZP is a media dependent, relative parameter. Thus, buffer concentrations can alter the quality of ZetaSizer phase plot data and distribution curves, despite still being capable of producing ZP measures proportional to measures taken in the same media. In this study, 1X DPBS was initially selected as the dilution buffer due to concerns of inducing hypoosmotic stress on sperm cells, and presented data are generally similar to results from samples measured in 0.1X DPBS (preliminary data not shown). In future studies, using 0.1X buffer may be advantageous for measuring ZP of sperm cells and could improve data quality and strength of correlations.

Regardless, using a small number of replicates we were able to demonstrate a correlation between ZP and basic measures of sperm quality. This association could be further improved by utilizing parameters such as DNA integrity, mitochondrial membrane potential, or the presence of apoptotic factors that are more strongly associated with embryo development and pregnancy outcomes [41,42].

Research in human IVF has focused on the development of a

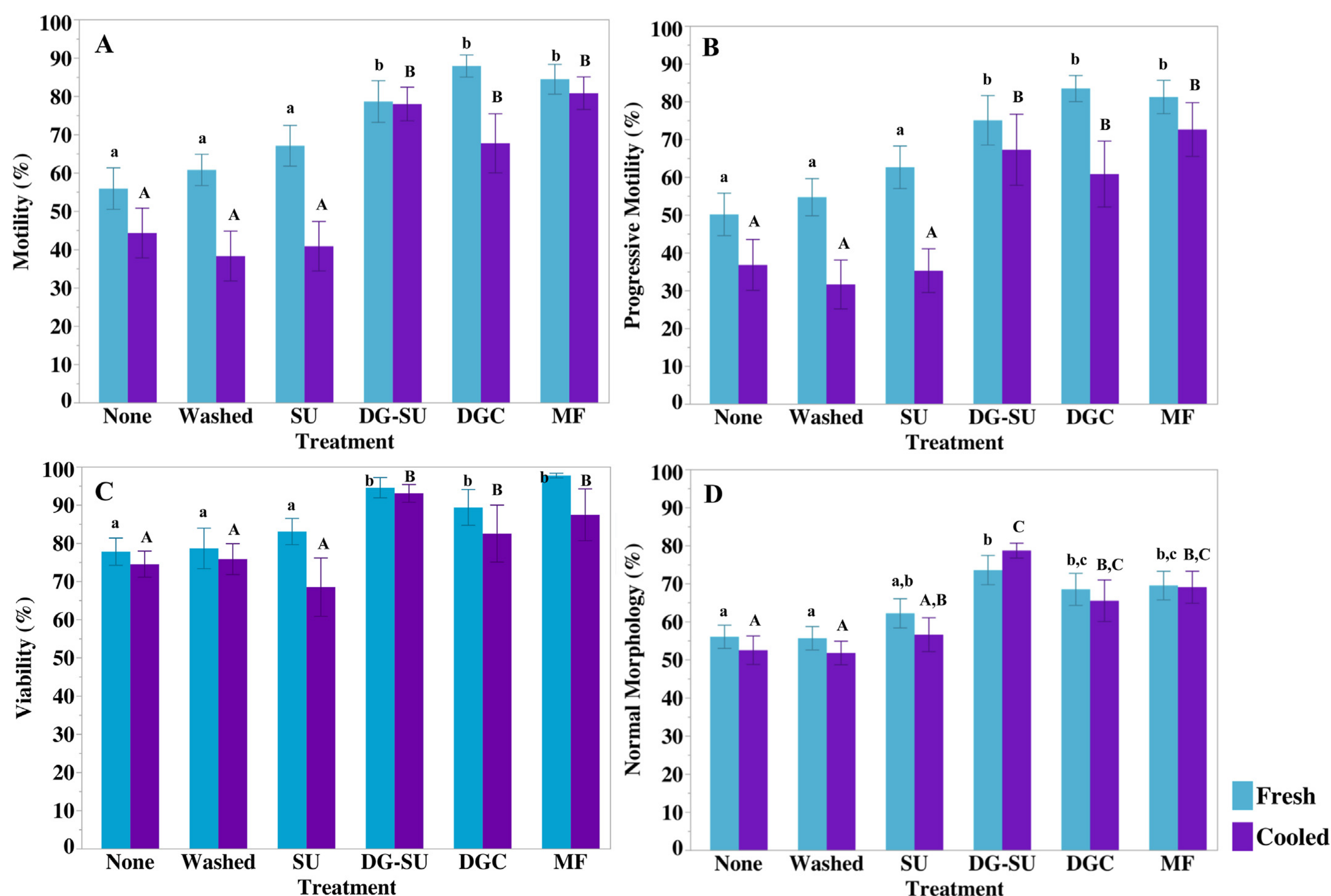


Fig. 3. Average sperm quality measurements by sperm selection technique. Motility (**A**), progressive motility (**B**), viability (**C**), and morphology (**D**) are given for unprocessed sperm (none) and sperm processed by washing, SU, DG-SU, DGC, and MF. Data are represented as mean ± standard error. Superscripts (*a*, *b*, *c*) denote statistical significance between fresh groups ($P < 0.05$) and (*A*, *B*, *C*) denote statistical significance between cooled groups ($P < 0.05$).

novel method of high quality sperm selection for IVP based on ZP [19–21]. In 2006, Chan et al. established a method of positively charging a glass centrifuge tube using static electricity generated by friction in order to bind more negatively charged sperm [4]. This method is used immediately following DGC and the combination of techniques will hereafter be referenced in total as the ZP selection method [4]. Upon its initial use, the ZP selection method almost doubled the percentage of morphologically normal sperm as compared to the original sample (19.3 ± 0.1 and 10.0 ± 0.1, respectively), as well as significantly increased hyperactive motility, progressive motility, and DNA integrity [4]. Total motility was not significantly increased and total recovery was just under 9% [4]. The ZP method was later implemented clinically with infertile couples in combination with ICSI. Again, ZP selection yielded improved DNA integrity as compared to results from a hyaluronic acid sperm binding method, percentages of normal morphology, and normal protamine content [20,21]. However, others showed that the ZP selection method increased percentages of Annexin-V and ubiquitin positive sperm as compared to DGC selected and untreated sperm, indicating either increased apoptosis or capacitation [20]. We did not perform Annexin-V staining in the current study. Additionally, ZP selection prior to ICSI significantly increases fertilization rates (52.39%–65.79%, $P < 0.05$) and numerically improves pregnancy rates ($P > 0.05$) over ICSI following DGC sperm selection [19]. A study with a similar experimental design observed significantly greater fertilization and pregnancy rates after ICSI utilizing ZP-selected sperm over ICSI utilizing DGC-selected sperm [3]. However, pregnancies reaching full term revealed a significantly skewed sex ratio, favoring XX offspring, which aligns with other observations of electrophoretic X-chromosome selection bias and X-chromosome bearing sperm possessing a more negative ZP [2,22,44,45].

As previously mentioned, the negative charge on sperm has been attributed to the presence of sialoglycoproteins embedded in the sperm membrane and extending outward to make up the sperm glycocalyx [6,7]. Glycoconjugate β-Defensin DEFB126 is a major contributing protein to the sperm glycocalyx that contains terminal sialic acid groups and is a largely homologous molecule between human and macaque sperm [46,47]. DEFB126 is highly expressed during epididymal maturation and heavily coats the sperm until capacitation [48]. Furthermore, the removal of DEFB126 from the glycocalyx is integral to tight sperm-zona binding [46], implying that the charged glycocalyx is integral to fertilization events. The negatively charged oligosaccharides of DEFB126, specifically terminal sialic acid residues, have been shown to play a role in active *in vitro* motility in the macaque. The removal of DEFB126 inhibits over 80% of successful navigation of periovulatory cervical mucus by disrupting progressive linear motion [7,48–50]. Similar to sperm, cervical mucus has been shown to possess a negative charge, likely repelling sperm and preventing adherence to the tract as well as driving progressive motion towards the site of fertilization [51,52]. Thus, it is no surprise that motility and progressive motility were correlated with ZP in the present study, although the exact relationship between the

properties *in vitro* still requires further investigation.

Properties of the glycocalyx, specifically electrical charge, have not only been shown as essential for the movement of the sperm through the female tract but also are important in the survival of sperm and immune evasion in the female tract, and sperm-zona binding [6,7,10–13]. The association between the survival rate of sperm and electrical charge could be an explanation for our finding that sperm assayed after a 24-h cooling period showed correlations between viability and ZP. Potentially, the integrity of the glycocalyx and thus the preservation of a negative charge protects the underlying sperm plasma membrane, both increasing survival and improving preservation of sperm quality. A lack of correlation between viability and ZP in fresh samples may be simply due to a shortened time between ejaculation and quality measurement.

Results from the current study also reinforced the efficacy and potential of MF sorting as a clinical method of sperm selection for equine IVP. Not only was MF capable of selecting high quality sperm, but MF selected for a population of sperm from a cooled ejaculate that possessed higher motility and progressive motility than all other selection methods. Viability and morphology parameters were numerically higher in MF selected sperm than sperm selected by all traditional methods other than DG-SU for both fresh and cooled sperm. The MF method was also user friendly, not requiring expensive incubators or centrifuges. Sperm selected by MF also pose an advantage over sperm selected by DGC and DG-SU methods because centrifugation causes DNA damage to sperm [53,54] as well as increasing the number of apoptotic like changes [55]. In fact, Percoll®, the silica coated particle solution used to create gradients that is commonly used in animal DGC, has been shown to be cytotoxic. Specifically, Percoll® has been shown to cause inflammatory responses in the female reproductive tract, and ultrastructural and endotoxic injury to sperm resulting in inferior embryo development [22,56–60]. As a result, Percoll® is prohibited for use in humans, and the equine industry often substitutes Percoll® with Equipure™ to reduce instance of sperm injury, which works similarly to increase motility, normal morphology, and pregnancy rates [61–63]. Equipure™ was not utilized in this study because similar results were observed between Equipure™ and Percoll® during initial optimization. Regardless of the cytotoxic effects of gradient particles on sperm injury, the lack of a centrifugation step is still an appealing feature of the MF method.

One drawback to the MF selection method, despite selecting sperm with extremely high quality, is that it selected for lower concentrations of sperm than the DGC method (data not shown). Although the efficacy of the selection method is highly dependent on the initial sample, the tight meshwork of the MF device may limit the number of viable sperm that are able to pass through the membrane over the course of a 30-min time period. Increasing the time period of sperm selection or introducing additional selection fluid over the device membrane after the initial time period for a secondary selection may increase sperm yield. However, due to the small number of sperm required for ICSI, it is potentially better to have a smaller pool of higher quality sperm for selection than it is to have a larger pool of inferior quality, centrifuged sperm. Although this study serves as the first comprehensive method comparison for equine sperm selection, future studies may focus on factors more directly associated with sperm fertility and future embryo developmental outcomes such as DNA integrity, membrane integrity, mitochondrial membrane potential, and a lack of apoptotic markers [41,42].

5. Conclusions

In conclusion, we have been able to verify a net negative surface charge, through ZP measurements, on equine sperm that is correlated with both motility and viability parameters. The identification of a negative ZP can be applied to the development of a future equine sperm selection technique to optimize IVP outcomes. Additionally, MF has been shown as an effective method of sperm selection for equine IVP purposes, selecting high quality sperm without exposing sperm to potential injury from forces applied during centrifugation. In providing groundbreaking approaches for sperm characterization and selection for implementation within the equine breeding industry, our results could spark future innovations for the improvement of IVP.

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CRediT authorship contribution statement

Morgan F. Orsolini: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Margo H. Verstraete:** Investigation. **Machteld van Heule:** Investigation. **Daniela Orellana:** Investigation. **Alyssa Ortega:** Resources. **Stuart Meyers:** Conceptualization, Writing – review & editing, Supervision. **Pouya Dini:** Funding acquisition, Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

All of the authors have agreed to the submission of this manuscript and to be responsible for its contents and declare no conflicts of interest.

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References

[1] Leemans B, Gadella BM, Stout TAE, De Schauwer C, Nelis H, Hoogewijs M, et al. Why doesn't conventional IVF work in horses. Reproduction 2016;152: R233–45.
[2] Ishijima SA, Okuno M, Mohri H. Zeta potential of human X- and Y-bearing sperm. Int J Androl 1991;14:340–7.
[3] Nasr Esfahani MH, Deemeh MR, Tavalaee M, Sekhavati MH, Gourabi H. Zeta sperm selection improves pregnancy rate and alters sex ratio in male factor infertility patients: a double-blind, randomized clinical trial. Int J Fertil Steril 2016;10:253–60.
[4] Chan PJ, Jacobson JD, Ju Corselli, Patton WC. A simple zeta method for sperm selection based on membrane charge. Fertil Steril 2006;85:481–6.
[5] Ohshima H, Kondo T. Electrophoresis of large colloidal particles with surface charge layers. Position of the slipping plane and surface layer thickness. Colloid Polym Sci 1986;264:1080–4.
[6] Calzada L, Salazar EL, Pedron N. Presence and chemical composition of glycoproteic layer on human spermatozoa. Arch Androl 1994;33:87–92.
[7] Tollner TL, Bevins CL, Cherr GN. Multifunctional glycoprotein DEFB126—a curious story of defensin-clad spermatozoa. Nat Rev Urol 2012;9:365–75.
[8] Orsolini MF, Meyers SA, Dini P. An update on semen physiology, Technologies,

and selection techniques for the advancement of in vitro equine embryo production: section I. Animals 2021;11.
[9] Tecle E, Gagneux P. Sugar-coated sperm: unraveling the functions of the mammalian sperm glycocalyx. Mol Reprod Dev 2015;82:635–50.
[10] Ma X, Pan Q, Feng Y, Choudhury BP, Ma Q, Gagneux P, et al. Sialylation facilitates the maturation of mammalian sperm and affects its survival in female uterus. Biol Reprod 2016;94:123.
[11] Levinsky H, Singer R, Malik Z, Sagiv M, Cohen AM, Servadio C, et al. Distribution of sialic acid in human sperm membranes. Arch Androl 1983;10: 209–12.
[12] Velásquez JG, Canovas S, Barajas P, Marcos J, Jiménez-Movilla M, Gallego RG, et al. Role of sialic acid in bovine sperm-zona pellucida binding. Mol Reprod Dev 2007;74:617–28.
[13] Schröter S, Osterhoff C, McArdle W, Ivell R. The glycocalyx of the sperm surface. Hum Reprod Update 1999;5:302–13.
[14] Veres I. Negative electrical charge of the surface of bull sperm. Mikroskopie 1968;23:166–9.
[15] Yanagimachi R, Noda YD, Fujimoto M, Nicolson GL. The distribution of negative surface charges on mammalian spermatozoa. Am J Anat 1972;135: 497–519.
[16] Magdanz V, Gebauer J, Sharan P, Eltoukhy S, Voigt D, Simmchen J. Sperm–particle interactions and their prospects for charge mapping. Adv Biosyst 2019;3:1900061.
[17] Giuliani V, Pandolfi C, Santucci R, Pelliccione F, Macerola B, Focarelli R, et al. Expression of gp20, a human sperm antigen of epididymal origin, is reduced in spermatozoa from subfertile men. Mol Reprod Dev 2004;69:235–40.
[18] Ionov M, Gontarek W, Bryszewska M. Zeta potential technique for analyzing semen quality. MethodsX 2020;7:100895.
[19] Kheirollahi-Kouhestani M, Razavi S, Tavalaee M, Deemeh MR, Mardani M, Moshtaghian J, et al. Selection of sperm based on combined density gradient and Zeta method may improve ICSI outcome. Hum Reprod 2009;24:2409–16.
[20] Zarei-Kheirabadi M, Shayegan Nia E, Tavalaee M, Deemeh MR, Arabi M, Forouzanfar M, et al. Evaluation of ubiquitin and annexin V in sperm population selected based on density gradient centrifugation and zeta potential (DGC-Zeta). J Assist Reprod Genet 2012;29:365–71.
[21] Razavi SH, Nasr-Esfahani MH, Deemeh MR, Shayesteh M, Tavalaee M. Evaluation of zeta and HA-binding methods for selection of spermatozoa with normal morphology, protamine content and DNA integrity. Andrologia 2010;42:13–9.
[22] Orsolini MF, Meyers SA, Dini P. An update on semen physiology, Technologies, and selection techniques for the advancement of in vitro equine embryo production: section II. Animals 2021;11.
[23] Zaferani M, Cheong SH, Abbaspourrad A. Rheotaxis-based separation of sperm with progressive motility using a microfluidic corral system. Proc Natl Acad Sci USA 2018;115:8272–7.
[24] Ko YJ, Maeng JH, Hwang SY, Ahn Y. Design, fabrication, and testing of a microfluidic device for thermotaxis and chemotaxis Assays of sperm. SLAS Technol 2018;23:507–15.
[25] Nagata MPB, Endo K, Ogata K, Yamanaka K, Egashira J, Katafuchi N, et al. Live births from artificial insemination of microfluidic-sorted bovine spermatozoa characterized by trajectories correlated with fertility. Proc Natl Acad Sci U S A 2018;115. E3087-e96.
[26] Cho BS, Schuster TG, Zhu X, Chang D, Smith GD, Takayama S. Passively driven integrated microfluidic system for separation of motile sperm. Anal Chem 2003;75:1671–5.
[27] Parrella A, Keating D, Cheung S, Xie P, Stewart JD, Rosenwaks Z, et al. A treatment approach for couples with disrupted sperm DNA integrity and recurrent ART failure. J Assist Reprod Genet 2019;36:2057–66.
[28] Asghar W, Velasco V, Kingsley JL, Shoukat MS, Shafiee H, Anchan RM, et al. Selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species. Adv Healthc Mater 2014;3:1671–9.
[29] Nosrati R, Vollmer M, Eamer L, San Gabriel MC, Zeidan K, Zini A, et al. Rapid selection of sperm with high DNA integrity. Lab Chip 2014;14:1142–50.
[30] Gonzalez-Castro RA, Carnevale EM. Use of microfluidics to sort stallion sperm for intracytoplasmic sperm injection. Anim Reprod Sci 2019;202:1–9.
[31] Dascanio JJ. Missouri artificial vagina. Equine Reproductive Procedures2014. p. 329-331.
[32] Equine reproductive procedures. second ed. ed.
[33] McCue PM. NucleoCounter® evaluation of sperm concentration and viability. Equine Reproductive Procedures2014. p. 363-365.
[34] Dascanio JJ. Computer-assisted sperm analysis. Equine Reproductive Procedures2014. p. 368-372.
[35] Brito L. Evaluation of sperm morphology. Equine Reproductive Procedures2014. p. 377-383.
[36] Brito LFC. Evaluation of stallion sperm morphology. Clin Tech Equine Pract 2007;6:249–64.
[37] Younglai EV, Holt D, Brown P, Jurisicova A, Casper RF. Sperm swim-up techniques and DNA fragmentation. Hum Reprod 2001;16:1950–3.
[38] Morrell JM, Dalin AM, Rodriguez-Martinez H. Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine Vet J 2009;41:53–8.
[39] Volpes A, Sammartano F, Rizzari S, Gullo S, Marino A, Allegra A. The pellet swim-up is the best technique for sperm preparation during in vitro fertilization procedures. J Assist Reprod Genet 2016;33:765–70.
[40] Wongtawan T, Dararatana N, Oonkhanond B, Kornmatissuk S. Zeta potential of bovine X and Y sperm and its application for sperm sorting. Thai J Vet Med 2018;48:25–6.
[41] Paasch U, Grunewald S, Glander HJ. Sperm selection in assisted reproductive techniques. Soc Reprod Fertil Suppl 2007;65:515–25.
[42] Alvarez Sedó C, Bilinski M, Lorenzi D, Uriondo H, Noblía F, Longobucco V, et al. Effect of sperm DNA fragmentation on embryo development: clinical and biological aspects. JBRA Assist Reprod 2017;21:343–50.
[43] Pertoft H, Laurent TC, Låås T, Kågedal L. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). Anal Biochem 1978;88:271–82.
[44] Engelmann U, Krassnigg F, Schatz H, Schill W-B. Separation of human X and Y spermatozoa by free-flow electrophoresis. Gamete Res 1988;19:151–60.
[45] Kaneko S, Oshio S, Kobayashi T, Iizuka R, Mohri H. Human X- and Y-bearing sperm differ in cell surface sialic acid content. Biochim Biophys Acta Biomembr 1984;124:950–5.
[46] Tollner TL, Yudin AI, Treece CA, Overstreet JW, Cherr GN. Macaque sperm release ESP13.2 and PSP94 during capacitation: the absence of ESP13.2 is linked to sperm-zona recognition and binding. Mol Reprod Dev 2004;69: 325–37.
[47] Perry AC, Jones R, Moisyadi S, Coadwell J, Hall L. The novel epididymal secretory protein ESP13.2 in Macaca fascicularis. Biol Reprod 1999;61: 965–72.
[48] Yudin AI, Generao SE, Tollner TL, Treece CA, Overstreet JW, Cherr GN. Beta-defensin 126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. Biol Reprod 2005;73:1243–52.
[49] Yudin AI, Treece CA, Tollner TL, Overstreet JW, Cherr GN. The carbohydrate structure of DEFB126, the major component of the cynomolgus macaque sperm plasma membrane glycocalyx. J Membr Biol 2005;207:119–29.
[50] Tollner TL, Yudin AI, Treece CA, Overstreet JW, Cherr GN. Macaque sperm coating protein DEFB126 facilitates sperm penetration of cervical mucus. Hum Reprod 2008;23:2523–34.
[51] Nasir ud D, Hoessli DC, Rungger-Brändle E, Hussain SA, Walker-Nasir E. Role of sialic acid and sulfate groups in cervical mucus physiological functions: study of Macaca radiata glycoproteins. Biochim Biophys Acta 2003;1623: 53–61.
[52] Yurewicz EC, Matsuura F, Moghissi KS. Structural studies of sialylated oligosaccharides of human midcycle cervical mucin. J Biol Chem 1987;262:4733–9.
[53] Muratori M, Tarozzi N, Carpentiero F, Danti S, Perrone FM, Cambi M, et al. Sperm selection with density gradient centrifugation and swim up: effect on DNA fragmentation in viable spermatozoa. Sci Rep 2019;9:7492.
[54] Amiri I, Ghorbani M, Heshmati S. Comparison of the DNA fragmentation and the sperm parameters after processing by the density gradient and the swim up methods. J Clin Diagn Res 2012;6:1451–3.
[55] Brum AM, Sabeur K, Ball BA. Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. Theriogenology 2008;69:1041–55.
[56] De Vos A, Nagy ZP, Van de Velde H, Joris H, Bocken G, Van Steirteghem A. Percoll gradient centrifugation can be omitted in sperm preparation for intracytoplasmic sperm injection. Hum Reprod 1997;12:1980–4.
[57] Arcidiacono A, Walt H, Campana A, Balerna M. The use of Percoll gradients for the preparation of subpopulations of human spermatozoa. Int J Androl 1983;6:433–45.
[58] Strehler E, Baccetti B, Sterzik K, Capitani S, Collodel G, De Santo M, et al. Detrimental effects of polyvinylpyrrolidone on the ultrastructure of spermatozoa (Notulae seminologicae 13). Hum Reprod 1998;13:120–3.
[59] Fishel S, Jackson P, Webster J, Faratian B. Endotoxins in culture medium for human in vitro fertilization. Fertil Steril 1988;49:108–11.
[60] Söderlund B, Lundin K. The use of silane-coated silica particles for density gradient centrifugation in in-vitro fertilization. Hum Reprod 2000;15:857–60.
[61] Macpherson M, Blanchard T, Love C, Brinsko S, Varner D. Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. Theriogenology 2002;58:317–20.
[62] Varner D, Love C, Brinsko S, Blanchard T, Hartman D, Bliss S, et al. Semen processing for the subfertile stallion. J. Equine Vet. Sci.. 2008;28:677–85.
[63] Edmond AJ, Teague S, Brinsko S, Comerford K, Waite JA, Mancill S, et al. Effect of density gradient centrifugation on quality and recovery of equine spermatozoa. Anim Reprod Sci 2008;107:318.