Microfluidic Selection, Zeta Potential Characterization, and Sperm Transcriptomics for the improvement of Equine Embryo Production

By

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Abstract

In horses, *in vitro* embryo production is limited to a technique called Intracytoplasmic Sperm Injection (ICSI), in which a sperm is manually selected and injected into the oocyte. Therefore, it is essential to select a sperm that is functionally intact and capable of both fertilizing an embryo and maximizing developmental potential by relying on measurable parameters indicative of sperm quality. However, sperm quality is often oversimplified to prioritize parameters such as motility and morphology, which do not necessarily account for the true ability of the cell to fertilize an oocyte and produce a viable offspring. Thus, understanding more complex properties of sperm is highly beneficial to maximizing the success of ICSI. In non-equid species, researchers have investigated a novel property called Zeta Potential (ZP). ZP is an estimation of surface charge, thought to originate from charged proteins within the sperm glycocalyx, that has been correlated with basic sperm quality parameters, fertilization, and embryo development in other species including humans. This parameter has not been investigated in stallions and is characterized, for the first time, in this thesis.

In addition to understanding sperm quality, it is common to select for a population of more fertile sperm based on sperm quality parameters using established sperm selection techniques. Equine breeding facilities utilize sperm selection techniques to enrich the population of sperm portraying superior fertility parameters, including motility and morphology, but also accounting for characteristics such as cell viability,

mitochondrial potential, and DNA integrity. Conventional selection techniques include swim up (SU), density gradient centrifugation (DGC), and DGC-SU combination (DG-SU). A novel technique used in humans that has not yet been studied for stallion sperm selection is the microfluidic chip (MF), which selects sperm based upon their rheotactic motion similar to that seen within the female tract. In this work, we will present a thorough comparison of conventional sperm selection methods to MF.

Lastly, the sperm transcriptome has become a focus of fertility research, as sperm RNAs are thought to contribute to regulation of the female environment as well as to early embryo development. However, recovery of sperm RNAs is often low, which makes downstream analysis more difficult. As there is success in sperm RNA extraction in non-equine species, we aim to compare sperm RNA extraction methods for both sperm RNA yield and quality.

In this thesis, we will be discussing normal sperm morphology and physiology, as well as conventional and novel selection techniques, we will characterize ZP as a novel parameter of equine sperm fertility, and we will compare RNA extraction methods for optimal yield and quality of sperm specific transcripts.

Chapter 1

An update on semen physiology, technologies, and selection techniques for the advancement of *in vitro* equine embryo production: Section I*

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Simple Summary: Male fertility is often estimated by simple sperm assessment, and therefore it is crucial to establish species-specific baselines for normal sperm parameters. In this paper, sperm physiology, function, and common abnormalities in stallions will be reviewed.

Abstract: As the use of assisted reproductive technologies (ART) and *in vitro* embryo production (IVP) expand in the equine industry, it has become necessary to further our understanding of semen physiology as it applies to overall fertility. This segment of our two-section review will focus on normal sperm parameters beginning with development and extending through the basic morphology of mature spermatozoa, as well as common issues with male factor infertility in IVP. Ultimately, the relevance of sperm parameters to overall male factor fertility in equine IVP will be assessed.

Keywords: stallion; fertility; sperm; assisted reproductive techniques

1. Introduction

During natural breeding, a stallion will deposit millions of sperm within the intra-uterine environment of the mare [1]. Among this population of sperm there is a wide array of sperm "quality", which represents the ability of the sperm to fertilize an oocyte

and produce viable offspring [2]. Although some variation in sperm morphology and physiology between either individuals of the same species or within an ejaculate will not affect fertilization and embryo development outcomes, some parameters are correlated with fertilization, embryo development, and pregnancy outcomes.

This significant diversity in sperm fertility within an ejaculate becomes more pertinent when applied to *in vitro* embryo production (IVP), during which a smaller number of sperm are generally selected for either *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Thus, it becomes necessary to understand which physiological and functional parameters are the biggest contributors to sperm fertility. This allows us to select the highest quality sperm within an ejaculate for IVP.

Studies of equine sperm fertility encompass sperm biogenesis [3, 4], motility and metabolism [5, 6], morphology [7], sperm ultrastructure [8], and biochemical elements of sperm function [9-12], including sperm interactions with accessory sex gland secretions [13-15]. The wholistic picture of sperm fertility is integral to the maximization of IVP outcomes, and therefore, in Section I of this review we will focus on equine spermatogenesis, sperm morphology, and common sperm abnormalities leading to infertility.

2. Spermatogenesis

Adequate production of high-quality sperm by the male is critical to both natural and artificial reproductive processes. Therefore, it is critical to understand the pathways by

which male gametes are derived. This process, known as spermatogenesis, occurs in the germinal epithelium of the seminiferous tubules of the testis, and is initiated during puberty (Figure 1) [3]. Cross sections of the seminiferous tubules reveal adjacent cellular associations that produce sperm in a cyclic manner, repeating approximately every 12 days in the stallion for the constant production of spermatozoa [3, 16-18].

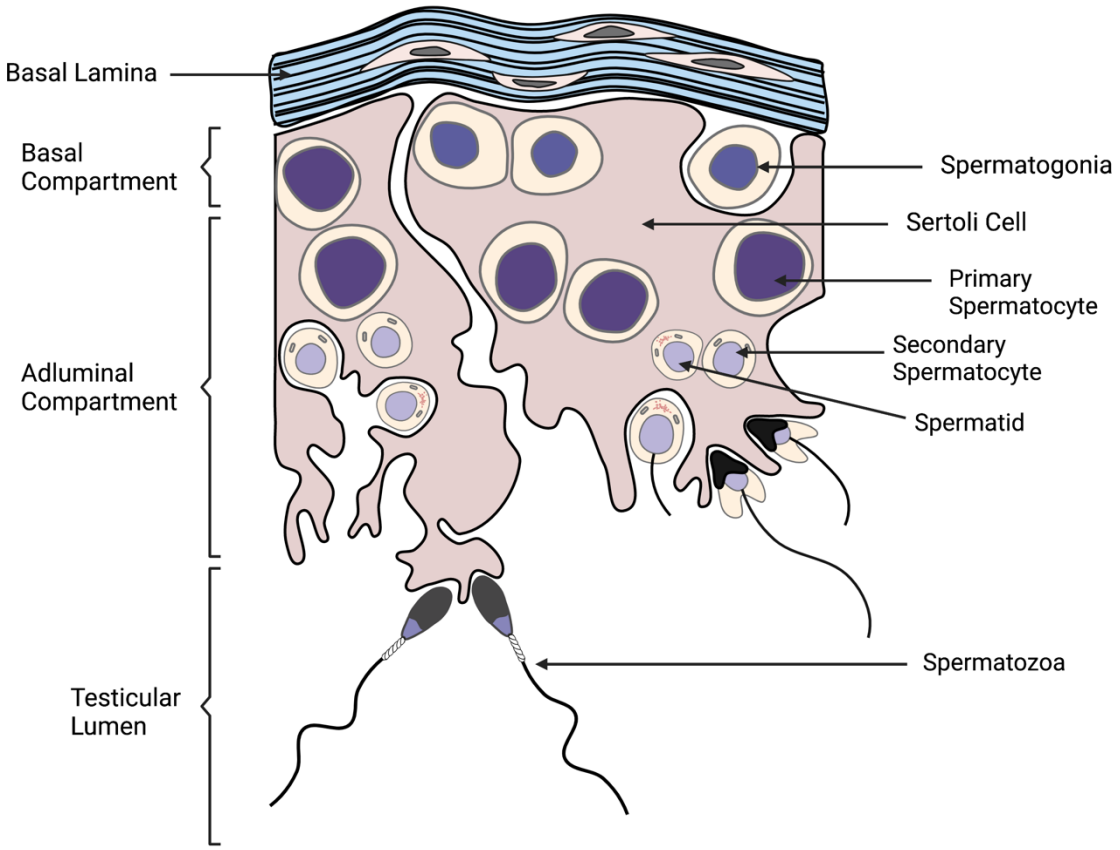

Figure 1. Schematic presentation of spermatogenesis. Facilitated by the nurturing Sertoli cells, basal spermatogonia replicate and differentiate into primary spermatocytes, and sequentially develop into secondary spermatocytes, spermatids, and the morphologically distinct spermatozoa during spermatogenesis. Spermatozoa are released into the lumen of the seminiferous tubule of the testis during spermiation.

The seminiferous tubule is divided into a basal and an adluminal layer, which is fully surrounded by a basal lamina [3, 19]. Leydig cells, which are stimulated by Luteinizing Hormone (LH) to produce sex hormones including testosterone, are key for regulating spermatogenesis as well as being responsible for the male phenotype [20, 21]. Leydig cells occupy the interstitial space of the testes between adjacent seminiferous tubules and serve as a key regulator of Sertoli cell function [21]. Within the seminiferous tubules, Sertoli cells span both the basal and adluminal layers, and their role is to host germ cells as they undergo meiosis and differentiation [22-24]. Specifically, Sertoli cells are stimulated by pituitary Follicle Stimulating Hormone (FSH) and secrete a variety of proteins that play a role in germ cell nourishment and development [22, 24]. In the stallion, it has been shown that seasonal fertility is partially attributed to changes in the number of Sertoli cells in the testis, which is directly correlated with the numbers of spermatozoa ultimately produced [25, 26].

The process by which mature spermatozoa are generated is a highly regulated process spanning across multiple domains of the testis. A non-committed store of A-spermatogonial cells exists at the basal layer and remains undifferentiated due to the expression of the Neurogenin 3 (*NGN3*) gene [27-29]. However, A-spermatogonia exist both to replenish the population of gametic stem cells and differentiate for continuation of spermatogenesis, and therefore a subpopulation of A-spermatogonia become committed to differentiation [27, 30]. The basal store of cells begin as single cells (A_{single})

and either undergo a complete mitotic division forming two single daughter cells or several rounds of incomplete mitosis in order to create paired (A_{paired}) and aligned ($A_{aligned}$) cell groups connected by intercellular bridges [30]. $A_{aligned}$ spermatogonia then undergo differentiation into committed A_1-spermatogonia, which also reside in the basal compartment [31]. However, A_1 cells do not express *NGN3* and therefore will undergo several rounds of mitosis and differentiation while remaining connected by intercellular bridges (A1, A2, A3, B1, B2 stages) [3, 4, 27, 32]. This period of cell replication is known as spermatocytogenesis and ultimately produces preleptotene primary spermatocytes [4]. These primary spermatocytes then enter the meiosis phase, where they pass into the adluminal compartment and participate in two meiotic divisions, first becoming haploid secondary spermatocytes and ultimately producing haploid spermatids [3, 4].

Following spermatocytogenesis, the final stage of spermatogenesis is the morphological shift denoted as spermiogenesis. Here, the sperm cell acquires its characteristic shape, including a species-specific streamlined head containing penetrative enzymes, a structured midpiece, a propelling tail, and the condensation of the male genome [4]. In most cells, nuclear DNA is organized by histone proteins [33]. However, during spermiogenesis, somatic histones are replaced by protamines: the dominant nuclear proteins of mature spermatozoa [33]. The strict compaction of protamine-DNA complexes prevents transcription, provides translational control, and promotes stability in the genome until penetration of the oocyte [33]. This final form produced via

spermiogenesis is known as a spermatozoon and is released into the lumen of the seminiferous tubule during the event of spermiation [4, 32]. The entire process of spermatogenesis takes approximately 57 days in the stallion [3, 7].

Following spermatogenesis, spermatozoa are exposed to a variety of proteinic and non-proteinic substances secreted by the accessory sex glands which aid in the acquisition of mature male fertility and sperm survival during transportion through the male tract and into the female tract [13-15, 34]. However, the remainder of this review will be focusing on the mature ejaculated spermatozoa and its relation to IVP, a process during which seminal plasma is largely removed, and thus we will not be elaborating on the significance of accessory sex glands and their secretions.

3. Sperm Morphology

The length of the equine spermatozoa from head to tail is approximately 60 μm [35]. A spermatozoa consists of three main components: a headpiece, midpiece, and tail which are fully encapsulated by a plasma membrane (Figure 2) [7]. The sperm head is an elongated, oval shape that is also relatively flat, with some variation on an individual basis [7, 36, 37]. The head consists of the acrosome, the post-acrosomal lamina, and the nucleus. The acrosome covers the anterior portion of the sperm head and contains hydrolytic enzymes which are released in order for the sperm to penetrate an oocyte [35]. In addition, it is theorized that the proteases and hydrolases contained within the acrosome play a role in the penetration of the oocyte cumulus complex, in addition to the

zona pellucida [38, 39]. The post-acrosomal lamina covers the caudal nucleus, which contains the highly condensed male genome [7, 35]. Species specific traits of the stallion sperm head include a characteristic asymmetrical head, a paraxially inserted tail, and a small acrosome relevant to other species [40].

The neck piece connects the sperm head to the tail and is made up of the connecting piece, the proximal centriole, and mitochondria. The neck serves as a connection point as well as orienting the tail distally [7]. The midpiece is made up of the cytoskeletal axoneme, which contains cylindrically arranged contractile microtubule doublets with attached dynein arms, which serve to facilitate tail movement. Each doublet is surrounded by dense fibers, which are in turn surrounded by a double spiral of mitochondria. The mitochondrial helix is critical for supplying energy to the sperm tail, allowing for the motility that is necessary in fertilization events. The end of the midpiece is defined as the caudal end of the mitochondrial sheath where the annulus, a ring of dense filaments, is located to separate the mitochondria and the sperm tail [7].

The principal piece of the propelling tail consists of the continuation of the axoneme and tapering dense fibers. The distinguishing feature of the principal piece is a protein-rich fibrous sheath that provides structure and flexibility for tail movements. The end of the fibrous sheath indicates transition from the principal piece to the end piece, which solely consists of the axoneme. All of these components are covered superficially by the sperm plasma membrane. Although parameters of a morphologically normal sperm may vary

significantly on an individual basis, abnormalities in the sperm anatomy may be an indication of subfertility or a problem with spermatogenesis [7].

The outer plasma membrane can be partitioned into the acrosomal, post-acrosomal, neck, midpiece, and principal piece domains [41]. Each region of the membrane can be characterized by a phospholipid bilayer of heterogeneously expressed lipids, proteins, carbohydrates, and cholesterol that is primarily established during spermatogenesis [33, 41-43]. The cell surface is additionally covered by a glycocalyx, a network of glycoproteins and glycolipids attached to a matrix of oligo- and polysaccharides, that is known to aid in the proper function of sperm as well as survival as it passes through the female reproductive tract

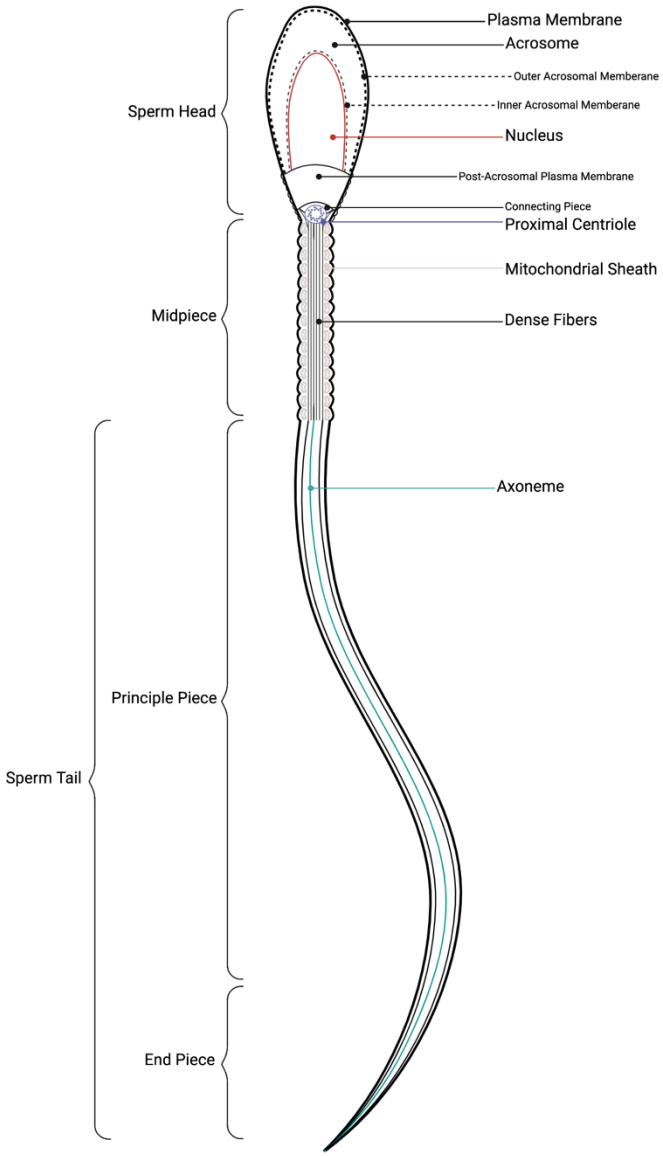

Figure 2. Anatomy of spermatozoa**.** A spermatozoa consists of three major components: head, midpiece and tail. The sperm head is overlaid by a plasma membrane, and an acrosomal compartment containing enzymes to aid in fertilization. The nucleus, surrounded by the nuclear envelope, contains the compacted male genome. The head is connected to the midpiece by the connecting piece. The midpiece consists of the proximal centriole, the mitochondrial sheath, and an inner dense fiber structure. The tail points distally, is also covered by a plasma membrane, surrounding the structural axoneme.

[43, 44]. However, spermatozoa in several species, including the ram, bull, rat, boar, buck, man, and stallion have been documented to undergo significant remodeling to the lipid and protein compositions during epididymal maturation [41, 45-50].

Due to the compaction of the sperm genome and the reduction in transcription, significant changes in protein, lipid, and sugar contents are thought to be a result of the uptake of epididymal epithelial secretions [51]. Although the mechanisms of proteomic alteration are not well understood, several corresponding hypotheses exist including (a) the reorganization of proteins into membrane specific domains [52], (b) the secretion of soluble proteins into the epididymal lumen by the epithelium and their subsequent absorption and integration into the plasma membrane [52], (c) the release of extracellular vesicles such as epididymosomes and proteasomes from the epididymis contributing micro and transfer RNAs as well as proteins [53-55], and (d) the potential direct anchoring of sperm heads to the epididymal epithelium for protein transfers via an unknown mechanism [56]. Specific proteomic changes to the sperm have been associated with various sperm functions including motility (flagellar, signaling cascade, and metabolic modifications) [57-60], capacitation (uptake of capacitation linked kinases) [61], acrosome reaction (modifications to the scaffolding proteins involved in acrosomal fusion and synapse) [62], and fertilization (facilitation of sperm-zona pellucida and sperm-oocyte interactions) [51, 63-66].

In the stallion, remodeling of the plasma membrane has been partially described through the domain-specific patterning of filipin-sterol complexes acquired during epididymal maturation as well as changes in intermembrane proteins [40]. Changes in protein composition have been thoroughly described in several species, and a majority of studies focus on the acquisition of epididymal secretory proteins between the caput and caudal epididymis [41]. Through freeze-fracture analysis, altered quantities and distributions of various intramembrane particles were observed over the course of epididymal transit in the equine testis, which is hypothesized to play a role in the establishment of various functional domains [50, 67]. It is hypothesized that functional domains assist the sperm cell in adapting to new conditions in the seminal plasma and female reproductive tract [41]. Specifically, changes in the binding affinity between sugar-binding lectins and the sperm glycocalyx indicate an altered exposure of terminal saccharide residues in the sperm membrane – thus altering the ability of the sperm to interact with its environment, such as within the uterus and oviduct, or with an oocyte [43, 68].

One of the physiological outcomes of membrane protein modifications is the overall change in net surface charge. This characteristic can be estimated through a measure of zeta potential, or electrophoretic mobility: an electrostatic potential at the slipping plane of the cell [69, 70]. The slipping plane can be described as the distance from the cell at which surrounding fluid particles are no longer bonded or attached to the cell, but are

completely mobile and free, and the charge at this location is proportional to surface-charge density [71, 72]. Zeta potential of sperm cells has been investigated in men, rats, bulls, rabbits, golden hamsters, guinea pigs, and mongoose [69, 73-76]. The source of the net negative charge is due to the addition of negatively charged sialoglycoproteins to the glycocalyx, such as the bipolar glycopeptide CD52, that appear in the sperm membrane during epididymal maturation [43, 69, 77, 78]. These proteins, as well as the total glycoproteic population in the plasma membrane, undergo compositional changes throughout maturation, capacitation, and acrosome reaction, and are thought to play a physiological role in these processes as well as in fertilization [41, 77, 79]. Thus, membrane charge is both a revealing and complex trait to accurately measure and interpret.

4. Bioenergetics and Generation of Motility

As previously mentioned, the mitochondrial helix is the primary grouping of organelles responsible for active motility and metabolism in the sperm cell. The number of mitochondrial gyres in the midpiece of the equine spermatozoa varies between 40-50, and their organization, or more specifically a disrupted organization, has been shown to play a role in the fertility of stallions through localized ATP production for sperm flagellar movement [80-82]. In fact, mitochondrial function, which can be approximated by mitochondrial membrane potential and electron transport chain (ETC) activity, are known to be positively correlated with overall sperm function [82-85].

ATP production occurs on the inner mitochondrial membrane within the intermembrane space between inner and outer membranes [6, 86]. In the process of oxidative phosphorylation, the primary mechanism of ATP generation in stallion sperm, a mitochondrial membrane potential is established as electrons are passaged through the respiratory enzyme complexes of the ETC of the inner membrane and energy is stored in the form of a proton gradient [82, 87-89]. Ultimately, ATP synthase uses the proton gradient to generate ATP [6, 88]. The mitochondrial membrane potential must be maintained, as reduced polarization can lead to an ATP shortage and cellular damage and hyperpolarization may produce an over-abundance of reactive oxygen species (ROS) and cause lipid peroxidation, which can be detrimental to overall cell integrity [6, 90]. It is also noteworthy that oxidative phosphorylation (the primary method of ATP generation in stallion sperm) coupled with mild oxidative stress is beneficial to sperm functional pathways such as hyperactivation, capacitation, acrosome reaction, and fertilization [89]. Lesser amounts of ATP may be produced by glycolysis under oxygen depleted conditions for maintenance of high sperm velocity [91, 92]. Additionally, research in stallions has shown correlations between ROS and motility, viability, and mitochondrial function [87, 91, 93], and thus it is highly beneficial to understand mitochondrial mechanisms as they relate to sperm fertility.

5. Common Abnormalities and Issues with Fertility

Sperm analysis is a significant method of infertility diagnoses and is critical in order to maximize IVP outcomes. Common issues in patients with male factor infertility can be either obvious or indiscernible to the human eye, and thus the depth of analysis by a technician depends on the technology immediately available to them. Due to the ease of analysis, sperm motility, viability, and morphology are the most common sperm assessments.

Sperm motility is essential for *in vivo* fertilization and *in vitro* fertilization (IVF), and is not necessarily required for ICSI where the sperm is manually injected [94-97]. Sperm motion can be described as either motile or hyperactivated; the latter being a result of capacitation that is required for oocyte penetration. Generally, clinics use Computer Assisted Sperm Analysis (CASA) or similar technologies to reduce subjective errors. CASA can also analyze more complicated motion parameters including the amplitude of lateral head displacement, average path velocity, straight line velocity, curvilinear velocity, linearity of the curvilinear path, and beat-cross frequency [98]. Sperm motility measures are widely considered to be indicative of fertility based on obvious biological functions, despite variable correlations with other sperm quality parameters [97, 99]. In the stallion, progressive motility is used as a general estimate of fertility, with less than 50% progressively motile in raw semen or less than 10% progressively motile two hours post collection being an indicator of potential subfertility [100]. However, stallion fertility

may be poor even with a highly motile population [101], and thus it is critical to understand other common sperm abnormalities.

Sperm viability is a generalized term that can be used to describe a number of traits, including an intact membrane, metabolic activity, and overall physiological health of the cell [102]. Generally, in sperm analysis, viability of the population is estimated by determining the percent of intact membranes using a fluorescent dyes such as propidium iodide (PI) and Hoechst 33528 [103, 104]. Although Hoechst is permeable with all cells, PI is only able to penetrate cells with disrupted plasma membranes. Thus, staining with two nuclear dyes is necessary for the identification of the non-viable population. Another double staining fluorescent methods for viability used in the equine industry is SYBR-14 and PI for viability [103, 105]. Assessment of sperm viability can also be indicative of early apoptotic changes, which could also be correlated with other severe sperm abnormalities or infertilities. Rather than, or in addition to, a viability stain with a permeable cell marker, another fluorescent dye may be added to expand upon the assessment of sperm integrity or function. Common fluorescent dyes used for equine sperm assessment include JC-1 [106, 107] or rhodamine 123 [108, 109] for mitochondrial membrane potential (an estimate of mitochondrial function), fluorescently conjugated Annexin-V (detection of apoptosis) [110, 111], or fluorescein isothiocyanate-PNA-Lectin (FITC-PNA) (assessment of acrosomal status) [112, 113]. Fluorescent dyes are a common method of

sperm quality assessment and a more extensive discussion of their use in ARTs can be found in Section II of this review.

Common morphological abnormalities seen in equine spermatozoa may include bent, coiled, or broken tails, misshapen heads, flattened or thickened acrosomal matrices over the apex of the sperm head, nuclear vacuoles, the presence of proximal droplets (an indication of immaturity), swollen or disrupted midpieces, and double heads or tails (Figure 3) [7]. In humans, morphology has been identified as an indicator of quality, and worsened morphology is specifically correlated with poor motility, DNA fragmentation, chromatin immaturity, high levels of ROS, a decreased ability to bind to the oocyte zona pellucida, and an overall decreased fertilization potential [114-118]. Similarly, studies in stallions have identified correlations between morphologic features, motility, and pregnancy outcomes [80, 119], indicating that there may be other sperm parameters associated with morphological abnormalities.

Prior to fertilization, the acrosome undergoes a calcium-dependent exocytotic reaction (acrosome reaction) as a result of sperm-oocyte binding that is essential for the subsequent penetration of the oocyte [120]. In equine spermatozoa, the precursor to the acrosome reaction is sperm activation, or capacitation, which occurs in the female reproductive tract as the spermatozoa approaches the oocyte. Capacitation can be generally characterized by the acquisition of both hyperactive motility and the ability to undergo the acrosome reaction through various molecular pathways and protein

phosphorylation cascades [51, 121, 122]. Capacitation has been successfully performed *in vitro* in numerous species, including humans and horses [122]. The acrosome reaction has also been achieved *in vitro* for the horse by using various components, including calcium (Ca^{2+}), calcium ionophore, bicarbonate (HCO_3^-), lysophosphatidylcholine, and progesterone leading to calcium oscillations [11, 112, 122-124]. Interestingly, sperm from stallions classified as fertile based on their breeding history are more likely to undergo the acrosome reaction *in vitro* when incubated with progesterone than sperm from subfertile stallions [125]. In humans, *in vivo*-derived inducers of calcium oscillations leading to the acrosome reaction include follicular fluid, cumulus oophorus, and the presence of granulosa cells; however, these methods are not well understood in the horse [122, 126-129].

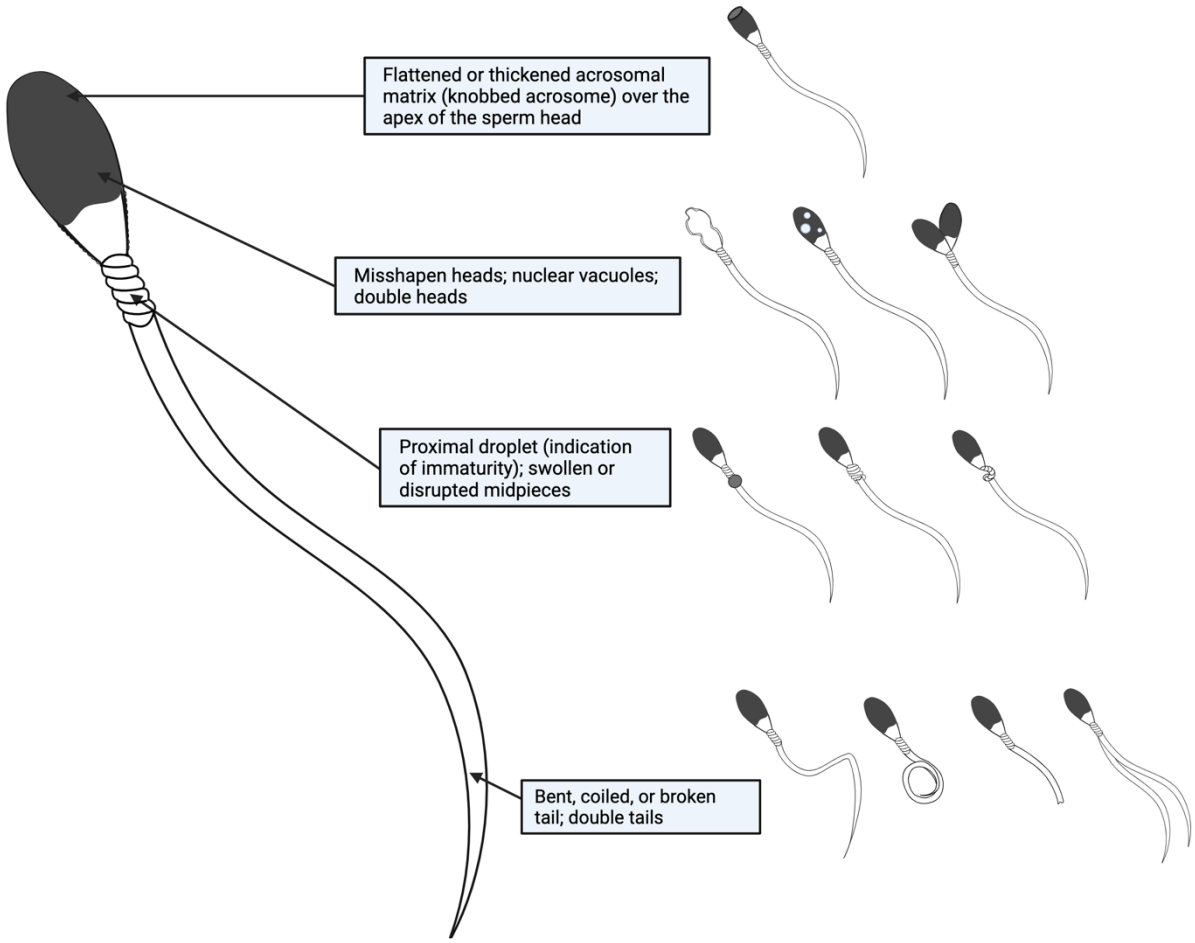

Figure 3. Common abnormalities of equine spermatozoa. Visualizable sperm abnormalities can be broken down by anatomical region: acrosome, head, midpiece, and tail.

In the context of fertilization, capacitation involves calcium oscillations that trigger a complex cascade of intracellular events leading to the binding of specific zona ligands on the outer plasma membrane with the zona pellucida of the oocyte [130, 131]. Subsequently, the acrosome reaction, or the fusion of the outer acrosomal membrane with the sperm plasma membrane, is marked by the exocytosis of proteolytic and hydrolytic enzymes from the acrosomal compartment [132, 133]. These enzymes aid in the digestion

of the zona pellucida so the sperm can penetrate the zona pellucida using hyperactivated motility acquired during capacitation. This results in the entrance of the sperm into the perivitelline space and the fusion of the inner acrosomal membrane and the equatorial region of the sperm head with the oolemma [133-135]. However, if a sperm cell undergoes the acrosome reaction prematurely, which can occur during cryopreservation or *in vitro* processing, it loses its ability to penetrate the cumulus oophorus and zona pellucida for fertilization [136, 137]. In human *in vitro* experiments, a premature acrosome reaction precluded the binding of sperm to the oocyte, and sperm that were able to bind were less successful in penetration [131, 138]. In horses, it has been demonstrated that sperm from subfertile stallions binds less frequently to the zona pellucida of the oocyte than sperm from fertile stallions, and that sperm from subfertile stallions are less likely to undergo acrosome reaction after binding [139], indicating discrepancies between fertile and subfertile sperm membrane affinities and compositions. Therefore, it is of interest to remove the prematurely acrosome reacted spermatozoa during selection procedures.

The mitochondrial helix is a sensitive structure that can be easily damaged under extreme environmental conditions, including cryopreservation [6]. Disruption of mitochondrial integrity, including the depolarization of the membrane, can disrupt ATP production and cause a sperm cell to become immotile and non-functional [6, 90]. Alternatively, hyperpolarization of the mitochondrial membrane will lead to lipid peroxidation and an over-abundance of ROS, leading to cellular damage [6, 90]. Although

exact mechanisms of cryoinjury to equine sperm are poorly understand, potential targets include disrupted plasma and mitochondrial membranes, increased ROS production, and generation of apoptotic factors [6, 93, 140].

Apoptosis is also a common issue seen in sperm samples, especially those that undergo thermal, oxidative, or osmotic stressors from extending, cooling, or cryopreservation [141, 142]. These stressors, as well as abnormal morphology, can initiate a variety of negative effects such as membrane and mitochondrial damage, plasma membrane restructuring (including the externalization of proteins such as phosphatidylserine), generation of ROS, and subsequent DNA damage [10, 12, 141-143].

DNA integrity assessment is one of the most valuable assessments of sperm fertilization potential due to the strong correlation with sperm reproductive competence; in fertilization as well as in subsequent embryo development and offspring phenotype [144]. Poor DNA integrity of sperm, or sperm with increased DNA fragmentation, can thus have detrimental effects on reproductive outcomes. DNA fragmentation is an all-encompassing term that includes both single- and double-stranded breaks, single base deletions or modifications, various non-desirable cross linkages, and mispackaging errors [145]. Causes of DNA fragmentation may include the mispackaging of chromatin during spermatogenesis [146], apoptosis [147], excessive ROS [146, 148], and other environmental factors [144]. The use of spermatozoa with damaged DNA has been associated with compromised fertilization both *in vivo* and *in vitro* as well as negative

effects on embryo development, such as worsened embryo quality and blastocyst rates [144, 149]. This could potentially lead to both miscarriages and altered offspring phenotypes including genetic diseases, such as Apert syndrome or achondroplasia, conditions thought to arise due to replication error mutations, and cancers [144, 150-153]. Thus, DNA integrity of semen can be a good indication of fertilization potential and potential effects on embryo development and offspring characteristics.

Surface composition and the resulting membrane charge are also of interest in sperm fertility studies. A greater net negative zeta potential, a parameter determined by surface composition as described previously, is acquired during epididymal maturation through extensive membrane remodeling and has been correlated with sperm quality in men [154, 155]. The acquisition of a net negative charge is primarily based on the extrusion of sialic acid (sialoglycoproteins) and other charged proteins to the outer membrane of the head region during epididymal maturation [44, 69, 77, 156]. The charge may also change significantly as a sperm changes environments, or when it undergoes capacitation or acrosome reaction [41, 79]. Specifically, membrane charge increases, or becomes less negative, when the sperm undergoes capacitation [157]. Externalization of sialoglycans by the sperm has been shown to play a role in avoidance of the uterine immune systems, as well as playing roles in capacitation and being an important component of sperm-zona pellucida binding, and therefore fertilization, and thus charge is a significant factor in sperm fertility [44, 77, 158, 159]. Extrapolating from these data, selecting sperm with a

greater net negative zeta potential will theoretically select for mature, functional, and viable spermatozoa.

5. Conclusions

Thorough interpretation of sperm physiology, despite its complexity, is the best method for assessing male fertility. In particular, furthering our understanding of the relationships between sperm morphology, viability, biological composition, and metabolism for equine sperm will be extremely beneficial in understanding fertility in stallions; as well as shedding light on associated mechanisms. In addition, characterization of new biophysical properties, such as zeta potential, will not only aid in our understanding of what makes a fertile sperm, but will also allow for the development of new semen selection technologies. For a review of current and prospective sperm selection technologies, please refer to Section II of this review. In conclusion, sperm physiological assessment is an invaluable tool for the equine breeding industry and merits continued consideration in clinical and research settings.

Institutional Review Board Statement: Not Applicable.

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Chapter 2

An update on semen physiology, technologies, and selection techniques for the advancement of *in vitro* equine embryo production: Section II*

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Simple Summary: In order to improve fertilization and pregnancy rates within artificial insemination or *in vitro* fertilization techniques in horses, producers may choose to select for the best sperm within an ejaculate. In this paper, we review conventional and novel methods of sperm selection.

Abstract: As the use of assisted reproductive technologies (ART) and *in vitro* embryo production (IVP) expand in the equine industry, it has become necessary to further our understanding of available semen selection techniques. This segment of our two-section review will focus on the selection of spermatozoa based on quality and sex for equine intracytoplasmic sperm injection (ICSI), as well as current and future developments in sperm sorting technologies. Ultimately, novel methods of semen selection will be assessed based on their efficacy in other species and their relevance and future application towards ARTs in the horse.

Keywords: stallion; fertility; sperm; assisted reproductive techniques

1. Introduction

The use of assisted reproductive techniques (ARTs) is expanding the field of equine reproduction, providing valuable opportunities to produce foals from animals which were unable to breed, conceive, or carry a pregnancy to term due to either sub-fertility or

logistical management issues. Techniques such as artificial insemination (AI) and embryo transfer (ET) are practiced with consistent success, and have already become an indispensable part of equine reproduction [1]. Newer techniques, such as transvaginal oocyte aspiration (TVA) and *in vitro* embryo production (IVP), are also being implemented worldwide, allowing veterinarians and farm managers to maximize the reproductive performance of horses [1-4]. Combining IVP with the use of sexed semen, the use of frozen-thawed sperm, observation of early embryo development, utilization of pre-implantation genetic testing, and cryopreservation of produced embryos further expands the potential of IVP in horses, despite some of these methods being in their infancy [5-10].

Most of the efforts for optimizing IVP outcomes are focused on oocyte maturation protocols, such as monitoring oocytes for meiotic progression [11, 12], microtubule and spindle assembly [13, 14], chromosomal abnormalities [15-17], nuclear maturation [18, 19], and cumulus cell expansion [20, 21]. Oocyte competence is critical in cases of subfertility, in part because the number of available oocytes at any given time is significantly less than the number of available sperm cells [11, 22]. However, sperm quality should not be overlooked as an indicator of fertilization and development potential, because there are wide ranges of sperm quality within an ejaculate that may influence ideal embryo production [23].

In horses, IVP is currently limited to a technique called intracytoplasmic sperm injection (ICSI) due to the failure of conventional *in vitro* fertilization (IVF) to produce foals [24]. One of the main advantages of ICSI is the low number of spermatozoa required per procedure; ultimately only a single male gamete needs to be selected for each oocyte. Therefore, ICSI has become the primary technique for producing embryos from men with low sperm count or viability [25]. Likewise, semen availability is one of the main reasons to perform ICSI in horses, allowing for the production of greater numbers of offspring from a reduced number of spermatozoa [26]. In general, frozen-thawed semen from stallions usually contains a limited number of viable spermatozoa and an overall worsened post-thaw quality [27]. However, frozen semen is ideal for ICSI because it can be stored and shipped easily, has a decreased dependency on proximity and frequent collection of stallions, requires only a fraction of a frozen ejaculate to be thawed to obtain sufficient numbers of spermatozoa for fertilization, and allows for the use of a variety of stallion genetics regardless of whether or not the stallion is healthy or deceased [28]. Additionally, it has been demonstrated that using frozen-thawed sperm for ICSI results in similar fertilization and embryo development rates to fresh semen, with some individual variation [6]. Other studies have shown that freeze-dried or air-dried sperm adequately maintain chromosome integrity and are also capable of producing live offspring after ICSI [29], [30-34]. However, little is known about the potential effects of

drying on overall sperm competence and embryo development and it is not a standard practice. Therefore, frozen-thawed sperm is the current ideal choice for equine ICSI.

Despite the numerous benefits of ICSI, is important to note that the manual selection of a sperm for ICSI bypasses the natural selection of viable spermatozoa that would naturally occur in the female reproductive tract, and to a lesser extent during conventional *in vitro* fertilization (IVF) procedures [35]. Consequently, the absence of natural sperm selection may represent a barrier to optimal fertilization and development [35, 36]. In particular with ICSI, it is possible to select a visually normal spermatozoa possessing damaged DNA or internal structure, which could lead to abnormal embryo development or miscarriage as observed in humans [37, 38]. Therefore, the use of artificial selection techniques to select for the most competent spermatozoa in a sample is a critical step in the optimization of ICSI outcomes.

Sperm selection has become an integral part of ARTs for both humans and animals [39, 40]. Spermatozoa from domestic mammals, as well as humans are generally evaluated and selected based on factors associated with their fertilization potential, which is then used to define sperm quality [41-43]. Various techniques to select the fraction of "high quality" viable spermatozoa in a sample rely on motility, morphology, DNA integrity, surface charge, and biochemical markers as indicators of potential fertility [44] and will be covered later in this review. However, there is increasing evidence that common selection parameters are insufficient indicators of fertility [41, 45]. Conventional

sperm selection techniques have been shown to be effective for the positive selection of motile and morphologically normal spermatozoa from a sample, yet they do not *directly* select for other important factors associated with fertilization and development, such as DNA fragmentation, membrane integrity, and spermatozoal ultrastructure [39, 46-48]. Although conventional selection methods for the enrichment of motile, morphologically normal spermatozoa within a population has been correlated with improved DNA integrity and consequently improved fertilization, blastocyst, and pregnancy rates, there is still room for improving the margin of error within sperm selection techniques [49, 50].

This section (Section II) of our review will focus on the ultimate selection of spermatozoa through advanced ARTs. Methods of sperm selection for ICSI based on both quality parameters and sex-chromosome will be presented and assessed based on their efficacy in horses. Ultimately, novel sperm selection methods used in non-equine species will be discussed in regard to their future application towards ARTs in the horse.

2. Sorting Semen: Significance and Method

In vivo, it is believed that sperm are naturally "selected" as they navigate through the female reproductive tract, resulting in only the most competent spermatozoa reaching the oviduct and ultimately fertilizing an ovum [35, 36]. However, these natural sorting procedures are bypassed during IVP, which could contribute to the suboptimal outcome of these techniques. According to a meta-analysis done in humans, IVP is only about one-

fifth as efficient as natural reproduction due to damage from cryopreservation and overall worsened gamete competence [51]. Inefficiencies of IVP have been documented in many species, beginning with the ability of *in vitro* culture conditions to disturb fertility and developmental competence [24, 52-55]. In addition, many have reported specific issues with fertilization and development events. For example, in cattle, fertilization rates after ICSI are extremely low potentially due to a failure in pronuclear formation without supplemental activation or due to physical disruption from the ICSI procedure [56-59]. In porcine IVF there is a high rate of polyspermia due to a reduced ability of *in vitro*-matured oocytes to invoke their mechanism of zona blocking against polyspermia [60, 61]. In horses, only two cases of IVF have been reported; this lack of success in conventional IVF is most commonly attributed to the inability of the sperm to penetrate the thick zona of the oocyte due to incomplete capacitation [24, 62]. Thus, ICSI is the only practical method of IVP in horses [5]. Despite the concentrated efforts in refining equine ICSI, blastocyst and pregnancy rates leave room for improvement [1]. Within a single study, reported blastocyst rates varied from 10%-70%, dependent on the source of oocytes (pre-ovulatory oocyte vs. immature), oocyte maturation protocols, and culture conditions [1, 63]. Therefore, blastocyst, pregnancy, and foaling rates are subject to significant improvement and standardization across the equine industry.

Although it is possible to produce healthy embryos and offspring from low quality sperm samples, it is preferable to process and select morphologically and functionally

superior sperm to maximize the chances of successful fertilization and embryo development [40, 64]. It is also noteworthy that there is limited information regarding the relationship between "good quality" sperm parameters and fertility [40]. Conventional sperm selection techniques generally rely on assessment of motility and morphology, which are factors that are positively correlated with fertilization and pregnancy rates for IVP [65-68]. However, spermatozoa with good motility and morphology will not always exhibit optimal viability, and may instead have poor DNA integrity, apoptotic factors, and disturbed mitochondrial integrity [48, 69]. Additionally, centrifugation steps required for specific processing methods are capable of generating injurious levels of ROS and ultrastructural damage in mammalian sperm [39, 69-71]. Thus, advancements in selection methods are likely to reduce sperm injury and to improve fertilization and pregnancy outcomes.

Many sperm selection methods have been developed in order to maximize the chances of selecting highly viable spermatozoa with variable success (Figure 4, Table 1) [40]. Commonly accepted and practiced methods of sperm selection include swim up (SU) and density gradient centrifugation (DGC) [39, 40, 47]. SU and DGC are widely used due to their simplicity and cost efficiency, and are known to select for improved motility, morphology, and nuclear maturity in a semen sample [39, 47].

2.1. Density Gradient Centrifugation

DGC works by overlaying a single or double density colloid gradient (known as a continuous or discontinuous gradient, respectively) with a semen sample of mixed quality within a centrifuge tube [39, 72, 73]. The entire tube is then spun at a moderate *g*-force (300-600 x g) for 15-30 minutes in order to induce passage through the gradient and separation of the high and low quality spermatozoa [39]. In a double density gradient, the less dense, upper layer will filter out larger macromolecules, leukocytes, or other unwanted cellular debris [74]. Mature spermatozoa should be able to easily pass through the upper gradient, and upon reaching the second, denser colloid layer, the morphologically normal spermatozoa will possess a greater density and will be able to orient head-downwards, allowing them to swim downwards through the colloidal silicon gradient and form a pellet with the aid of centrifugation forces [39, 74, 75]. Percoll® DGC is the most common gradient in most animal industries, but is prohibited in human sperm preparation due to potential inflammatory, ultrastructural, and endotoxic effects of the PVP-coated silica particle, which is associated with cytoplasmic fragmentation and worsened embryo development [76-80]. In equine reproduction, the use of Equipure™ has become a common substitute for Percoll® in various laboratories. Results from Equipure™ centrifugations have yielded enriched motility, morphology, and pregnancy rates as compared to other centrifugation methods [81-83]. It has been shown that Equipure™ not only selects for progressively motile spermatozoa, but also enriches the population of viable sperm with good mitochondrial membrane integrity from frozen-thawed samples

[84]. The resulting pellet after either Percoll® or Equipure™ centrifugation has been generally known to enrich the population of motile, morphologically normal spermatozoa with an intact genome in both men and stallions [83, 85-87]. Alternatively, some report DNA damage to actually increase as a result of centrifugation in horse or human [75, 87]. Results likely vary due to individual, handling, or protocol variations. Increased DNA damage can lead to poor embryo quality, blastocyst rates, implantation rates, and pregnancy to term rates after IVP, and thus it is critical to understand the potential for inducing damage with centrifugation protocols [88, 89].

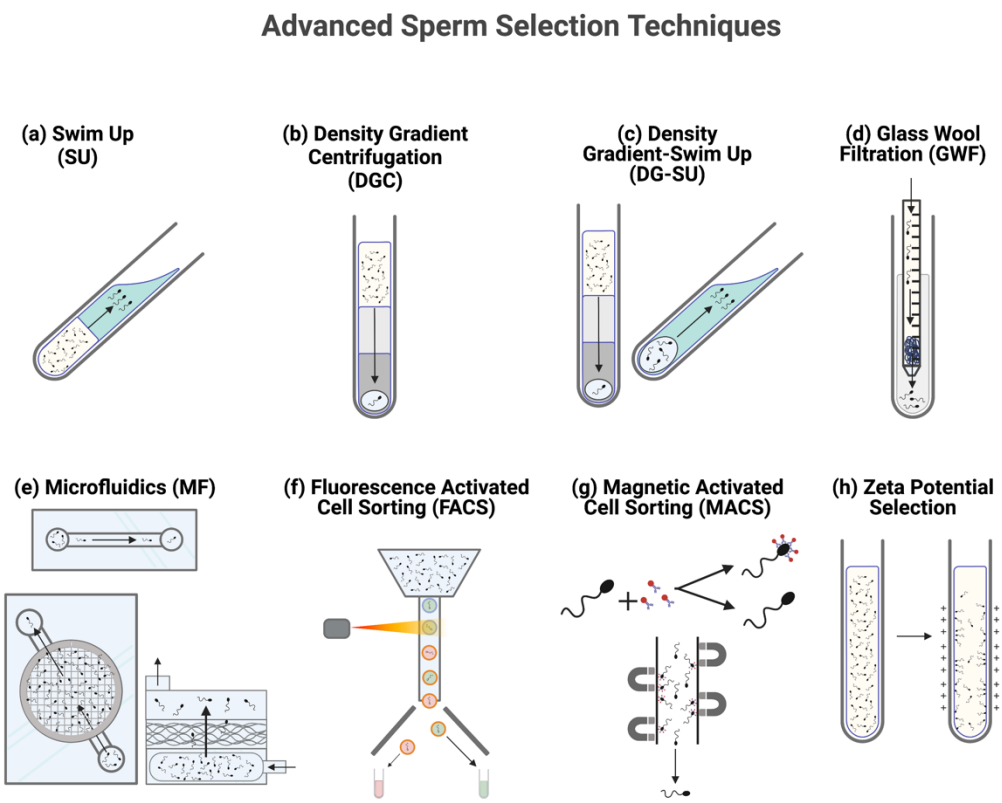

Figure 4. Conventional and novel methods of sperm selection. Selection techniques used to select superior quality sperm include (a) Swim Up (SU), (b) Density Gradient Centrifugation (DGC), (c) Density

Gradient-Swim Up (DG-SU), (d) Glass Wool Filtration (GWF), (e) Microfluidics sorting (MF), (f) Fluorescence Activated Cell Sorting (FACS), (g) Magnetic Activated Cell Sorting (MACS), and (h) Zeta Potential Selection.

2.2. Swim Up

In contrast to DGC, the SU procedure does not require a centrifugation step, and relies on the inherent progressive motility of sperm to swim upwards through a medium over a period of 30 to 60 minutes, with the top fraction ultimately being selected [39, 48]. SU has been shown to select for highly motile, morphologically normal, DNA-intact populations from human sperm samples, while also removing extraneous cell or protein debris [39, 40, 48, 90, 91]. Unfortunately, the recovery rates of SU are low, meaning SU is only a viable assay for highly concentrated samples or for procedures like ICSI that do not require a significant number of spermatozoa in the final selected fraction [39, 92].

Table 1. Sperm selection techniques select for a variety of sperm parameters and are capable of reducing percentages of negative factors within a sample, although some methods may have detrimental effects.

Method	Selects Based On:	Benefits:	Detriments
Density Gradient Centrifugation	Morphology Cell density Motility	Enriches for: Morphology; Motility; Viability; Mitochondrial membrane integrity; Pregnancy rates; DNA integrity Removes cell and protein debris	Toxicity of Percoll® Centrifugation causes DNA damage

Swim Up	Progressive motility	Enriches for: Motility; Morphology; DNA integrity Removes cell and protein debris	Low recovery rate
Density Gradient-Swim Up	Morphology Cell density Motility	Enriches for: Motility; Morphology; DNA integrity Removes pathogens	Toxicity of Percoll® Centrifugation causes DNA damage
Glass Wool Filtration	*In vivo* fertility Motility	Enriches for: Motility; Morphology; Chromatin Condensation; Membrane, Integrity; Cleavage rates; Blastocyst rates; Pregnancy rates High Recovery Rate	Possible damage to sperm head and acrosome ultrastructure Glass wool contamination of final product
Fluorescent Activated Cell Sorting	Variable physiological markers (membrane integrity, apoptotic markers, mitochondrial membrane potential, sex chromosome)	Enriches for: Pregnancy rates; Live birth rates Removes unwanted cells	May cause oxidative and DNA damage Mechanical Stress Time consuming High operating expenses Inability to select for numerous factors
Microfluidic Sorting	Motility Rheotactic, Chemotactic, and Thermotactic behavior	Enriches for: Motility; Viability; DNA integrity Reduced ROS generaton Removes extracellular debris Can be combined with IVF	May impose stress May reduce viability in some species
Magnetic Activated Cell Sorting	Variable physiological markers (removes sperm with apoptotic markers, acrosome reacted sperm)	Enriches for: Motility; Viability; Morphology; Survival, motility, and mitochondrial integrity after cryopreservation; Sperm binding rates; Fertilization rates; Embryo development rates	May possess cytotoxic effects
Zeta Potential Selection	Greater net negative membrane charges	Enriches for: Maturity; Morphology; DNA integrity; Protamine content; Fertilization rates; Pregnancy rates	Not shown to increase motility and viability

2.3. Combination Density Gradient-Swim Up

DGC and SU have also been used in combination (DGC-SU) by pelleting the sperm using DGC and then allowing sperm to swim upwards through an overlying media. DGC-SU has been shown to enrich for motility and morphology better than SU alone, to decrease the percentage of ultrastructural abnormalities in the selected sample, as well as select for the population with overall better DNA integrity as compared to DGC alone [93, 94]. This method has also been shown to be an effective way of removing pathogens, such as equine arteritis virus or bovine viral diarrhea virus, from contaminated semen while also selecting for undamaged spermatozoa [95, 96]. Therefore, this method could have significant benefits for other equine diseases transmissible through an ejaculate.

2.4. Glass Wool Filtration

Glass Wool Filtration (GWF) is another technique that has been used in both human and animal reproduction [71]. This method is intended to mimic the ability of the female reproductive tract to effectively filter out dead sperm, leukocytes, and infectious materials, by allowing spermatozoa to swim through and be filtered by a dense arrangement of glass wool fibers within a column [71, 97]. In humans, spermatozoa separated with GWF yield a sample enriched for motility, morphologically normal spermatozoa, and good chromatin condensation, as well as having a high recovery rate

and good cleavage and blastocyst rates [98, 99]. GWF was also shown to produce a sufficient number of cells, with better recovery than SU, for insemination with frozen-thawed spermatozoa [100]. In an experiment with bovine sperm comparing DGC and GWF, GWF was capable of enriching motility, membrane integrity, and GWF-selected sperm used for IVF resulted in higher cleavage and blastocyst rates than control samples [101]. In horses, the pregnancy rate from deep-horn inseminations with sperm separated by GWF was elevated above the rate when using an absolute number of sperm, and similar to that of insemination with Percoll® separated sperm, showing that GWF is a valuable technique for many species [102]. It is noteworthy that some reports claim that traditional GWF can damage the sperm head and acrosome ultrastructure [103], and glass wool fibers could appear in the filtered product, which poses a problem for artificial insemination procedures [104].

All of the techniques described thus far work on the basis of selecting highly motile, morphologically normal, and intact spermatozoa, and are moderately successful in doing so. However, the ability of these techniques to improve motility and morphology parameters does not always correlate to the selection of spermatozoa with the best DNA integrity and overall fertilization potential [48]. In response, other techniques have been developed in order to select sperm based on viability and biophysical markers.

2.5. Fluorescent Activated Cell Sorting

Flow cytometry has been utilized in order to characterize various quality parameters within an ejaculate, including membrane integrity, ROS generation, capacitation, acrosome reaction, mitochondrial status, apoptotic markers, and DNA integrity [105]. Although general characterization of a cell population by flow cytometry requires the use of a fixative, fluorescence activated cell sorting (FACS), has been adapted in order to recover live cells. FACS utilizes a variety of fluorescence stains and dyes which are biologically compatible or conjugated to bind to sperm based on specific sorting parameters and primarily works by allowing for the removal of damaged cells from a sample (negative selection). The cell's affinity for the stain or dye allows for it to emit a readable fluorescent or non-fluorescent signal (more complex probes can be sorted based on the degree of fluorescence) when the cell is excited by a laser and the probe is activated. Subsequently, the droplet is charged and sorted into an appropriate subpopulation by deflection plates [71]. The use of a stain or dye targeting indicators of poor sperm quality results in the negative selection of the high-quality population which can then be used for a variety of *in vitro* procedures. Live sperm have been flow-sorted based on membrane permeability and apoptotic markers [106-108], mitochondrial membrane potential [109], and even sex chromosomes [110].

One of the early stages of apoptosis involves a change in the membrane where phospholipid phosphatidylserine is transferred to the sperm's outer membrane and

displaces phosphatidylcholine [111, 112]. A method to enrich the population of non-apoptotic spermatozoa in a sample is to tag and remove apoptotic spermatozoa from the population by FACS with fluorescently labeled Annexin-V; the antigen of phosphatidylserine [106, 107]. Sperm negatively selected via Annexin-V FACS have been shown to possess intact chromatin [108]. Additionally, in a human clinical trial, spermatozoa from the population that did not bind Annexin-V during FACS were used for ICSI resulted in improved pregnancy and live birth rates, as well as reduced miscarriage rates over embryos generated with sperm selected from SU [113].

Sex selection using flow cytometry is perhaps the most common application of FACS to sperm selection and has been successful in separating sperm from many species including humans, cattle, horses, pigs, sheep, goats, dogs, cats, deer, elk, and water buffalo, as reviewed by [114]. In horses, sexed semen has been used to produce live foals, sexed with over 90% accuracy [115-118]. More information on sex selection can be found below (Sexing Semen).

Despite the advantages of FACS sorting, it has been shown that FACS in equine sperm induces significant oxidative and DNA damage to spermatozoa [119]. In addition, FACS can cause significant mechanical stress due to the high-pressure throughput, and laser and dye exposure may reduce mitochondrial activity and motility as demonstrated with bovine sperm [120, 121]. It can thus be extrapolated that mechanical and functional stress may be induced regardless of specific dyes, laser wavelengths, or flow pressures.

Additionally, flow cytometers are costly to maintain and operate, and selection can be time consuming due to the individual characterization of each cell; therefore, flow cytometry is not always an ideal method of selection [116, 117, 122].

2.6. Microfluidic Sorting

Another technique for high quality sperm selection is the microfluidic (MF) sorting method, which can select highly motile sperm based upon rheo-, chemo-, and thermotactic behaviors of viable spermatozoa, while also removing extraneous cellular debris, in an attempt to mimic aspects of *in vivo* sperm selection [123-126]. Various MF devices exist, including those that rely on the ability of the highly motile subpopulation to swim through a porous membrane [127-129] or combinations of channels and collection chambers to be selected [126, 130-135]. MF sorting with human and bovine sperm has shown to select for sperm with overall enriched motility, viability and DNA integrity, as well as reduced ROS generation, when compared to centrifugation methods [127, 128, 133].

In porcine IVF, polyspermia is a large contributor to developmental incompetence in early embryos; thus, a microfluidic-IVF combination device known as a Microfluidic Sperm Sorter (MFSS) has been developed to combine the sperm selection and IVF processes [131]. In a study by Sano et al., (2010), MFSS-generated embryos showed reduced cases of polyspermia and improved developmental competence as compared to

embryos generated by traditional IVF [131]. This method has also been used in cattle IVF to improve developmental competence and blastocyst rates [132]. Other variations among microfluidic devices include the use of hydrostatic pressures to facilitate rheotactic behaviors [134] or highly viscous medias [135] to more closely mimic *in vivo* fertilization.

In a recent study with horse sperm, microfluidic devices containing a porous membrane that allows only motile spermatozoa to swim through resulted in a selected population enriched for sperm with normal morphology, and improved motility, viability, and DNA integrity parameters [129]. This study also showed that MF yielded similar results to DGC and was superior over results from SU, but no clinical outcomes were investigated [129]. Yet, despite the widely reported benefits of MF sorting, it has also been demonstrated that some microfluidics may impose stress upon boar, but not bull, spermatozoa and negatively impact viability [136]. As boar spermatozoa are often used as a model for human spermatozoa, this phenomenon, as well as potential injuries to spermatozoa from other species, requires further investigation.

2.7. Magnetic Activated Cell Sorting

Another relatively new technique for selection of viable spermatozoa is the use of magnetic nanoparticles (MNP) to select for various parameters of quality, otherwise known as Magnetic Activated Cell Sorting (MACS) [137, 138]. Nanoparticles, defined as

being less than 100 nm in diameter, can be coated with a variety of magnetic compounds and subsequently conjugated to a variety of biomarkers for physicochemical properties of the sperm [139, 140]. Applications of the iron oxide (Fe3O4) MNP are diverse, and consequently this is a common choice of magnetic conjugate [139, 141]. Magnetized biologically relevant conjugates can be incubated with spermatozoa and then passed through a magnetic field for sorting [137, 138]. Previously, MACS has been used for high quality sperm selection with samples from humans, pigs, cows, and donkeys by selecting for characteristics of apoptotic and prematurely acrosome reacted sperm, which results in improved fertilization and embryo development [137, 138, 140, 142-144].

Specifically, Annexin-V conjugated MNP have been used to eliminate human spermatozoa in the early stages of apoptosis from a population using a paramagnetic microbead conjugated to Annexin-V in order to bind to phosphatidylserine and negatively select for an intact population [138, 144]. In early studies, several groups were able to successfully reduce the percentage of apoptotic sperm within their sample without any observable negative effects [138, 145]. Later studies further found that Annexin-V MACS prior to cryopreservation resulted in significant improvements in survival, motility, and mitochondrial integrity after thawing as compared to an untreated control [143, 144]. Paasch et al. (2003) compared binding between known infertile patients and donors, and found that infertile patients had much higher binding rates to the Annexin-V MNP with strong specificity for apoptotic cells. In another human clinical

trial, Annexin-V MACS and DGC selection combined was the most optimal method of selecting sperm with improved motility, viability, and morphology, and a reduction in early apoptotic markers, over that of DGC alone or MACS alone [146].

In animals, MACS has been used in pigs to remove both apoptotic and acrosome reacted spermatozoa, via MNP conjugated with Annexin-V and Lectin, respectively [140]. MACS selection resulted in an enriched motile population, and no negative effects have been observed when negatively-selected sperm were used for AI, which negates concerns over potential toxicities of nanoparticles in sows [140, 147]. In donkeys, peanut agglutinin (PNA)-lectin conjugated nanoparticles have been used to remove acrosome damaged spermatozoa, while simultaneously improving progressive motility, and in some cases membrane viability [142]. Additionally, MACS has been proposed as an alternative to flow cytometry sex sorting and has been successfully used to enrich the population of X spermatozoa with 90% accuracy [148]. MACS sex-sorted semen also demonstrated good viability and motility without premature capacitation or DNA damage [148]. MACS has not been widely used in the equine breeding industry, but may be suitable for stallions with subfertility.

2.8. Zeta Potential Selection

Another novel sperm selection technique is the zeta potential sorting method. In humans, a greater net negative zeta potential, has also been reported in mature,

morphologically normal, DNA intact sperm, thus making zeta potential a potential marker of sperm quality, or fertilization potential as well as playing a functional role in *in vivo* selection [149-157]. As described in section I of this review, zeta potential is the electrostatic potential at the slipping plane of the cell and is an estimation of the surface charge of the cell [153, 158]. Due to the nature of zeta potential measurements being dependent on fluid dynamics, there is no defined optimal measurement for this parameter. Therefore, zeta potential measures must be performed under identical conditions in order to be compared. For example, a sample with a greater net negative charge is theorized to be better quality than a sample with a more positive zeta potential under identical conditions. Thus, membrane charge is both a revealing and complex trait to accurately measure and interpret.

Regardless of complexity, several selection methods have been developed in order to separate sperm based on membrane surface charge [151, 154, 156]. An existing zeta potential-based selection method involves inducing a positive charge on a glass centrifuge tube using friction and allowing the more negatively charged spermatozoa to bind. This method has been used in human IVF to successfully select sperm with overall improved DNA integrity, morphology, and protamine content compared to unprocessed semen [154, 156]. In turn, this resulted in improved fertilization and pregnancy rates [150, 153, 154, 156]. In another study, selection of human spermatozoa based on morphology, motility, and viability resulted in a significantly increased net negative charge of the

sample; for example, morphologically normal semen possessed an average zeta potential of -7.79 mV whereas morphologically abnormal semen read an average -5.37 mV [150]. An alternative method of zeta quality selection utilizes electrophoresis to drive the movement and isolation of high quality spermatozoa that also possess a greater net negative charge. Subsequently, this method is able to improve measures of morphology, and select against spermatozoa with DNA damage, although motility and viability parameters did not change significantly from the original sample [151]. The selection of high quality equine semen based on zeta potential has yet to be reported, and could have pronounced effects on outcomes of equine IVP.

3. Sexing Semen

Selection of spermatozoa based on the presence of an X or Y chromosome is of significant interest for many species [117]. Although not used in human applications, sex selection is especially important in equine and agricultural industries where female or male phenotypes may be better suited for sporting or production outcomes, as well as appealing to owner preferences. In horses specifically, female or male phenotypes are desirable in different forms of recreation [117]. For example, females are used almost exclusively in Polo sports and are also the preferred sex to be used as cutting horses and in Quarter horse racing [117, 159, 160]. However, males are more desirable as reining horses, and are preferred for Thoroughbred racing, dressage, and show jumping, as well

as fetching higher prices at Thoroughbred auctions [117, 159-161]. In addition, the ability to determine fetal sex may aid in making key breeding management decisions, as well as make it easier to sell pregnancies with known fetal sex for a higher price [117]. Due to the strong preferences of many industries for one sex or the other, it is of key interest to be able to pre-determine sex, primarily starting with sexed semen. However, in order to select for one sex or the other, clinicians must rely on the physiological differences between X and Y chromosome bearing spermatozoa.

Although X and Y spermatozoa are essentially equivalent in regard to functionality, there are notable differences. Most notably, the human X spermatozoa contains approximately 2.8% more genetic material than the Y spermatozoa, and differences for livestock species range 3-4.2% [162]. This principle has become the foundation for sex sorting with flow cytometry.

Currently, flow cytometry is the only vetted method for separation of X and Y chromosome-bearing spermatozoa. Sex sorting with flow cytometry utilizes the Hoescht 33342 fluorescence stain (which preferentially binds to AT rich regions along the minor groove of DNA) to categorize individual sperm based on differences in sex chromosome mass [as reviewed by [114, 118, 122]]. Sperm are individually run through the flow cytometer, and their respective droplet is charged according to relative fluorescence and separated [116]. Sex selection using flow cytometry has been successful in separating X- and Y-bearing sperm from a variety of species (as reviewed by [114]. In horses, sexing

technologies have been used to produce live foals, sexed with over 90% accuracy [115-118].

Although various technical advancements have been made with flow sorting, the method is inefficient in producing doses adequate for AI and can cause damage to the sperm including reduced motility, generation of reactive oxygen species, acrosomal and membrane damage, and reduced longevity of the sperm (as reviewed by [116, 118, 120, 122, 163]). This results in pregnancy rates as low as 10-50% [115, 160, 164, 165]. Injury to the sperm cells may be due to a number of the following: staining, high pressure flow, charging, deflection, and specific handling methods. These effects are worsened in frozen-thawed samples, making flow sorting undesirable for procedures like ICSI where frozen-thawed samples are primarily used [114, 116, 117, 120, 166, 167]. Additionally, flow cytometry is expensive and time consuming, making it unappealing or inaccessible to many commercial operations [116, 117, 122]. In stallions the unique head shape of spermatozoa, makes distinguishability of X and Y-sperm difficult, and significant variation between individuals has prevented the optimization of the method [118, 120, 168]. Therefore, the development of an alternative sexing method that reduces sperm injury and is more affordable and practical for the equine industry and individual consumers would be beneficial.

Interestingly, some studies with human sperm have revealed that the zeta potential quality selection method has a selection bias for X-chromosome sperm [158], although

others have observed no sex-bias in electrophoretic selection [169]. This implies that using this technique in horses might lead to a bias towards XX embryos, which could be beneficial in industries such as polo in which females are more desirable. Specifically, in a comparison between DGC and a combined DGC/zeta potential selection method prior to human ICSI, the DGC/zeta potential selected group resulted in 63.6% of XX babies delivered, whereas the DGC-only group only resulted in 38.5% of XX babies [158].

An altered sex ratio during electrophoretic sorting aligns with observations of a membrane charge differential between X- and Y-chromosome bearing spermatozoa; with X-chromosome bearing human sperm exhibiting approximately a -20 mV charge and Y-chromosome bearing human sperm exhibiting a charge of -16 mV [153, 158]. Thus, several methods utilizing zeta potential as a basis for sex-sorting semen have been developed and are reviewed in this section.

Zeta potential sex-selection techniques include electrophoretic separation, in which fluid flow mediates sperm to swim perpendicular to an electric field. This method has been able to separate spermatozoa with a population containing almost 80% X-chromosome bearing spermatozoa [170]. Using free-flow electrophoresis, X and Y spermatozoa from humans, mice, and bulls have been successfully separated based on this external charge differential [171-173]. However, due to the increased net negative charge in X chromosome-bearing sperm, there have been observed biases towards the selection of X chromosome-bearing sperm due to their increased electrophoretic mobility

[170, 171]. A later study called the method of sex-chromosome identification via electrophoresis into question, and reported no differences in sex ratio when using electrophoretic separation [169]. No data regarding differences in zeta potential between X and Y chromosome-bearing sperm in horses has been reported.

Another recent study in donkeys observed that X chromosome bearing sperm could be successfully isolated with 90 ± 5% accuracy based on the membrane zeta potential differential between sexes and a subsequent specificity when conjugated directly to MNP under precise conditions [148]. This study utilized a modified MACS technique in which MNP were adhered to the surfaces of Y-spermatozoa using specific environmental conditions and negatively selected for X-spermatozoa [148]. Collectively, this suggests that sperm sex chromosome-dependent membrane potential, despite the poor understanding of its mechanisms, is a conserved trait across species and can be used for sex selection with equine sperm.

Regardless of the apparent conservation of sex chromosome-dependent sperm charges across species, it is unfortunate that sperm membrane charges are subject to change under a variety of physiological processes and environmental conditions. Sperm membrane composition and charge are known to change and increase, respectively, during capacitation and acrosome reaction [149, 174]. It is not uncommon to see premature capacitation, or capacitation-like changes, in cryopreserved semen, and capacitation can be artificially induced using media components [175]. In addition, the

interaction between sperm and specific fluids and medias may promote alternative membrane changes which can alter the surface charge [176-178]. Therefore, it is necessary to be consistent and critical when measuring and interpreting sperm zeta potential measures, particularly when trying to characterize minute differences between X- and Y-bearing sperm.

4. Conclusions

Although a variety of techniques exist to separate and select for spermatozoa based on quality and viability, as well as sex chromosome, not all methods have yet been translated to the equine industry. As the implementation of IVP in horses expands, so does the need for highly affordable and efficient semen selection techniques. The inconsistent efficacy of traditional sperm selection methods, such as SU and DGC, presents a barrier to the optimization of IVP. The introduction of techniques, such as MF, that are used clinically in other species may consequently improve IVP in the horse. There is also a need to assess the efficacy of newly developed sperm selection methods, such as viability sorting with MNP, zeta potential quality sorting, and zeta potential sex sorting, in the stallion to determine their potential for use in clinical settings. Ultimately, the rapid expansion of novel semen selection techniques provides many opportunities for improved fertilization, embryo development, and pregnancy rates within the equine breeding industry over the following decade.

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Chapter 3

Selection and Characterization of High-Quality Stallion Spermatozoa Using Microfluidics*

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Abstract: 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 essential to employ superior methods of selecting high quality sperm. Therefore, in this study 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, and normal morphology were evaluated for both unprocessed fractions and post-selected fractions. 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 ($P<0.001$), and of similar to DG-SU and DGC without the use of potentially harmful centrifugation steps. In conclusion, we identified MF as a novel effective method of equine sperm selection for IVP.

Keywords: Stallion, sperm, sperm selection, microfluidic chip

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 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.

In addition to furthering our understanding of sperm physiology, it is necessary to find relevance of sperm parameters 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 [2-5]. 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 [6-9]. 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 [6, 7, 10]. 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 [10]. 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 [10].

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). We hypothesize 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 [11]. 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 4ºC for a period of 24 hours prior to analysis and processing [12]. 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 [13]. A small fraction of the sample was diluted into a lysis reagent (Reagent S100 Lysis Buffer, ChemoMetec) to measure total concentration and into PBS (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) (CASA, HTM CEROS, Version 12.2 g; Hamilton Thorne Biosciences, Beverly, MA, USA) and Sperm Vision® SAR software (Minitübe, Tiefenbach, Germany) [14]. For CASA motility analysis, 3 μL of semen 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; minimum 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 microscopy under 100X magnification. 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 [15, 16].

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) [17], Density Gradient Centrifugation (DGC) [18], Density Gradient-Swim Up Combination DG-SU [19], or Microfluidics (MF) [10] 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 32-175 M/mL, with an average of 142.45 M/mL.

Wash: Semen diluted in extender was centrifuged for 8 minutes at 300 x 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 minutes 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 minutes at 300 x 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 minutes at 300 x 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, 850µL, 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 minutes 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). 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.

2.6. Variation in quality of sperm selected by different methods

Each ejaculate was divided and evaluated as one of 6 treatment groups. Quality parameters and ZP were compared among unprocessed, wash-only, and DGC, SU, DG-SU, and MF selection methods (Figure 5).

When comparing selection efficacy by total motility, both unprocessed and washed groups possessed significantly less motile populations than MF ($P < 0.0001$; $P < 0.0001$), DG-SU ($P < 0.0001$; $P < 0.0001$), and DGC ($P < 0.0001$; $P < 0.0001$). Motility of the SU group was also significantly lower than MF ($P < 0.0001$), DG-SU ($P < 0.0005$), and DGC ($P < 0.0005$) selected groups. Numerically, the average motility of sperm selected by MF

(mean motility$_{MF}$ = 82.922%) was greatest, followed by DGC (mean motility$_{DGC}$ = 78.712%), and DG-SU (mean motility$_{DG-SU}$ = 78.432%).

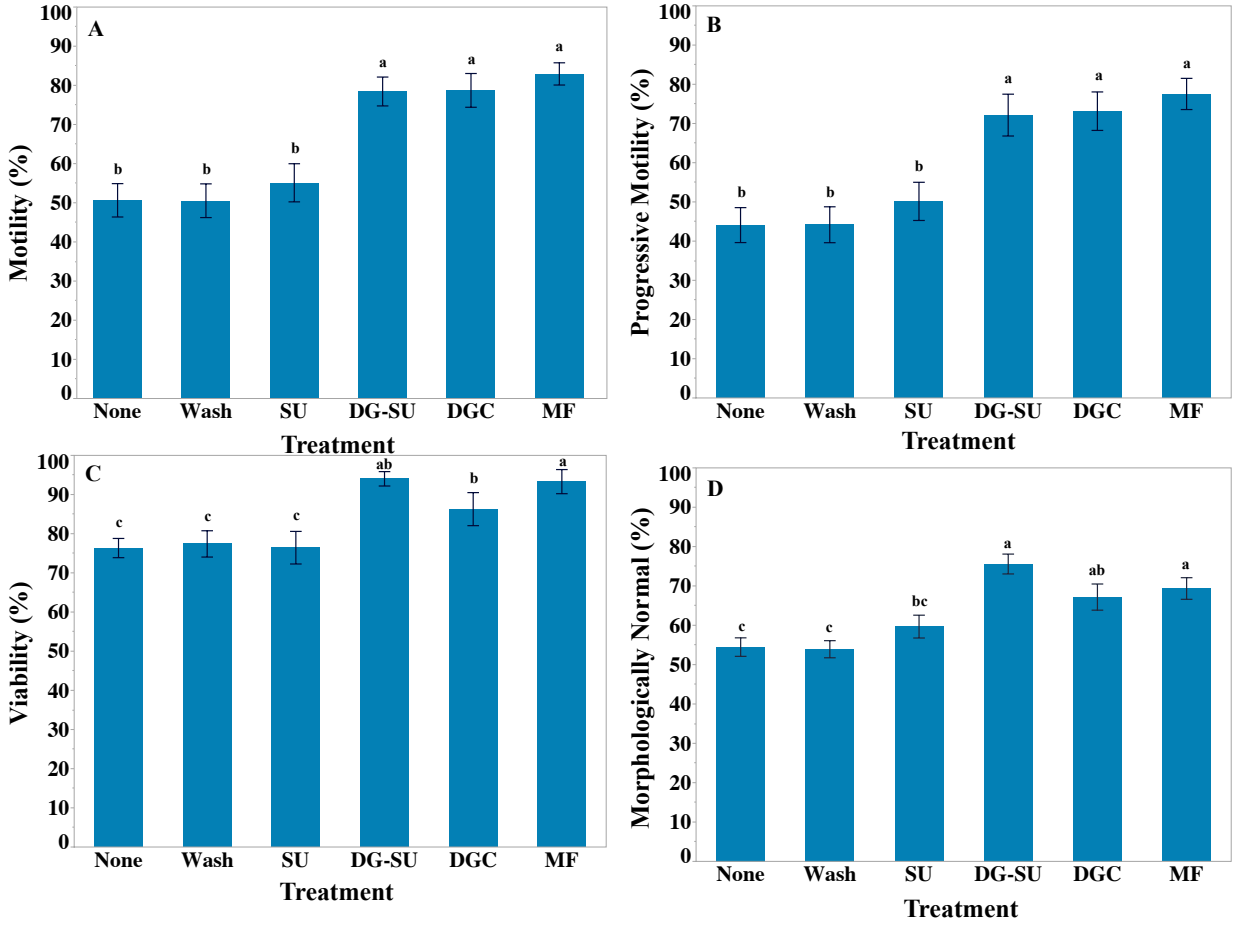

Figure 5. Average sperm quality measurements by sperm selection technique. Motility (**A**), progressive motility (**B**), viability (**C**), and morphology (**D**) are given for unprocessed sperm (none) and sperm processed by washing, SU, DG-SU, DGC, and MF. Superscripts (*a*, *b*, *c*) denote statistical significance between groups ($P < 0.05$).

Similarly, analysis of progressive motility showed that the unprocessed, washed, and SU groups demonstrated significantly lower percentages of progressively motile

populations than MF ($P < 0.0001$; $P < 0.0001$; $P < 0.0005$), DGC ($P < 0.0001$; $P < 0.0001$; $P < 0.0005$), and DG-SU ($P < 0.0005$; $P < 0.0005$; $P < 0.005$). Numerically, MF (mean progressive $motility_{MF} = 77.532\%$) selected the highest average progressive motility, followed by DGC (mean progressive $motility_{DGC} = 73.141\%$) and DG-SU (mean progressive $motility_{DG\text{-}SU} = 72.142\%$).

Viability analysis also showed that unprocessed, washed, and SU groups had significantly lower population viability than MF ($P < 0.0001$; $P < 0.0001$; $P < 0.0001$), DGC ($P < 0.0005$; $P < 0.001$; $P < 0.005$), and DG-SU groups ($P < 0.0001$; $P < 0.0001$; $P < 0.0001$). MF also selected for a more viable population than DGC ($P < 0.05$). The average viability of the DG-SU group was the highest (mean $viability_{DG\text{-}SU} = 94.017\%$), followed by MF (mean $viability_{MF} = 93.298\%$) and DGC (mean $viability_{DGC} = 86.274\%$).

In selecting for morphologically normal sperm, DG-SU ($P < 0.0001$; $P < 0.0001$), MF ($P < 0.0005$; $P < 0.0005$), and DGC ($P < 0.005$; $P < 0.001$) selected for a significantly higher percentage of morphologically normal sperm than unprocessed and washed groups. Morphology of sperm selected by SU was significantly lower than DG-SU ($P < 0.0005$) and MF ($P < 0.05$) groups. Numerically, DG-SU selected for the highest percentage of morphologically normal sperm (mean normal $morphology_{DG\text{-}SU} = 75.571\%$), followed by MF (mean normal $morphology_{MF} = 69.348\%$) and DGC (mean normal $morphology_{DGC} = 67.167\%$).

3. Discussion

Results from the current study reinforce 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 that possessed numerically 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. 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 [20, 21] as well as increasing the number of apoptotic like changes [22]. 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 [9, 23-27]. 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 [28-30]. 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. 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-minute 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 [31, 32].

4. Conclusions

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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Chapter 4

Characterization of High-Quality Stallion Spermatozoa Using Cell Membrane Charge*

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Abstract: 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. 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 (ZPfresh = -11.91 ± 2.13, mean ± s.d.; ZPcooled = -12.20 ± 1.73) and also correlated with motility (ϱ = -0.27, P < 0.05 and ϱ = -0.26; P < 0.05, for fresh and cooled samples, respectively) and progressive motility (ϱ = -0.25, P < 0.05 and ϱ = -0.29; P < 0.05, for fresh and cooled samples, respectively). The ZP of cooled samples was also correlated with viability (ϱ = -0.31; P < 0.05). 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 human IVF labs, ZP-based selection techniques have already been developed and implemented. Therefore, to improve sperm selection for equine IVP, future studies should focus on the adaptation and development of novel methods of sperm selection based on ZP. In conclusion, we identified an average net negative ZP on equine sperm and correlations between ZP and other measures of sperm quality.

Keywords: stallion; fertility; sperm; assisted reproductive techniques;

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1. Introduction

Zeta Potential (ZP), or electrophoretic mobility, is an estimation of cellular surface charge [1]. 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 [Figure 6.A]), that is proportional to cell surface-charge density [1-4]. 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 (Figure 6.A) [1, 5-8]. 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 (Figure 6.B). 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 [5, 6, 8-12]. 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 [13-15].

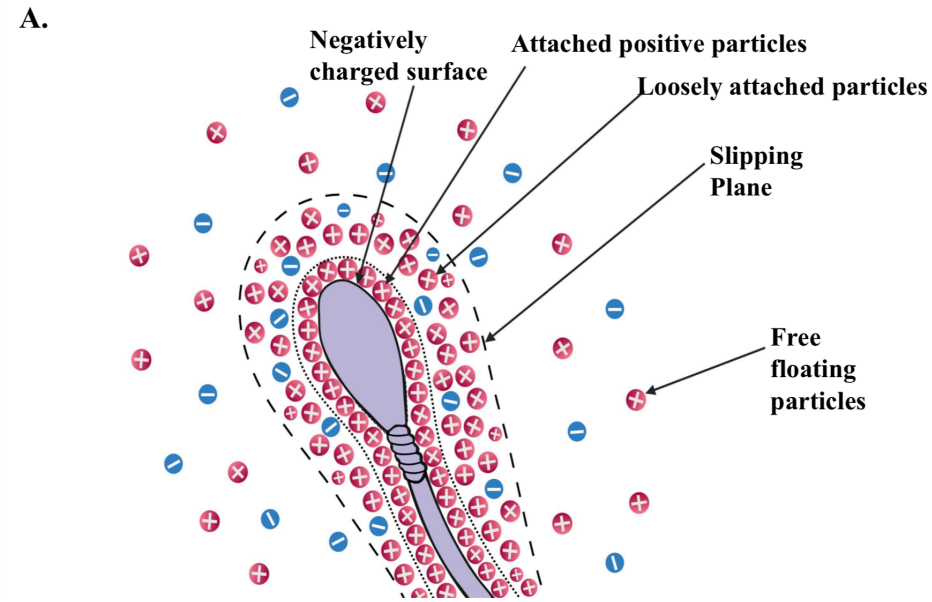
In humans, a greater net negative ZP, largely attributed to the composition of the glycocalyx, has been correlated with increased percentages of morphologically normal sperm as well as those possessing high DNA integrity [1, 5, 16]. Additionally, the selection of sperm by conventional methods (selecting for motility and viability) also selected for a more negative population [17]. Based on these observations, multiple techniques to select sperm based upon ZP have been developed and implemented in human clinics [3, 5, 18-21]. However, no studies on equine sperm ZP or its potential applications to sperm selection have been published to date.

In this study, 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.

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.



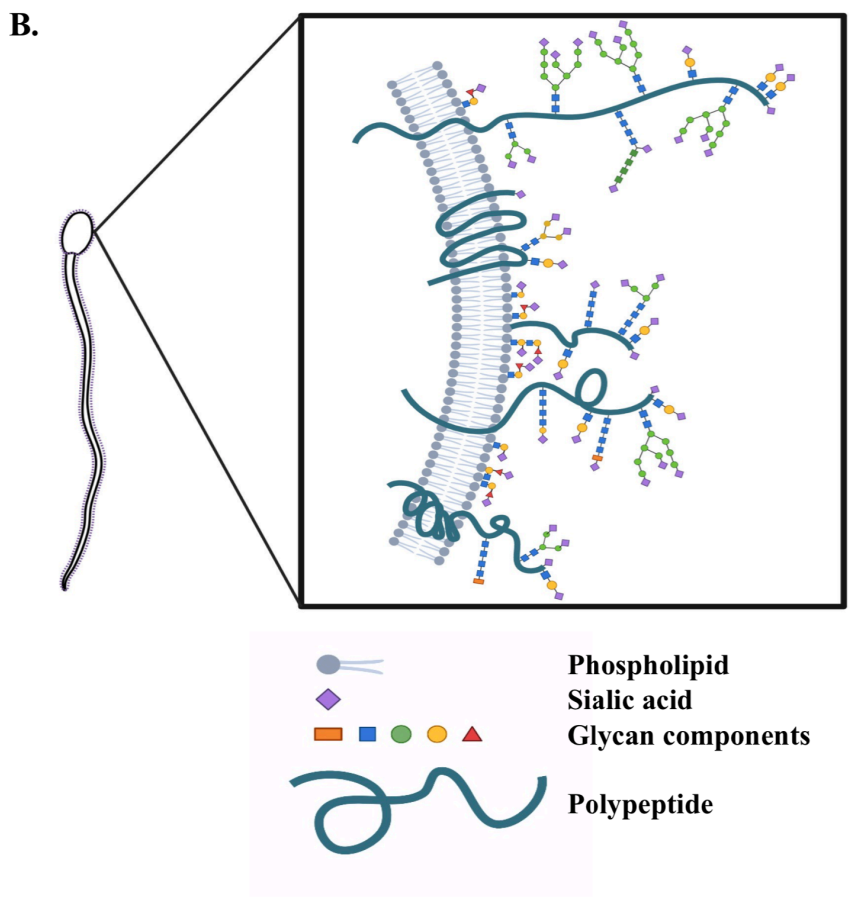


Figure 6. Schematic diagram of Zeta Potential (ZP). **A.** Diagram of the slipping plane of a sperm cell, the point at which fluid particles are not bound, but free-floating around the cell, and where cell surface charge can be estimated as ZP. Positively charged particles are bound to the negatively 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.

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 [22]. 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 4ºC for a period of 24 hours prior to analysis and processing [23]. 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 [24]. A small fraction of the sample was diluted into a lysis reagent (Reagent S100 Lysis Buffer, ChemoMetec) to

measure total concentration and into PBS (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) (CASA, HTM CEROS, Version 12.2 g; Hamilton Thorne Biosciences, Beverly, MA, USA) and Sperm Vision® SAR software (Minitübe, Tiefenbach, Germany) [25]. For CASA motility analysis, 3 μL of semen 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; minimum 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 microscopy under 100X magnification. 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 [26, 27].

Lastly, zeta potential (ZP) was measured as described by Ionov et al. [17] 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) [DPBS (1X), Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA]. Each individual sample was run through the zeta potential analyzer, and the total run time included a 2-minute 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) [28], Density Gradient Centrifugation (DGC) [29], Density Gradient-Swim Up Combination DG-SU [30], or Microfluidics (MF) [31] 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 32-175 M/mL, with an average of 142.45 M/mL.

Wash: Semen diluted in extender was centrifuged for 8 minutes at 300 x 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 minutes 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 minutes at 300 x 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 minutes at 300 x 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, 850µL, 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 minutes 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.

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 -

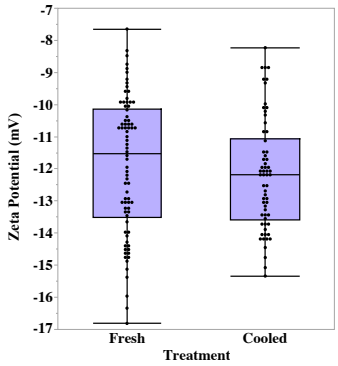
11.91 ± 2.13 (mean ± s.d.; −16.81 to −7.65 mV, min-max) and the combined average of cooled samples was -12.20 ± 1.73 (mean ± s.d.; −15.34 to −8.23 mV, min-max) (Figure 7.A).

3.2. ZP correlations with sperm quality

The associations between ZP measurements for each sample and other known measures of quality (motility, progressive motility, viability, and normal morphology) were assessed (Figure 7.B). 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 correlated with both motility ($\varrho = -0.27$; $P < 0.05$), and progressive motility ($\varrho = -0.25$; $P < 0.05$). ZP of fresh sperm was not correlated with normal morphology or viability ($P > 0.05$).

In cooled sperm, ZP was significantly correlated with motility ($\varrho = -0.26$; $P < 0.05$), progressive motility ($\varrho = -0.29$; $P < 0.05$), and viability ($\varrho = -0.31$; $P < 0.05$). ZP of cooled samples was not correlated with normal morphology ($P > 0.05$).

A.



B.

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

Figure 7. 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 $P < 0.0001$.

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 [1, 17, 32]. 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. This 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 [17]. 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 [17]. The authors also showed that ZP was correlated with viability ($P < 0.05$) and morphology ($P < 0.05$) [17], unlike our data in which viability was only correlated with ZP in cooled sperm and no correlation was observed between morphology and ZP. 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 [33]. 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.

Research in human IVF has focused on the development of a novel method of high quality sperm selection for IVP based on ZP [18-20]. 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 [3]. This method is used immediately following DGC and the combination of techniques will hereafter be referenced in total as the ZP selection method [3]. 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 [3]. Total motility was not significantly increased and total recovery was just under 9% [3]. 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 [19, 20]. 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 [19]. We did not perform Annexin-V staining in the current study. Additionally, ZP selection prior to ICSI significantly increases fertilization rates (52.39% to 65.79%, $P < 0.05$) and numerically improves pregnancy rates ($P > 0.05$) over ICSI following DGC sperm selection [18]. 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 [2]. 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 [1, 21, 34, 35].

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 [5, 6]. Glycoconjugate β-Defensin DEFB126 (Defensin Beta 126) 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 [36, 37]. DEFB126 is highly expressed during epididymal maturation and heavily coats the sperm until capacitation [38]. Furthermore, the removal of DEFB126 from the glycocalyx is integral to tight sperm-zona binding [36], 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 [6, 38-40]. 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 [41, 42]. 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 [5, 6, 9-12]. The association between the survival rate of sperm and electrical charge could be an explanation for our finding that sperm assayed after a 24-hour 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.

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.

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Chapter 5

Extraction and Confirmation of Equine Sperm RNAs

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Abstract: Efficient extraction of sperm RNAs could have profound impacts on our understanding of sperm function and male fertility. Few studies have successfully extracted equine sperm RNAs and no studies to date have optimized the extraction protocol in order to establish relationships between stallion fertility and the sperm transcriptome. In this study, we aimed to compare two known methods of RNA extraction for stallion sperm, including a Qiazol phase separation-based protocol and a column-based method (Qiazol method) with a TCEP-supplemented lysis reagent (TCEP method). Results show that both methods are capable of extracting sperm RNA through confirmation by RT-qPCR, and that the TCEP method yields an increased concentration over the Qiazol method. By optimizing equine sperm RNA extraction, we will also improve downstream RNA characterization methods such as PCR, microarray, and RNA sequencing.

Keywords: Stallion, sperm RNA, RNA extraction

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1. Introduction

The sperm cell is notorious for its highly condensed genome that functions to conserve genetic integrity as the sperm cell traverses the female tract and fertilizes an oocyte. Unlike somatic cell DNA histone methylation, in which loosely bound histone proteins wind and unwind to regulate gene specific transcription, sperm utilize protamines as

their primary nuclear binding protein to inactivate and protect its genetic material. Due to the strict binding of the protamine, along with the sperm cell's decreased rate of development post spermatogenesis, it has been assumed that mature sperm are transcriptionally silent. Regardless of the transcription status, various RNAs have been shown to exist using RT-PCR and *in situ* hybridization, both within the sperm of plants, mice, rats, humans, and extracellularly within the seminal plasma [1-7]. Through further sequencing and microarray analyses, RNA profiling in humans has led to the identification of multiple diagnostically relevant transcripts that are now used as a viable method of clinical fertility assessment [8, 9]. Although the origin and function of sperm RNAs are highly debated, it is inarguable that the identification and characterization of sperm RNAs may lead to the discovery and understanding of previously unknown mechanisms of male fertility and embryo development that could be crucial to the equine breeding industry.

The presence of RNAs in human sperm, as well as their expression patterns, differ between sperm in pre- and post-meiotic periods, sperm of varying motilities, before and after capacitation, and before and after cryopreservation [10, 11]. During testicular maturation, mouse spermatogonia contain both large and small RNA types. As demonstrated in the mouse, small non-coding RNAs (sncRNAs) include primarily PIWI-interacting RNAs (piRNAs), as well as smaller proportions of micro RNAs (miRNAs) and tRNA-derived small RNAs (tsRNAs). These small RNAs can be found in the sperm head

as part of RNA-DNA bound complexes as well as in the sperm tail [12-14]. As spermatids complete spermatogenesis and become spermatozoa to be matured in the epididymis, there is a reduction in the proportion of piRNAs and an increase in tsRNAs [9, 15]. These small RNAs are thought to potentially play a role in early embryo development, as they are potentially delivered with the sperm nuclear contents. Larger RNA transcripts found within sperm include fragmented rRNA, long non-coding RNAs (lncRNAs), and intact mRNAs that have regulatory potential [9]. These long RNAs are generally embedded into the membrane of the sperm cell, and it has been shown that membrane-removing detergent treatment results in a 66% loss of long RNA products [16].

Due to the generally accepted transcriptional cessation in the nucleus of a mature sperm, other theories for transcript origin include the retention of RNAs from spermatogenesis or from the mitochondria of the mature sperm [17, 18]. Specifically, early spermatids are known to transcribe gene products for sperm Protamine 1 and 2 (PRM1 and PRM2) [7], and thus transcriptional products identified in mature sperm could be retained from transcriptional activity during spermatogenesis. Additionally, mitochondria may retain transcriptional capabilities contributing to the small RNA population in the sperm body [18].

If not retained or transcribed, sperm RNAs could also be acquired during post-spermatogenesis maturation. For example, epididymosomes excreted during epididymal maturation are believed to carry small RNAs that can influence both sperm maturation

as well as embryo development. These vesicles fuse with the sperm and transfer their RNA products [19, 20]. Extracellular vesicles produced by the accessory sex glands, and added to seminal fluid at the time of ejaculation, may also contribute to total sperm RNA.

The purpose of RNAs in sperm are also speculative. RNAs extracted from a pool of multiple human ejaculates, purified of somatic cells, contained subsets of mRNAs with known functions in embryo development [21]. Sperm RNAs have also been implicated as a form of epigenetic inheritance in mice. In a study by Gapp et al. (2014), the injection of total RNA from sperm of experimentally stressed males caused significant behavioral modifications of offspring; suggesting that sperm carry important paternal RNAs that dictate offspring development [22]. Similarly, dietary changes or restriction can similarly affect the transcriptome of a mature sperm [19].

Although sperm RNAs have been studied in humans and model species, there is a startling lack of peer-reviewed research regarding the equine sperm transcriptome. The most influential study from the field is by Das et al. (2013), in which an unmodified extraction kit was used prior to PCR, microarray, and RNA-sequencing (RNAseq) [23]. This study revealed many sperm-specific transcripts as well as differentially expressed genes between sperm and testis. However, no direct relation to sperm fertility was made and it is unclear if the extraction method they used was best suited for breaking apart the lipid saturated membrane of sperm to access and report all transcriptional products.

In this study, we will directly compare two methods of RNA extraction previously reported for sperm. Firstly, we will utilize a Qiagen RNeasy extraction kit supplemeted with tris(2-carboxyethyl)phosphine (TCEP) [24] to further lyse the sperm plasma membrane, which will be referenced as the TCEP method. Secondly, we will utilize a phase separation, hereto referenced as the Qiazol method (Balasubramaniam, K.).

By comparing RNA extraction methods, we aim to identify the optimal method to extract a high RNA yield and quality from equine sperm. Subsequently, we will identify the method suited for downstream analysis such as RNAseq. Overall, the optimization of stallion sperm RNA extraction would allow us to use transcriptomics as a significant biomarker in stallion fertility as well as optimizing our characterization of sperm for assisted reproductive technology.

2. Methodology

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 medium 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=2) were collected using a phantom mount and a Missouri artificial vagina, as previously described [25]. Gel fractions were removed using a mesh filter (Disposable Nylon Mesh Semen Gel Filters, ARS Inc, Ontario, CA, USA), and the remaining gel free fraction was extended 1:1 with a commercial equine semen extender (INRA96®, IMV Technologies, L'Aigle, France).

2.3. Sperm Processing:

Sperm were processed in one of two ways to remove somatic cells from the ejaculate prior to RNA extraction.

2.3.1. Method I: Density Gradient Prior to RNeasy Column Extraction:

Firstly, to remove somatic cells, this method utilized density gradient centrifugation (DGC), in which 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 (300 ± 10 mOsm/kg). Unprocessed semen was then carefully deposited above the upper gradient using a serological pipette. The tube was centrifuged for 30 minutes at 300 x g. The resulting pellet was removed using a fine-tip aspiration pipet, resuspended in 1 mL of BWW, and homogenized by gently flicking the tube. The sperm concentration in the final homogenized solution was determined using the NucleoCounter SP-100 (ChemoMetec Allerød, Denmark), as previously described [26]. Sperm were then diluted in BWW to make individual aliquots of 80 million cells (M)/mL, 60 M/mL, 40 M/mL, and 20 M/mL that were used for downstream RNA extraction.

2.3.2. Method II: Triplicate Wash Prior to Qiazol Phase Separation:

This method included washing the sperm by first pelleting the sperm at 2934 x g (4000 rpm) for 5 minutes. The supernatant was then removed and then sperm were resuspended in 2 mL of DPBS [DPBS (1X), Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA]. The sperm were then pelleted again using 300 x g over 5 minutes, suspended in 2 mL of DPBS, and then re-pelleted at 300 x g for 5 minutes. The final pellet was again suspended in 2 mL of DPBS and homogenized. Sperm concentration was measured and sperm were diluted into a concentration gradient as described above.

2.4. RNA Extraction and Sequencing:

2.4.1. Method I: TCEP supplemented RNeasy Column Extraction:

Each sample of a different sperm concentration was processed using a 0.5 mL volume. Sperm were added to an Omni Tube (Omni, Kennesaw, GA, USA) containing 1 mL of QIAzol Lysis Reagent (QIAzol® Lysis Reagent, Qiagen, Hilden, Germany) supplemented with 0.1 M tris carboxylethatris(2-carboxyethyl)phosphine (TCEP) (Millipore Sigma, Burlington, MA, USA). Samples were then homogenized using an Omni Bead Ruptor 4 (Omni) at a speed of 5 for 30 seconds. Homogenization was performed three times with a 2 minute incubation interval between cycles to allow for cooling. Samples were then incubated at room temperature for five minutes, and fluid was transferred to an RNase-free 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). 140 μL of chloroform (Sigma-Aldrich, Saint Luis, MO, USA) was added and the sample was vortexed and incubated again for three minutes before a 15 minute centrifugation at 12000 x g at 4°C.

The aqueous upper phase was then transferred to a sterile 1.5 mL tube and one volume of 70% ethanol (Sigma-Aldrich) was added and mixed. The volume was then added to the inner spin column of an RNeasy extraction kit (Qiagen) and the remaining steps followed manufacturer instructions. The final extracted volume was 35 μL.

2.4.2. Method II: Qiazol Phase Separation:

The following protocol is adapted from a standard phenol-chloroform extraction, with some modifications, for the extraction of total equine sperm RNAs. After washing

and dilution steps, samples were homogenized using Omni tubes as described above. Following a five-minute incubation at room temperature, samples were removed from the Omni tube and placed in an RNase-free 1.5 mL microcentrifuge tube (Eppendorf). Tubes were then centrifuged at 12,000 x g at 4°C for 10 minutes. Samples were then vortexed, and 0.2 mL of chloroform was added before an additional vortex. Samples were incubated for 5 minutes at room temperature and then centrifuged again at 4°C at 12,000 x g for 15 min. The aqueous phase was then transferred to a new 1.5 mL RNase free tube and 0.5 mL of isopropyl alcohol was added and incubated at room temperature for 5 minutes. Following incubation, samples were centrifuged at 12,000 x g at 4°C for 10 minutes. Supernatant was discarded and 1 mL of 75% ethanol was added before a final centrifugation at 12,000 x g for 5min at 4°C. Supernatant was discarded. Samples were air-dried over low heat until and 50µl of RNase -free water was added. An aliquot of all samples was taken for assessment using a Tapestation 4200 (Agilent Technologies, Santa Clara, CA, USA) and the remainder was stored at -80°C.

2.5. Tapestation quality analysis

Initial quality of RNA was assessed using Tapestation 4200 software (Agilent Technologies) to visualize electropherogram peaks, estimate concentration and determine RIN^e values as per manufacturer instructions.

2.6. Reverse Transcription and quantitative PCR

For production of cDNA, 10 μL of RNA was used with a TaqMan™ Reverse Transcription Reagent kit (Invitrogen, Waltham, MA, USA) and reversed transcribed as per manufacturer instructions. Reverse transcription products were then used for qPCR analysis of PRM1, ACTB, and GAPDH, with 1 uL of cDNA being diluted in 4 μL of ddH_2O and combined in a 96 well plate with 6.25 ug of SYBR Green Master Mix (Applied Biosystems, Bedford, MA, USA), 0.75 μL of ddH_2O, 0.25 μL of forward primer and, 0.25 μL of reverse primer. Forward and reverse primers for PRM1 (Forward – 5'GAGGTAAAAAGGGTGGAGCGG'3; Reverse – 5'CTTCTCCTACACCTCAGGACA'3), ACTB (Forward – 5'CGACATCCGTAAGGACCTGT'3; Reverse – 5'CAGGGCTGTGATCTCCTTCT'3) and GAPDH (Forward – 5'AGAAGGAGAAAGGCCCTCAG'3; Reverse – 5'GGAAACTGTGGAGGTCAGGA'3) were used (Thermo Fisher). Total reaction volume was 25 μL per well. After loading, plates were spun at 500 x g for two minutes. Samples were analyzed in duplicate on a QuantStudio3 PCR Machine (Applied Biosystems) using the following conditions: a 10-minute hold at 95°C, and 40-45 cycles of 15 seconds at 95°C and 1 minute at 60°C. The melting curve was added and finally samples were held at 4°C. A PCR reaction with the same conditions was run with both unprocessed RNA (serving as a control for the residue of gDNA) and reverse transcribed cDNA of the same sample to screen for DNA contamination.

All data is reported as the negative ΔCT relative to the average of GAPDH and ACTB controls, with testis cDNA as a positive control [27].

3. Results

3.1. RNA Concentration and Quality

TapeStation 4200 data was used to determine sample concentration and quality. No RIN^e values were available as RNA profiles were missing required marker peaks. Estimated concentrations of sample RNAs are reported in Table 2.

Table 2. TapeStation 4200 reported concentrations and RIN^e values from TCEP and Qiazol extraction methods. Values are represented as either an individual concentration or a mean ± standard deviation if the extraction was done in duplicate (n = 2). An RIN^e value was only obtained for a single replicate of the 40 M/mL concentration, and is thus representing a single value rather than a mean.

Sample	TCEP – Estimated Concentration (pg/μL)	Qiazol – Estimated Concentration (pg/μL)	RIN^e
20 M/mL	232 ± 28.3	43.3	-
40 M/mL	328 ± 188.1	55.4	2.5
60 M/mL	213 ± 72.12	61.7	-
80 M/mL	193	82.3	-
343 M/mL	-	39.5	-

From the TCEP extraction, the concentration was highest in the 20 M/mL sample, followed by 40 M/mL and 80 M/mL samples (Figure 8). The Qiazol extraction resulted in

an inverted trend in RNA concentration, with 80 M/mL being the most concentrated, followed by 60 M/mL and 40 M/mL. All samples extracted by the Qiazol method were reportedly less concentrated than the same concentrations extracted by the TCEP method.

3.2. Confirmation of Sperm RNAs using RT-PCR

Following RT, qPCR was run on cDNA samples in order to determine relative expression of sperm RNA. PRM1 was detected in both TCEP and Qiazol cDNA samples. TCEP-cDNA reported low expression levels at all cell concentrations, while the Qiazol method only reported PRM1 cDNA at the highest sperm cell concentration. No PRM1 DNA was detected in RNA samples extracted by the TCEP method, however, low expression of PRM1 was detected in the RNA from the highest sperm concentration using the Qiazol method, indicative of DNA contamination (Figure 9).

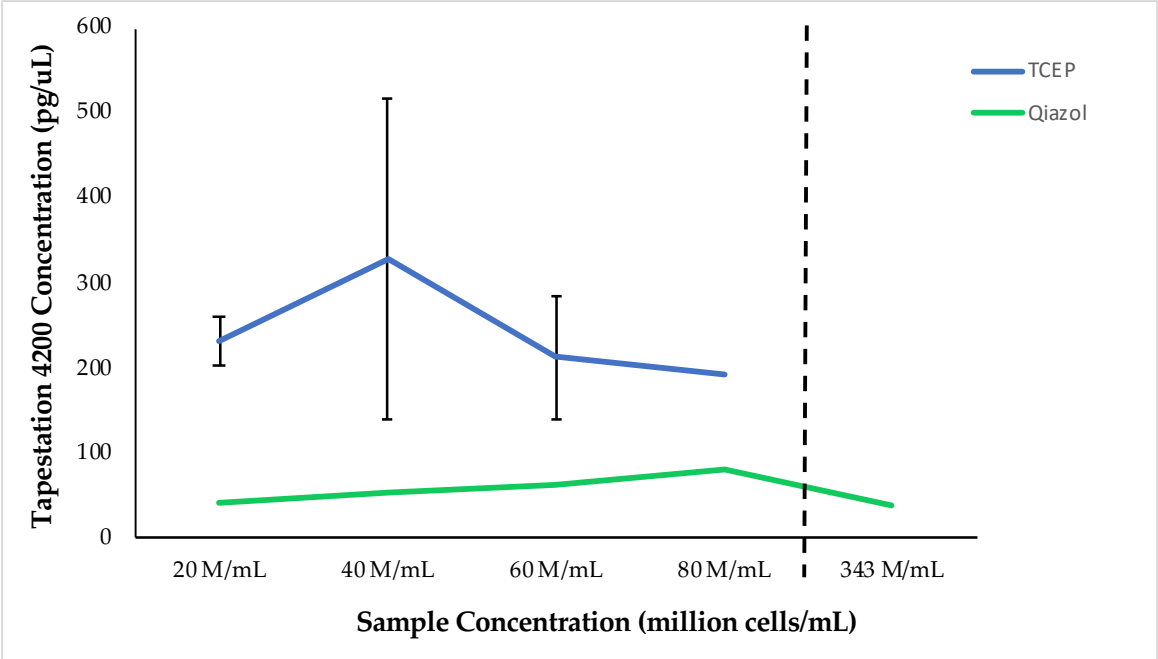

Figure 8. Visual representation of RNA concentrations (pg/μL) at each sample concentration. Blue represents samples extracted using the TCEP method, and green represents the samples extracted using the Qiazol method. Inverse trends are observed from samples using TCEP and Qiazol methods. Values are represented as either an individual concentration or a mean ± standard deviation if the extraction was done in duplicate (n = 2).

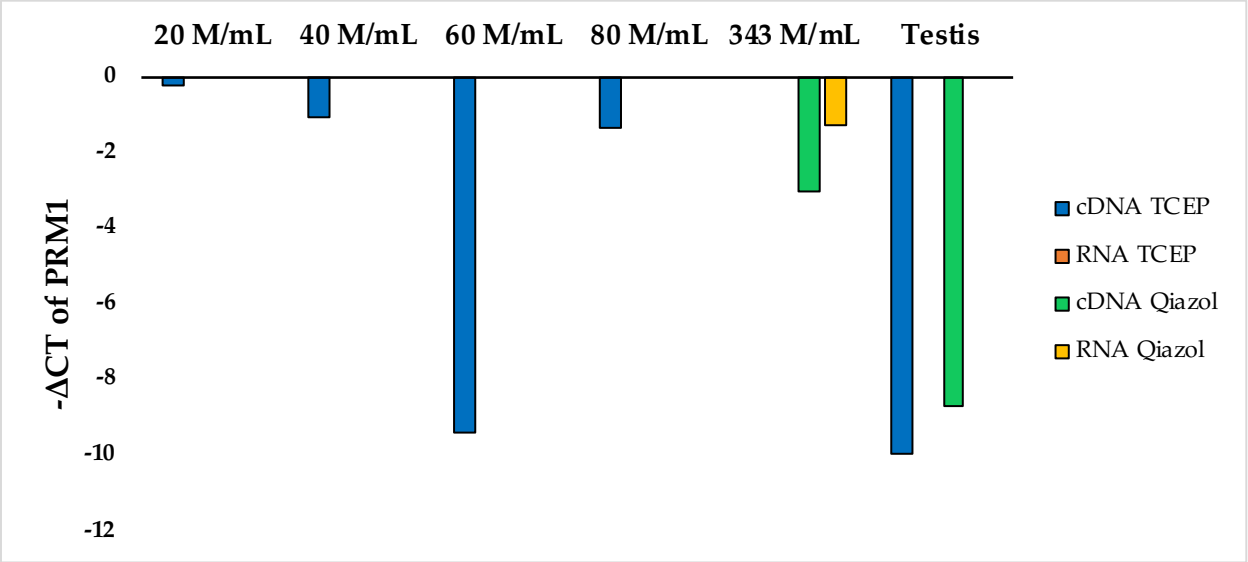

Figure 9. Relative expression of PRM1 from qPCR in sperm samples and testis sample using -ΔCT. Blue represents samples extracted and reverse transcribed using the TCEP method, and green represents the samples extracted and reverse transcribed using the Qiazol method. Orange represents an RNA sample that was extracted using the TCEP method, and yellow represents an RNA sample that was extracted using the Qiazol method, neither of which were reverse transcribed. Testis cDNA was run alongside sample cDNA of each extraction method as a control. If no bar is visible, no expression was recorded.

4. Discussion

In this study, we have provided preliminary comparisons of RNA yield and quality from known RNA extraction methodologies. Through the positive identification of sperm specific PRM1 in both Qiazol and TCEP extraction methods, we have also successfully validated the presence of sperm specific transcripts as seen in previous studies.

Surprisingly, the concentration of RNA from the TCEP extraction was higher than that of the Qiazol extraction, despite the presence of a column membrane which could theoretically be clogged by lipids of the sperm plasma membranes. However, TCEP is known to reduce disulfide bonds such as those within the sperm plasma membrane, potentially indicating a superior lysis of the sperm cell as opposed to lysis in Qiazol not supplemented with TCEP. The final eluted volume of the Qiazol extraction was also almost double the volume eluted from the TCEP extraction, which likely contributed to the discrepancies in RNA concentration.

Of note, several previous methodologies used in other species have failed to perform DNase treatments or to confirm RNA profiles were not from gDNA, which may lead to the false identification of sperm RNAs. Here, we tested the presence of gDNA by analyzing the gene expression of PRM1 in total RNA samples (without reverse transcription) through PCR to confirm that the extracted RNA was not contaminated with gDNA. In comparing the TCEP and Qiazol methods, the TCEP method yielded low expression of PRM1 in all cDNA samples without identified expression in RNA samples,

indicating sample purity. In contrast, the Qiazol method failed to produce expression profiles for most sperm concentrations, and PRM1 expression seen in the Qiazol RNA sample of sperm concentration 343 M/mL may indicate the presence of gDNA in the cDNA sample of the same concentration. This invalidates low expression of PRM1 seen in Qiazol-extracted cDNA. Thus, the purity of RNA extracted by the TCEP method is greater than RNA extracted by the Qiazol method. Our preliminary results highlight the importance of DNase treatments in sperm RNA extractions.

Despite the use of a TapeStation high sensitivity RNA kit, our reported concentrations for both extraction methods were still below the desired minimum RNA concentration for bioanalysis, which may have contributed to poor expression profiles from both methods. As previously mentioned, sperm are thought to be largely transcriptionally silent, as well as physiologically streamlined due to the extrusion of the cytoplasmic droplet that normally contains many RNA products. Without addressing the origin of sperm RNAs, the morphological changes to the sperm imply that the majority of large, or intact, RNAs would be extruded with the cytoplasm and less detectable than they might be in somatic cells [28]. Additionally, sperm are known to carry many small RNA products including piRNAs, miRNAs and tsRNAs. An abundance of sncRNAs may lead to poor or absent RIN^e values, as seen here, and lower reported concentrations. As described previously, the sperm transcriptome is depleted of intact RNAs, and thus it is normal to see insufficient quality data [23].

Poor yield may cause issues with methods downstream of RNA extraction such as PCR or RNAseq. RNAseq utilizes high throughput transcriptomic technologies to identify and quantify all RNA products within a sample. Despite the wide applications of this technology, the minimum yield of sperm RNAs may lead to difficulties in subsequent sequencing. However, in a study by Das et al. (2013), sperm RNA was extracted using the RNeasy mini elute kit (Qiagen) with Trizol. This study also employed a DNase treatment and intron spanning primers to confirm a lack of genomic DNA prior to qRT-PCR, microarray assessment, and RNA-sequencing [23]. Somatic cell contamination was also not detected through the use of a somatic cell specific primer. Through microarray, the authors discovered novel sperm transcripts including 82 miRNAs. RNA-seq yielded the identification of 60 sperm up-regulated and 165 sperm-enriched transcriptional products in comparison to testis [23].

Although the identification of specific sperm transcripts holds immense implications towards mechanisms of sperm function and fertility, further research in equine sperm RNAs is warranted. Through our optimization of sperm RNA extraction methods, we aim to provide more details to labs attempting extraction for highly sensitive characterization methods like RNAseq. Future experiments that include RNAseq could compare expression profiles in sperm from high and low fertility samples, thus utilizing RNA expression as a diagnostic tool for utilization in horses. In humans, sperm RNA profiling has led to the discovery of transcripts that are now used as a viable method of

clinical fertility assessment [8, 9]. This includes the identification of transcripts specific to asthenozoospermia, teratozoospermia, oligozoospermia, and idiopathic infertility [29-32]. Associations have also been made with individual sperm parameters such as motility and cell viability [10, 11]. Thus, not only could RNA expression be matched to individual breeding statistics for individual stallions, but eventually it may be possible to compare expression profiles of individual ejaculates to better select equine assisted reproductive techniques.

5. Conclusion

In conclusion, RNA extraction, analysis, and expression profiling hold significant diagnostic value to equine breeders, veterinarians, and owners. By optimizing RNA extraction, we are moving closer to the application of RNA analysis to equine reproduction, which could provide significant insight into sperm and stallion fertility.

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Chapter 6

Final Conclusions

Understanding normal equine sperm parameters is inevitably useful in both the characterization of stallion fertility as well as when selecting sperm for *in vitro* embryo production, or intracytoplasmic sperm injection (ICSI). Despite ample evidence that conventional selection methods are able to select sperm capable of fertilizing an oocyte and producing a live foal, there is room for improvement in fertilization, pregnancy, and foaling rates. In this thesis, conventional and novel sperm selection techniques were discussed and compared, and microfluidics (MF) was introduced as a highly efficacious method of sperm selection for ICSI. In addition, the characterization of a net negative zeta potential (ZP) on equine sperm validates that ZP is a conserved physiological trait among species and can be used in future studies as a means of fertility assessment. Additionally, the adaptation of ZP-based selection techniques to stallions could provide breeders with an easily accessible selection method that also enriches the population of highly fertile sperm. This thesis also investigated methodologies of extracting equine sperm RNAs and represents the first comparison of RNA extraction methods used in stallions, to our knowledge. By optimizing RNA extraction methods, it will be possible to characterize transcriptomic differences between high and low quality spermatozoa and to gain insight

into potential mechanisms of stallion fertility. Overall, this collection of work serves to both strengthen our basic understanding of sperm physiology, as well as to address novel applications of sperm selection and characterization. Moving forward, the use of the novel MF method and the understanding of equine sperm ZP and transcriptomics could have pronounced effects on equine ICSI and the ultimate production of foals.