THESIS

EXTRACELLULAR VESICLES FROM THE EQUINE UTERUS: UPTAKE BY STALLION SPERMATOZOA AND EFFECT ON CAPACITATION PARAMETERS

Submitted by

Shelby K. Granier

Department of Clinical Sciences

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Master's Committee:

Advisor: Patrick McCue
Co-Advisor: James K. Graham

Jennifer Hatzel
Dawit Tesfaye

ABSTRACT

EXTRACELLULAR VESICLES FROM THE EQUINE UTERUS: UPTAKE BY STALLION SPERMATOZOA AND EFFECT ON CAPACITATION PARAMETERS

Fertilization in mammalian species relies on the activation of spermatozoa in the female reproductive tract by a consecutive series of events termed ‘capacitation’. *In vivo,* ejaculated equine spermatozoa are deposited directly into the uterus and eventually arrive in the ampulla of the oviduct, which is the site of fertilization. However, the roles of the uterus, oviduct, and their secretions have on equine sperm capacitation is largely unknown. Extracellular Vesicles (EVs), including microvesicles and exosomes, are membrane enclosed nanoparticles released from most cell types that carry cargos of biologically active molecules that can affect nearby or distant recipient cells. EVs have recently been identified as playing a role in reproductive functions including sperm capacitation. The aims of the present study were: 1) characterize EVs collected from the uterine lumen of mares in both the estrus and diestrus phases of their reproductive cycles; and 2) investigate the effect these uterine EVs have on stallion sperm function. Uterine fluid from 6 mares was collected during both estrus and diestrus using a low volume uterine lavage. EVs were isolated from the fluid by ultracentrifugation, and EV concentration determined by nano-tracker analysis. The concentration of EVs obtained from estrus fluids (EEV) was 235 ± 164.029 billion EVs/mL and tended to be higher ($p=0.07$) than those obtained in diestrus fluids (DEV) (83.67 ± 89.328 billion EVs/mL). The average size of EVs were similar ($p > 0.05$) with values of 148.633 ± 11.35 nm for

EEV and 146.183 ± 11.89 nm for DEV. Transmission electron microscopy delivered images of vesicles with characteristic cup-shaped morphology and size consistent with NTA results. Immunoblotting confirmed the particles contained exosome markers TSG-101 and CD-63, and were negative for cytochrome C, a mitochondrial organelle marker, indicating these vesicles were indeed EVs. To determine the effect EVs have on sperm, semen from 3 Quarter Horse stallions were cryopreserved, and EVs added to samples after thawing. In the first experiment, EVs or PBS void of EVs were fluorescently labeled and incubated with frozen-thawed stallion spermatozoa for one hour and uptake was evaluated by fluorescent microscopy. Fluorescence was observed only in sperm incubated with EVs, and a greater fluorescent intensity detected in EEV treated sperm. In a second experiment, spermatozoa from each stallion were co-cultured with EEV, DEV, and PBS void of EVs (control) for 90 minutes and sperm functions associated with capacitation, including hyperactivated motility, and acrosome reactions, were evaluated using a computer assisted semen analysis unit (CASA) and flow cytometry. The percentages of hyperactively motile sperm were higher ($p < 0.05$) for EEV treated sperm compared to control and DEV. In addition, the percentage of acrosome reacted sperm was higher ($p < 0.05$) for sperm treated with EEV and DEV when compared to control. In summary, these results confirm that: 1) EVs can be isolated from uterine fluid of mares, 2) uterine derived EVs can be taken up by stallion spermatozoa, and 3) uterine derived EVs have a biological effect on stallion spermatozoa function *in vitro*. Consequently, it is hypothesized that EVs from the mare reproductive tract will have similar biological effects on stallion sperm function *in vivo.*

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Capacitation

Freshly ejaculated mammalian spermatozoa cannot fertilize an oocyte, but require additional maturation within the female reproductive tract *in vivo* or under selected conditions *in vitro* [32]. These biochemical changes that enable sperm to bind to and penetrate the zona pellucida and subsequently fuse with the oocyte plasma membrane is termed “capacitation”. The process was first described by Chang [33] and Austin [34] in 1951, and the term coined by Austin a year later [35]. Despite research being conducted on capacitation for over half a century, much of the molecular basis of capacitation is still poorly understood, as are the roles that the uterus and oviduct play in sperm capacitation [36].

In most domestic species, capacitation and fertilization can be achieved *in vitro* using conditioned media [11]. The ability to capacitate sperm *in vitro* has allowed for detailed studies that form most of our current knowledge of the mechanisms involved [37]. To capacitate sperm *in vitro,* sperm are first washed by centrifugation to remove seminal plasma and incubated in a medium containing HCO_3^-, Ca^{2+} and albumin, which stimulates a series of events known as the capacitation cascade [38]. First cholesterol is removed from the sperm plasma membrane by a cholesterol acceptor, such as albumin [39]. The loss of cholesterol from the membrane increases the fluidity of the plasma membrane and allows the aggregation of lipid raft receptors, which make the sperm more permeable to ions such as bicarbonate and calcium [39]. The influx of

these molecules stimulates activation of second messenger systems, such as a form of soluble adenylyl cyclase (sAC) abundant in sperm, leading to the production of cAMP [40]. The production of cAMP results in the phosphorylation of tyrosine residues on sperm proteins and induces hyperactivated motility [40]. These events facilitate binding of the sperm plasma membrane with the outer acrosomal membrane, resulting in acrosomal exocytosis [41]. The “acrosome reaction” exposes the hydrolytic enzymes capable of locally dissolving the zona pellucida of the oocyte and is recognized as the final capacitation associated event [42].

However, *in vitro* fertilization (IVF) has historically been difficult to achieve with equine gametes despite attempts to induce capacitation with the addition of heparin [40], caffeine [44], and/or procaine [49] to *in vitro* maturation medias. In 2002, it was demonstrated that *in vitro* matured oocytes placed into the oviduct could be fertilized [43], while *in vivo* matured oocytes harvested from pre-ovulatory follicles could not be fertilized *in vitr*o [44], suggesting incomplete sperm capacitation was the root of the problem. In 2003, it was found that stallion spermatozoa consistently bind to the zona pellucida in IVF attempts, but fail to undergo the acrosome reaction necessary for oocyte penetration [40]. Finally, in 2022, successful in vitro fertilization in the horse was achieved by prolonged sperm pre-incubation with penicillamine, hypotaurine, and epinephrine prior to a 6-hour co-incubation with cumulus-oocyte complexes [52]. The prolonged struggle to effectively stimulate complete capacitation in equids highlights how little we know about equine sperm capacitation. Additional *in vivo* studies, and *in vitro* studies utilizing tissues and secretions derived from the female reproductive tract,

such as extracellular vesicles, may fill in the gaps of our knowledge and contribute to developing an effective method for equine *in vitro* fertilization.

It is commonly thought that capacitation needs to be inhibited in the uterus *in vivo* to preserve the viability of the spermatozoa before they attach to the oviductal epithelium, where they await the ovulated oocyte before completing capacitation and fertilization [48]. However, there are several studies which indicate that capacitation takes place in the uterus, at least to some extent, and is dependent on the stage of the estrous cycle [22, 27, 29-31, 33, 49]. Rabbit sperm undergo capacitation when inseminated into the estrous uterus *in vivo* or when incubated *in vitro* with fluids from estrous uteri, while capacitation appears to be inhibited when inseminated into the diestrus uterus [33, 49]. An equine *in vitro* study found that 31-35% of the viable spermatozoa recovered from the uteri of mares in estrus had undergone an acrosome reaction 6 hours post insemination, while only 5% of the sperm underwent an acrosome reaction 6 hours post insemination when inseminated into the uterus of mares 7 days after ovulation [22]. These findings indicate that a sub-population of spermatozoa capacitate in the mammalian uterus, but the mechanisms by which they do so are not clear. In recent years, extracellular vesicles derived from female reproductive fluids have been proposed as a mediator of capacitation.

Extracellular Vesicles

Extracellular vesicles (EVs) are bioactive nano-particles secreted by most cell types into the extracellular environment, where they can interact with recipient cells [1].

EVs have emerged as key mediators of cell-to-cell communication that regulate many physiological and pathological processes [2]. These nano-particles are produced by virtually all mammalian cell types and can differ markedly in size, composition, and biological function, depending on the cell of origin and physiological state of the cell [3]. EVs consist of a lipid bilayer membrane that encases an organelle-free cytosol and a cargo of biologically active molecules such receptors, proteins, lipids, and genetic materials (mRNA and miRNA) specific to the parent cell and it's physiological state [4]. Because their cargo and function relate to the parent cell, EVs are often named relative to their cell of origin. For example, EVs derived from the uterine epithelium may be called "uterosomes" and EVs derived from the oviductal epithelium may be called "oviductosomes" and so on.

Extracellular vesicles (EVs) can be characterized into three dominant classes: exosomes, microvesicles, and apoptotic bodies, which are generally distinguished by their size and biogenesis (Figure 1) [5]. All classes show specific structural and morphological characteristics on transmission electron microscopy such as a lipid bilayer, and a cup-shaped morphology produced by drying during the preparation for analysis [6]. Exosomes range from 30-120 nm in diameter and are released through the endocytic recycling pathway when multi-vesicular bodies fuse with the plasma membrane [7]. Exosome bio-markers include tetraspanin CD63, intramembrane proteins ALIX, TSG 101 and lipid raft flotillin-1, which can be detected by immunoblotting to confirm the presence of EVs in fluids processed for the vesicles [6]. Microvesicles range from 100-1000nm in diameter and bud directly from the cell plasma

membrane and are therefore composed of surface markers largely dependent on the cell of origin [7]. Finally, apoptotic bodies include 50-500 nm vesicles produced as a consequence of plasma membrane blebbing during programmed cell death [2, 8]. Each class of EVs can be uptaken by nearby or distant cells and cause changes in the recipient cells' biological function [9]. The nano-vesicles can be internalized by recipient cells through several pathways including direct membrane fusion, phagocytosis, clathrin-mediated endocytosis, lipid-raft mediated endocytosis, and macropinocytosis [4].

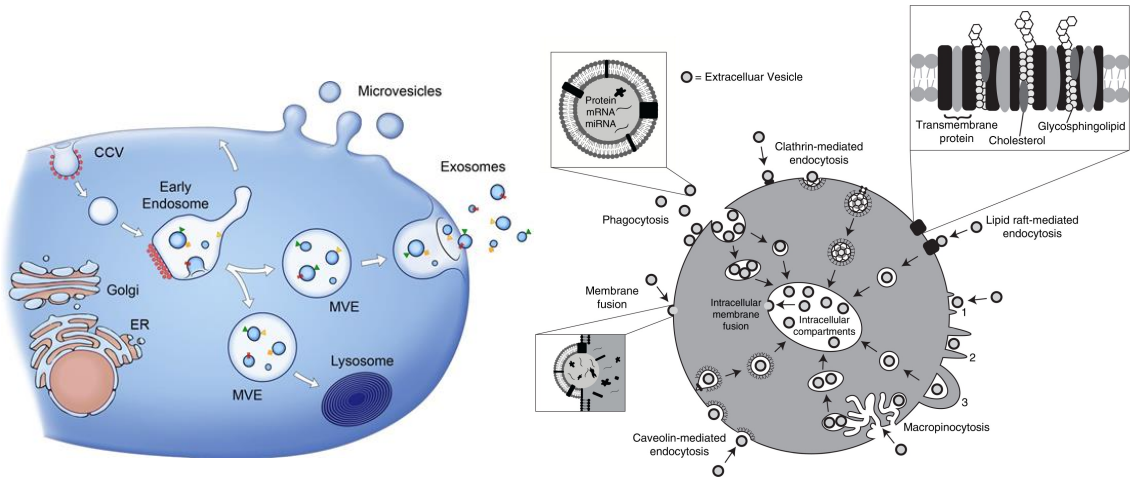


Figure 1: Proposed pathways of EV biogenesis by donor cells (left) [10] and pathways shown to participate in EV uptake by recipient cells (right) [4]. EVs originate from either the endosomal system or are shed from the plasma membrane of parent cells and tissues, and contain a cargo of biologically active molecules such as protein, lipids, mRNA, miRNA, receptors, and transporters [2]. The nano-particles have been shown to be internalized by recipient cells through several pathways including direct membrane fusion, phagocytosis, clathrin-mediated endocytosis, lipid-raft mediated endocytosis, and macropinocytosis [4].

Extracellular vesicle's role in reproduction

EVs present in the female reproductive tract have recently raised attention regarding their role in the fertility process and their potential to improve outcomes in

assisted reproductive technologies (ART) [9]. Upon natural mating, spermatozoa may encounter EV populations of vaginal (vaginosomes), uterine (uterosomes) and oviductal (oviductosomes) origin as they journey through the female reproductive tract to the site of fertilization (Figure 2) [9]. EV populations from all sections of the female reproductive tract have been shown to influence biochemical and physiological changes consistent with capacitation in several species [5]. Oviductosomes and their impact on sperm function have been studied most extensively due to the oviduct being recognized as the site of fertilization; however, uterosomes appear to have a similar influence on capacitation as oviductosomes when incubated with spermatozoa *in vitro* [11].

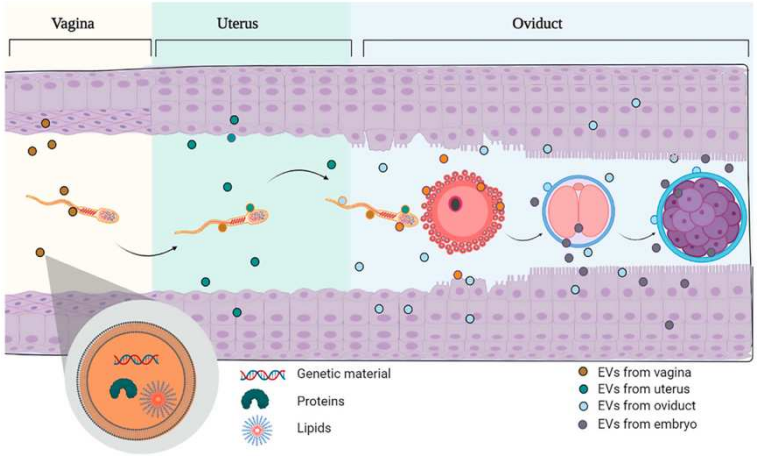


Figure 2: Schematic representation of sperm travel through the female reproductive tract. Sperm cells encounter different EV populations in the female reproductive tract produced by the vagina, uterus, and oviduct. EVs also interact with the oocyte and early blastocyst, contributing to successful fertility throughout several reproductive processes [9].

Equine spermatozoa bypass the vagina in natural mating by being deposited directly into the uterus where they reside for 4 hours before arriving to the site of fertilization in the oviduct [11-13]. There is evidence that capacitation can occur in the

equine uterus under the influence of estrogen, but the mechanisms are not well understood [22]. As described in the following studies, uterosomes are likely to have a role in this process. Uterosomes have been identified in the uterine fluid of other species including sheep, mice, cows, and women [19, 26-31]. Several of these studies subsequently evaluated the effect uterosomes have on spermatozoa [27, 29-31], while others focused on evaluating EV cargo throughout the female reproductive cycle [19, 26].

Human *in vitro* studies found that exposure of fresh sperm to uterosomes stimulates acrosomal exocytosis as early as 15 minutes, but the greatest increase observed at 2 hours [29, 30]. Furthermore, a significant increase in uptake efficiency was observed by flow cytometry in sperm incubated with fluorescently labeled EVs derived from the proliferative phase (estrogen dominated) compared to those derived from the secretory phase of women (progesterone dominated) [30]. Additionally, spermatozoa incubated with the uterosomes demonstrated a significant elevation in protein tyrosine phosphorylation and acrosome reactions [29, 30].

Oviductosomes have been identified in the oviductal fluid of several species including women [15], mice [15-17], cattle [18,19], pigs [20], wolves [21], and cheetahs [21]. In women and mice, uterine and oviductal EVs contain several proteins essential for fertilization, including PMCA4 and PMCA1, and are capable of transferring these proteins to the spermatozoa which facilitated capacitation [15]. In addition, expression of these proteins was higher in oviductosomes and uterosomes when the epithelium was

under the influence of estrogen than when under the influence of progesterone, and a 4-9 fold increase in expression of these proteins was found in oviductosomes compared to uterosomes [15].

A similar study evaluated the miRNA content in mouse oviductosomes throughout the estrous cycle and found that the majority of miRNAs had similar expression levels throughout the cycle, but 7% of miRNAs in estrus derived oviductosomes showed a 1.5 fold increase [16]. When incubated with murine spermatozoa, the oviductosomal miRNAs were incorporated into spermatozoa and distinctly localized, indicating that the miRNAs are sorted to regions where they function physiologically [16]. Bovine extracellular vesicles derived from the oviductal fluid induced capacitation associated events including the acrosome reaction in frozen-thawed spermatozoa over 90 minutes of incubation [17]. Additionally, the EVs induced an increase in sperm protein tyrosine phosphorylation compared to the control and maintained cell survival similar to the control [18]. The study also reported that fluorescently labeled EVs bind to frozen-thawed bull spermatozoa within a few minutes, and reach a maximum at 2 hours [18].

To date, no published studies report isolating oviductosomes directly from equine oviducts, likely due to practical and ethical limitations. Only recently have uterosomes been isolated and characterized from the equine uterus, with a focus on embryo-maternal interactions; and no studies have reported incubating uterosomes with equine spermatozoa [23-25]. However, there is a recent report in which extracellular vesicles

from *in vitro* cultured equine oviductal spheroids were incubated with fresh stallion spermatozoa [14]. Fluorescently labeled EVs were observed in the sperm head and mid-piece after one hour of co-culture and contributed to higher rates of acrosome reactions and fertilization [14].

Estrous cycle of the mare

The basic aspect of equine reproduction in the mare is the hormonally regulated estrous cycle, in which ovulation occurs spontaneously every 21 days during the physiological breeding season. The estrous cycle is composed of two phases: the estrus phase and diestrus phase. The estrus phase lasts 5 to 7 days, and is the period in which the mare is under the influence of estradiol (E2) produced by the growing dominant follicle [45]. During estrus, elevated concentrations of E2 and low concentrations of progesterone (P4) contribute to an increase in uterine edema [45, 46]. The ovarian follicle continues to mature until ovulation, which typically occurs 24-48h before the end of the estrous period and is triggered by a prolonged surge in luteinizing hormone. After ovulation, granulosa and theca cells of the follicle begin the process of lutenization to become an ovarian structure called a corpus luteum (CL) [45]. The luteal cells produce P4, the dominant hormone of the diestrus phase [45]. If the mare does not conceive, the hormone prostaglandin is released from the endometrium 12 to 14 days after ovulation and causes regression of the corpus luteum, and a new cycle begins [45]. The morphological changes that occur in the endometrium and ovaries can be appreciated via transrectal ultrasonography to accurately determine the phase of the estrous cycle and ovulation [47].

Summary

In summary, little is known about *in vivo* mechanisms of sperm capacitation, especially in the horse. EVs have recently raised attention in their contribution to fertility and have been isolated from the female reproductive tract of several species. The production and cargo of EVs is hormonally regulated and EVs are able to deliver their cargo to spermatozoa *in vitro* with physiological effects that support capacitation. An increase in protein tyrosine phosphorylation, acrosome reaction, and hyperactive motility were observed after incubation of spermatozoa with uterine or oviductal derived EVs, with the greater increase observed in sperm incubated with EVs obtained under the influence of estrogen. To date, no studies have evaluated stallion sperm capacitation parameters when co-cultured with uterosomes collected from mares.

Chapter 2: Materials and Methods (Study 1)

Animals and estrous cycle monitoring

Six Quarter Horse mares, 6 to 15 years old, were used for uterine fluid collections in June and July of 2021. Transrectal ultrasonography was performed daily by a veterinarian using a linear array ultrasound unit (SonoScape, Model S9 Portable Veterinary Ultrasound Machine) to monitor uterine edema, follicular development, ovulation, and formation of a corpus luteum. A double guarded uterine swab was used to collect a sample from the uterus for microbial culture and a double guarded brush was subsequently used to collect a sample from the uterus for cytologic evaluation. All uterine cultures were negative for microbial growth and no inflammatory cells were noted on uterine cytology.

Low-volume uterine lavage

Uterine contents were obtained using a low-volume uterine lavage (LVL). The first LVL was performed on each mare during estrus, when a dominant ovarian follicle ≥ 35 mm was identified and moderate uterine edema was present by infusing 250 mLs of lactated Ringer's solution (LRS) into the uterus. The fluid was moved throughout the uterus by transrectal uterine massage before recovery. Recovery of the fluid was facilitated by administration of IV oxytocin (20 units; intravenously) to induce uterine contractions and the effluent fluid was recovered in the original bag in s closed system. A second LVL was performed when the mare was in the diestrus phase, 7 days after ovulation, with the presence of a corpus luteum and no uterine edema on the ultrasound. The recovered uterine fluid obtained from each flush was transferred to 50

mL conical centrifugation tubes (Thermo Fisher Scientific, #339652) and centrifuged at 750 x *g* for 5 minutes to pellet a majority of the cellular debris. The supernatant containing EVs was collected and stored in a -80° C freezer until processed.

EV isolation procedure

Extracellular vesicles were isolated by a standard ultracentrifugation method [30]. Samples were thawed overnight at 4° C and all centrifugations were carried out at 4° C. Each sample was first centrifuged at 2,000 x *g* for 30 minutes to pellet residual cell debris and the supernatant was recovered and centrifuged at 25,000 x *g* for 30 min to pellet large microvessicles. The supernatant was filtered through a 0.2 µm sterile syringe filter (PALL, #4612) to remove particles greater than 200 nm and centrifuged by ultracentrifugation (Beckman Coulter Optima XPN-100 Ultracentrifuge, Beckman Coulter, Brea, CA) at 120,000 x *g* for 70 minutes using the Beckman SW 32 Ti Swinging-Bucket Rotor and Ultra-Clear Thin Wall 25 x 89 mm 38.5 mL Ultracentrifuge tubes. The supernatant was discarded, and the EV rich pellet was resuspended with 5 mL cold PBS before undergoing a second ultracentrifugation at 120,000 x *g* for 70 minutes using a Beckman SW 55 Ti Swinging-Bucket Rotor Package (Beckman Coulter, #342194) and SETON Open-Top Polyclear Ultra Tubes; 13 X 51 mm (SETON, #7022). The supernatant was removed and the EV pellets were resuspended with 500 µL of PBS and stored at -80° C until characterization was performed.

EV Characterization

Isolated EVs were characterized in accordance with MISEV guidelines [58] by nano-tracker analysis (NTA), bicinchoninic acid assay (BCA), western blot, and transmission electron microscopy (TEM).

Nano- tracker analysis (NTA)

EVs were characterized using a Z-NTA analyzer (ZetaView, Particle Metrix, Germany). EV samples from individual mares were thawed at 4° C, thoroughly mixed, and a volume of 1-3 µL from each sample was diluted with phosphate buffer solution (PBS) at a ratio of 1:1000 or 1:3000 (EVs to PBS) for analysis on the Z-NTA. The average size and concentration of EVs for each sample was determined before pooling the 6 samples from estrus and 6 samples from diestrus. Each of the 6 samples was equally represented in the final pooled sample. The final EV concentration was 200 billion EV/mL.

Protein Quantification

The protein content of the two pooled samples was determined using a Pierce™ BCA protein assay kit (#23225; Thermo Scientific, Waltham, MA, USA). A 9-point standard curve (range 0–2000 µg/mL) was developed using serial dilutions of bovine serum albumin (BSA) and a working BCA reagent. A volume of 100 µL from each sample was mixed with 2.0 mL of working reagent and incubated at 37° C for 30 min. The samples were cooled to room temperature, and the protein content determined using a calibrated spectrophotometer (DS-11; Denovix, Wilmington, DE, USA) to assess the absorbance at 562 nm.

Western Blot

Proteins contained in the uterosomes were characterized by Western blot analysis. A volume of 100 µL from each sample was resuspended with 50 µL of RIPA buffer (Marker Gene Technologies, #M2777) while a volume of 10 µL loading buffer was combined at a ratio of 9:1 4X Laemmli Sample buffer and 2-Mercaptoethanol (Bio-Rad, #1610747 and #1610710) and then added to 30 µL EV- protein lysate. The samples were incubated at 95° C for 5 minutes as the gel holding cascade was assembled with Mini-Protean TGX stainless Gels (Bio-Rad #4568084) and filled with 1X running buffer (100mL 10x Tris/Glycine/SDS Buffer (Bio-Rad, #1610772) and 900 mL Nanopure water). Precision Plus Protein Dual Color, 5 µL, (Bio-Rad, # 161-0374) was added to the first well followed by 40 µL of each protein lysate mixture in subsequent wells. The gels were run by electrophoresis at 4° C at 90 V for 15 min followed by 200 V for 30 minutes before being transferred to a Nitrocellulose membrane (Bio-Rad, #1620112) and filter papers (Thermo Scientific, #88600) for electrophoresis at 100 V for 1 hour at 4° C in a gel holding cascade filled with 1x transfer buffer blocking solution (100mL Tris/Glycine Buffer (Bio-Rad, # 1610771), 200 mL Methanol, and 700mL Nanopure water). A blocking solution was prepared by adding 2.5 g of dried milk powder to 50 mL of 1X Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) (Bio-Rad, #1706435, Bio-Rad, #1706531). When the transfer was complete, the membrane was placed in the blocking solution on a shaker in a cold room for 1 hour. The primary antibodies CD63, FLOT1, ALIX, (SBI, #EXOAB-CD63A-1, #EXOAB-FLOT1-1, #EXOAB-ALIX-1) and Cytochrome C (Sino Biological, #102139-T42) were prepared by diluting 1:100 in blocking solution. After 1 hour of blocking, the diluted primary antibody solutions were

poured on the membrane and left rocking in a cold room overnight. The next morning, the membrane was washed 5 times for 5 minutes with TBST on the shaker. Secondary antibodies were diluted 1:2000 in milk buffer and placed on the rocker for at least one hour followed by another 5 washes in TBST. Fresh TBST was poured on the membrane and imaging solution consisting of equal parts of SuperSignal West Pico PLUE Stable Peroxide and SuperSignal West Pico PLUS Luminol/Enhancer (Thermo Scientific, #34577) were mixed by vortex before imaging. The membrane was placed in a blot developing folder (VWR #490016-806) and imaging solution was added to the membrane before closing the folder. After sitting for 5 min, the immune blot was imaged on the ChemiDoc XRS+chemiluminescence system with Image Lab Software (BioRad, #1708265).

Transmission Electron Microscopy

Electron Microscopy (EM) was used to obtain high resolution images of the EVs. For negative staining of EVs, drops of 1% Alcinan Blue (AB) 8GX (Sigma, #33864-99-2) were dispensed on parafilm. Mesh copper grids with carbon-coated formvar film (EMS, #FCF 400-Cu) were placed on AB drops for 5 minutes with the film side down to achieve hydrofilicity, then rinsed in three water droplets for 15 seconds each. Next, excess fluid was removed from the grid, then the grid floated on a 5 micro-liter specimen drop for 10 minutes. Excess fluid was removed before fixing grid with sample in 2.4% glutaraldehyde (Sigma, #111-30-8) for 5 minutes. The grid was then rinsed in two water droplets for 15 seconds each, wicked of excess fluid, and placed on drops of 2% uranyl acetate (EMS, #22400) for 30 seconds. Excess fluid was removed and the

samples were dried and stored in the grid case until visualized and photographed on a JEOL JEM-1200 EX electron microscope.

Chapter 3: Materials and Methods (Study 2)

Semen collection and cryopreservation

Three Quarter Horse stallions were used for semen collection from June-August 2022 using a Colorado-style artificial vagina. Six billion spermatozoa from each ejaculate were diluted 1:1 in non-capacitating modified Whitten's (MW) medium (NCMW; 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM anhydrous glucose, 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, and 1.0 mM pyruvic acid) and centrifuged at 1000 x *g* for 25 minutes to remove the seminal plasma. The pellets were resuspended to 200 million sperm cells/mL in CryoMax LE freezing extender (Animal Reproduction Systems Inc, Chino, CA) and cryopreserved according to manufacturers instructions. The extended semen was loaded into 0.5 mL straws and placed on a floating freezing rack (Animal Reproduction Systems Inc #FSR-101) into a 4° C refrigerator for 30 minutes prior to floating the rack above liquid nitrogen for 15 minutes. The straws of semen were then fully submerged in liquid nitrogen and stored in a liquid nitrogen storage tank until further use.

EV staining

EV association with spermatozoa was assessed as described by Merdica et al. with modifications [30]. First, 100 µL of EVs (200 billion/mL) were stained with 1 µL stock Vybrant DiO stain (Thermo Fisher, V22886), mixed well by pipetting, and incubated for 30 min at 37° C in the dark, shaking every 5 minutes. The labeled EVs were then mixed with 5 mL PBS and centrifuged by ultracentrifugation (120,000 x *g* for

70 min) to pellet the stained EVs, and any unbound stain was discarded with the supernatant.

Sperm preparation

Two straws of frozen spermatozoa from each individual stallion were thawed in a 37° C water bath for 30 seconds. The thawed sperm were placed over 1 mL 45% Percoll and centrifuged (300 x *g* for 7.5 minutes) to remove the freezing extender and pellet the sperm. The supernatant was aspirated and discarded and the sperm pellet was resuspended to a concentration of 30 million sperm cells/mL with BSA-free MW medium (100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose [anhydrous], 22 mM HEPES, 4.8 mM lactic acid hemicalcium salt, and 1.0 mM pyruvic acid, 25mM $NaHCO_3$) at 37° C. The sperm suspensions were used in all of the subsequent evaluations described below.

EV uptake by spermatozoa

Spermatozoa were labeled with Hoechst stain (0.005 mg/mL) for 5 minutes and washed to remove excess dye. A volume of 200 µL of Hoechst-stained sperm were added to DiO labeled EVs or PBS (subjected to the same DiO staining and centrifugation protocol as EVs) at a concentration of 200:1 (EVs: sperm). Samples were mixed and incubated at 37° C for 1 hour before 6 µL samples were placed on a slide with a cover slip, and assessed using an Olympus BX63 microscope (Evident, Tokyo, Japan) with a 40x UPlanXAPO objective (Evident, Tokyo, Japan).

Sperm treatment with EVs for CASA and flow cytometric analysis

Three samples (250 µL) were made by adding spermatozoa to EVs from estrus, diestrus, or a control of PBS at a ratio of 200:1 (EVs to sperm) in polystyrene culture tubes, mixing the samples and incubating them at 37° C for up to 90 minutes.

Sperm motion assessment

Motility pattern of spermatozoa from each treatment was assessed on a Sperm Vision Therio CASA system (minitube, Tiefenbach, Germany). A sub-sample of 6 µL from each treatment was examined at time points 0, 15, 30, and 60 minutes. The percent of hyperactively motile sperm was detected by CASA and recorded.

Flow Cytometric analysis

At time points 0, 15, 30, 60, and 90 minutes, two 50 µL samples from each treatment (control, estrus EV, diestrus EV) were removed and stained for 1) membrane scrambling using Yo-Pro-1 and merocyanine and 2) acrosome reaction using propidium iodide and FITC PNA. After staining, samples were diluted to 3 million cells/mL with PBS and analyzed using an Accuri™ C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cells were passed through the instrument at 100 events per second and data collected for 10,000 cells. Propidium iodide fluorescence was detected with a 610-nm band pass filter (FL-3), FITC and Yo-Pro-1t fluorescence were detected with a 520-nm band pass filter (FL-1), and MC540 was detected with a 575 nm band pass filter (FL-2).

Acrosomal status and spermatozoa viability

The percentages of live- acrosome reacted sperm in each sample was determined by staining samples with 2 µL fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA, 1 mg/mL) and 1 µL propidium iodine (PI, 2.5 µg/ml) for 3 min prior to assessment. The percentage of viable (PI negative) acrosome-reacted sperm (FITC positive) and membrane integrity were determined at 0, 15, 30, 60, and 90 minutes.

Statistics

EV concentration and size were analyzed using a two-sample t-test in R Studio. All data are presented as the mean ± standard deviation. The remaining data were analyzed on R-software using a mixed model for grouped data considering fixed and random effects by a type III ANOVA test with Kenward-Roger's method on a mixed model. Data were considered to be statistically significant at $p < 0.05$.

Chapter 4: Study 1 Results

Nano-tracker analysis (NTA) (Concentration, Size)

The concentration of EVs collected from equine uteri during estrus (235 ±164 billion EV/mL) tended to be higher than the concentration of EVs in the samples collected in diestrus (83.67 ± 89 billion EV/mL) ($p<0.05$). The size of EVs in diestrus samples (146.183 ± 11 nm) and estrus samples (148.633 ± 11 nm) were similar ($p>0.05$).

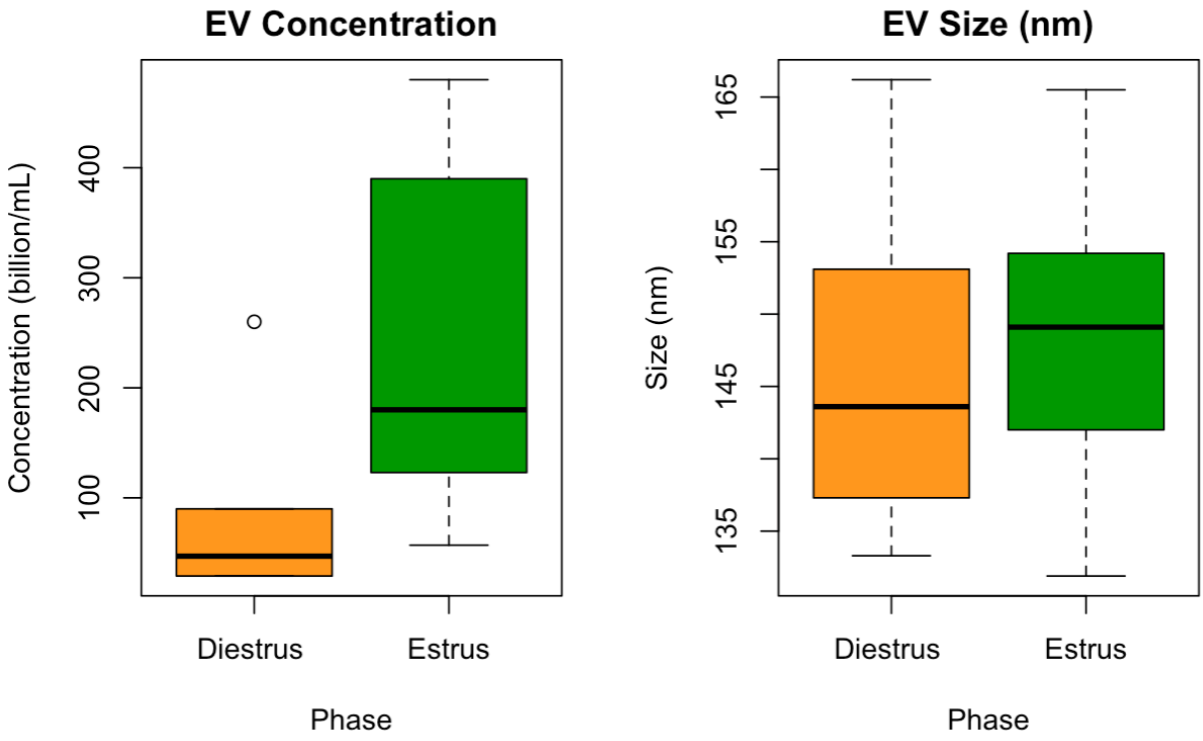


Figure 3: The concentration and size of EVs collected from the uterine flushings from estrus and diestrus mares (n=6).

Protein content and Immunoblotting

The protein content of the pooled samples (200 billion EV/mL) was determined to be 0.6 g/mL for DEVs and 0.534 g/mL for EEVs by BCA. Immunoblot analysis (Figure 4)

showed that both pools of EVs were positive for known exosome markers TSG-101 and CD-63, and negative for mitochondrial organelle marker, cytochrome C (positive control), indicating that the samples were indeed EVs.

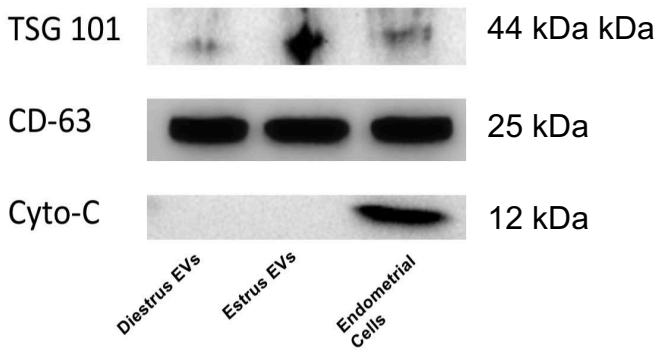


Figure 4: Images from Western blot analyses marked with the molecular weights on the right and proteins probed on the left.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy revealed that vesicles were approximately 140 nm in diameter, consistent with the size detected using NTA. The vesicles had distinctive cup-like morphology [23], resulting from the procedures which dehydrate the naturally spherical vesicles (Figure 5).

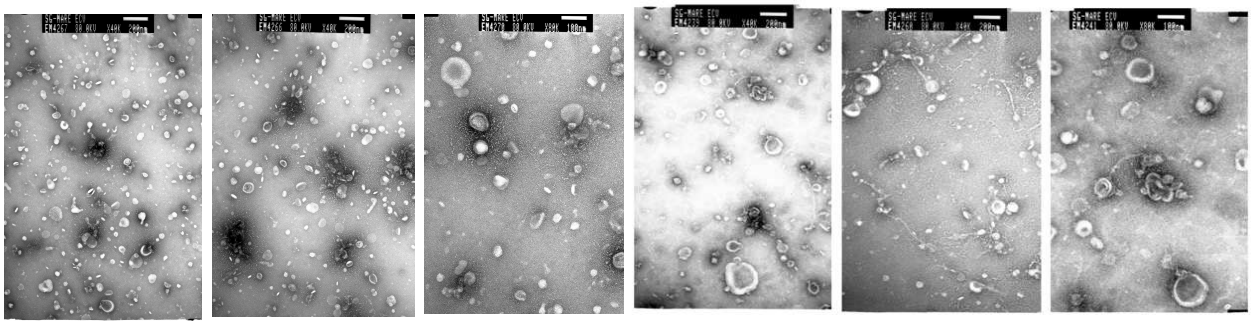


Figure 5: TEM photos of EEV (left 3) and DEV (right 3) depicting uterine derived extracellular vesicles of a size range consistent with nano-tracker analysis and a distinctive cup-like morphology.

Chapter 5: Results Study 2

EV uptake by stallion spermatozoa

Fluorescent microscopy revealed that DiO stained uterosomes associate with stallion spermatozoa, and sperm incubated with EEVs exhibited a higher fluoresce with EEVs than that in sperm incubated with DEV (Figure 6). No fluorescence was observed with “DiO labeled” PBS without EVs, indicating that passive fluorescence from unbound dye did not cause the fluorescence observed.

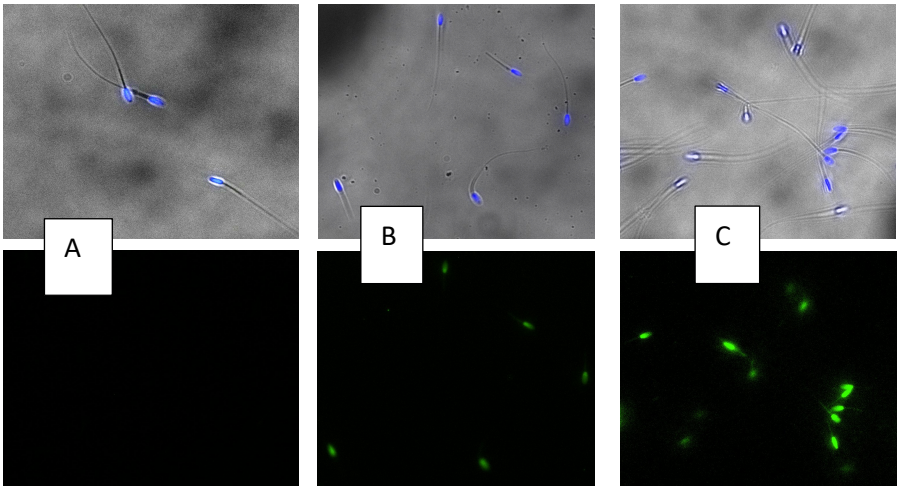


Figure 6: Microscopic images of EV uptake by sperm. Top row of photos are bright field overlayed with DAPI filter. The bottom row of photos show FITC associated with sperm after incubation with: A) Sperm incubated with PBS containing DiO label without EVs, B) Sperm incubated with diestrus EVs (DEVs), and C) Sperm incubated with estrus EVs (EEVs).

Viability

The viability of sperm decreased over the 90 minute incubation period for all treatments (Figure 7). At time=0, 15, 30, and 90 minutes, no treatments were

statistically different from one another. However, at time = 60 minutes, DEV was lower than the control (p=0.0194), while EEV was not different from the control (p=0.2336), and DEV and EEV was not different (p=0.5144).

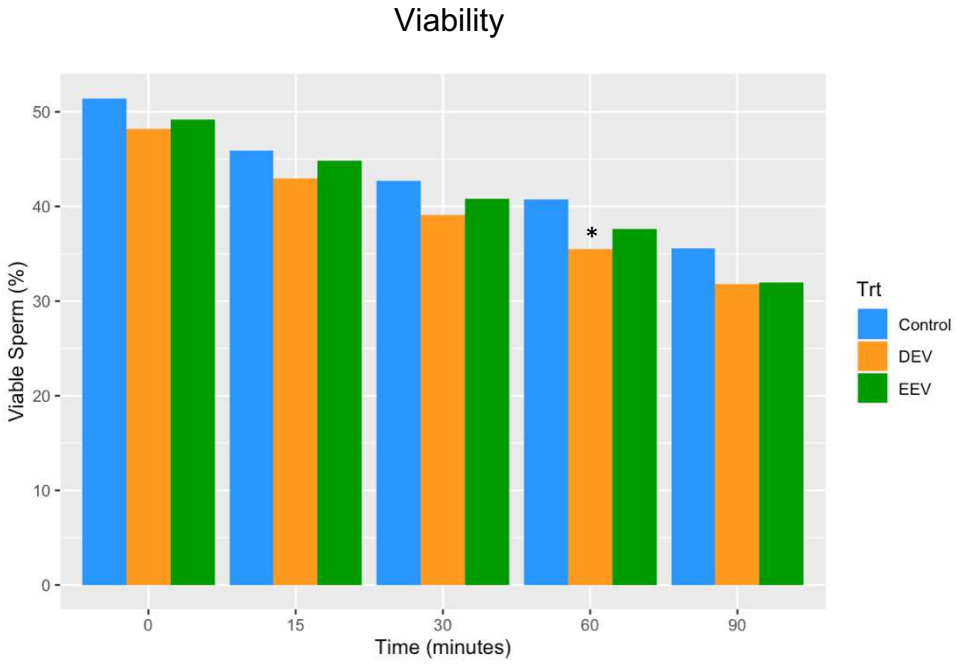


Figure 7: The percentages of viable (PI negative) sperm for each treatment 1) PBS, 2) DEV, and 3) EEV over 90 minutes of incubation. Statistical differnces are noted by an astrict (*).

Acrosome reaction

The percentage of viable, acrosome reacted spermatozoa over the 90 minute incubation period was elevated for spermatozoa treated with EEVs beginning at 15 minutes (Figure 8). At time=0 minutes, no treatments were statistically different from one another. At time=15 minutes, EEV had a higher incidence of the acrosome reaction than the control (p=0.05), while DEV was not different from the control (p=0.671), and EEV was not different from DEV (p=0.285). At time= 30 minutes, both DEV and EEV

were higher than the control (p=0.008) and (p=<0.0001), respectively, but DEV and EEV were not different from each other (p=0.1204). At time = 60 minutes, EEV was higher than the control (p=0.0001), DEV is not higher than the control (p=0.1808), and EEV was higher than DEV (p=0.036). At time= 90 minutes, EEV was higher than the control (p=0.007), DEV was not higher than the control (p=0.678), and EEV was higher than DEV (p=0.063).

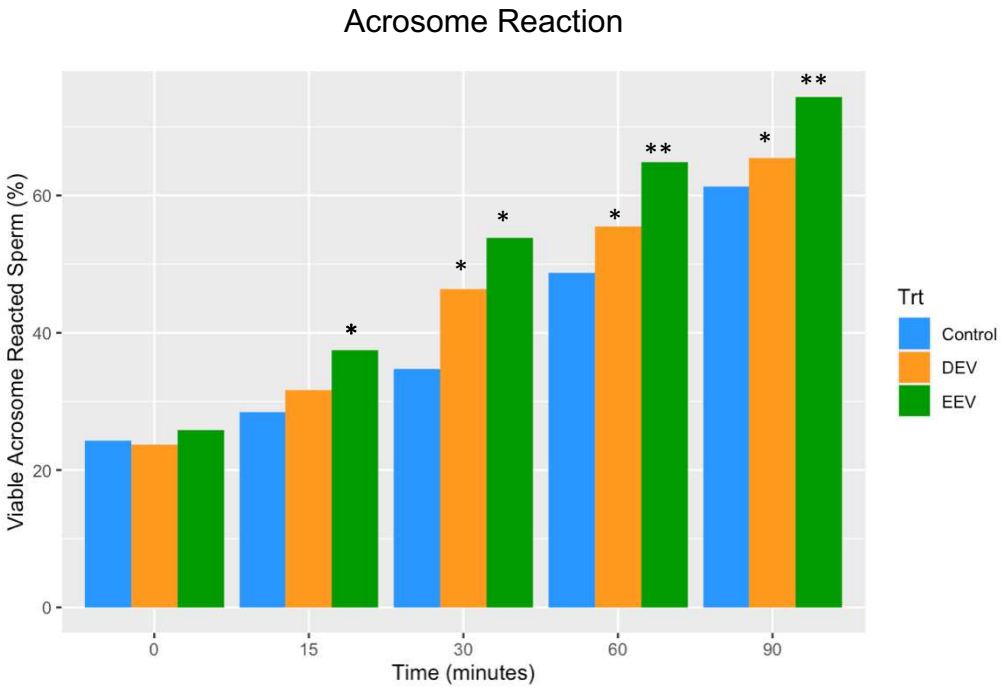
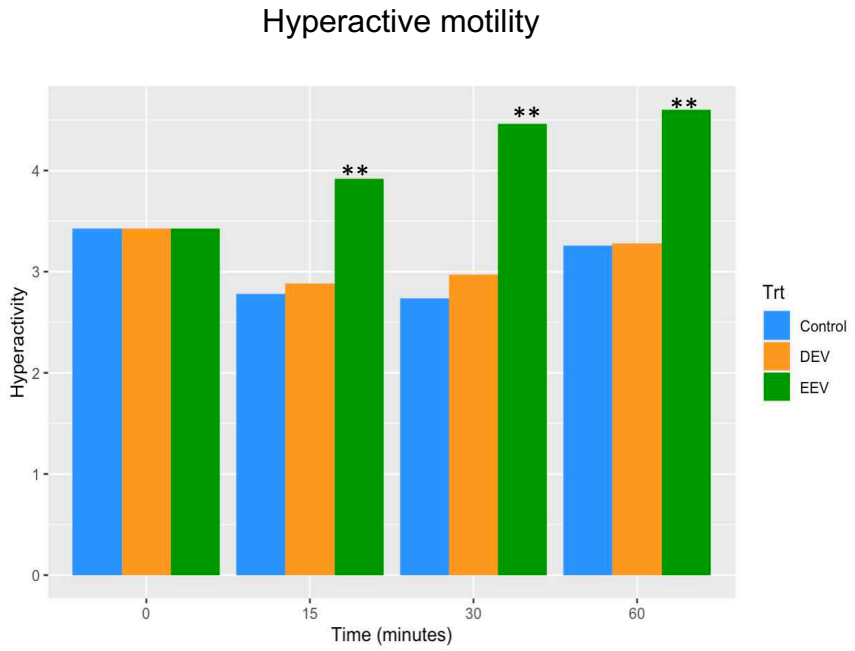


Figure 8: Percentages of live (PI negative) acrosome reacted (FITC positive) sperm after treatment with: 1) PBS, 2) DEV, and 3) EEV over 90 minutes of incubation. Statistical differnces are noted by an astrict (*).

Hyperactivity

For hyperactive motility assessment, only one measurement was taken at time =0 (Figure 9). At all following time points, EEV had a significantly higher occurrence of

hyperactivity than control (p=0.0088), (p=<0.0001),and (p=0.0014), respectively. EEV also had a higher occurance of hyperactivity than DEV (p=0.0190), (p=0.0004), and (p=0.0017), respectively. DEV and control did not differ from each other at any time points (p=0.9604), (p=0.8038), and (p=0.9982), respectively.



Figures 9: The percentages of hyperactive sperm detected by CASA after treatment with: 1) PBS, 2) DEV, and 3) EEV over 90 minutes of incubation. Statistical differnces are noted by an astrict (*).

Chapter 6: Discussion

Although *in vitro* sperm capacitation has been studied for over half a century, and *in vitro* fertilization has been successfully achieved in a majority of domestic animal species [53-56], the mechanisms and biochemical changes that encompass capacitation are still unclear. Furthermore, *in vitro* sperm capacitation and fertilization has not been reliably successful with equine gametes until recently [52]. The struggle to capacitate equine spermatozoa highlights how little we know about this complex process.

In equids, semen is deposited directly into the uterus at copulation, and are transported into oviduct within four hours, where spermatozoa can survive for long periods of time under the influence of estrogen. It has been established that fresh and frozen-thawed stallion sperm can be capacitated in an estrus mare's uterus [22]. In 1995, it was found that 31-35% of spermatozoa underwent the acrosome reaction after 6-hour incubation in the estrus mare uterus, while only 2-5% of the sperm underwent acrosomal exocytosis 6 hours after being inseminated into the uterus of a mare 7 days post- ovulation [22]. The authors of this study proposed the hormone estrogen to be responsible for these observations. Although estrogen does play a role in signaling for capacitation, other uterine components, such as extracellular vesicles, are also likely to be key players in sperm capacitation.

The present studies support the increasing evidence of the involvement of EVs in intracellular communication, and their emerging role in the fertility process. To date, several studies report EVs isolated from uterine and oviductal fluids support sperm capacitation [27, 29-31]. However, no studies have yet evaluated the effect equine reproductive tract derived EVs have on stallion sperm capacitation.

We confirm previous findings indicating that a heterogeneous population of extracellular vesicles can be isolated from the equine uterus with distinct cup-shape morphology positive for the exosomal markers TSG 101 and CD-63 as detected by immune-blotting [23, 25]. Both populations of vesicles had similar size distributions and averaged 140 nm in diameter. There was a tendency for a greater number of vesicles to be recovered when uterine flushes were performed in estrus mares than in diestrus mares, indicating the production of EVs may be up-regulated under the influence of estrogen to support sperm capacitation.

Human *in vitro* studies found that exposure of fresh sperm with uterosomes enhances sperm capacitation as soon as 15 minutes, with the greatest increase observed at 2 hours [28, 29]. Furthermore, one study found an increase in uptake efficiency in sperm incubated with fluorescently labeled EVs derived from the proliferative phase, under the influence of estrogen, compared to those derived from the secretory phase of women's reproductive cycles [29]. Additionally, spermatozoa incubated with the uterosomes from the proliferative phase demonstrated an elevation in protein tyrosine phosphorylation and acrosome reaction [28, 29].

The present study suggests equine uterosomes also have a profound and phase specific effect on equine sperm capacitation as soon as 15 minutes, as indicated by an increased occurrence of the acrosome reaction and hyperactive motility in the estrus-phase EV (EEV) treated groups compared to the diestrus-phase EV (DEV) groups and the control. Equine uterosomes likely contain capacitation factors, and these factors are up-regulated in EVs produced under the influence of estrogen. In women and mice, uterine and oviductal EVs contain proteins essential for fertilization, PMCA4 and PMCA1, and the EVs are capable of transferring these proteins to the spermatozoa inducing sperm capacitation [15]. Furthermore, the expression of these proteins was higher in oviductosomes and uterosomes obtained under the influence of estrogen compared to progesterone, and a 4-9 fold increase found in oviductosomes compared to uterosomes [15].

We demonstrated that fluorescently labeled EVs associated with stallion spermatozoa as visualized by fluorescent microscopy after 1 hour of co-culture, with a greater fluorescent intensity observed in the spermatozoa treated with estrus-phase derived uterosomes. Our group utilized a standard method involving incubation of cells with vesicles labeled with a fluorescent lipophilic dye [29]. To rule out the potential for dye to be passively transferred to spermatozoa through the medium that contains the EVs, we subjected EV-depleted PBS to the same lipophilic dye labeling procedure and for the assessment of cellular uptake. A possible drawback of the lipophilic dye employment is the potential diffusion of these fluorescent molecules from EVs onto the

cellular membrane, leading to an internalization pattern that could be caused by physiological recycling instead of EV capturing. However, this seems unlikely given the numerous studies reporting the activity of molecular inhibitors preventing the uptake of dye-labeled EVs [4, 30, 50].

In conclusion, our results show evidence that EVs released in the equine uterine tract can be taken up by stallion spermatozoa and may be connected to the molecular mechanisms involved in sperm capacitation. These discoveries not only expand our current knowledge on the *in vivo* mechanisms of equine sperm capacitation, but could be expanded in the future to potentially improve *in-vitro* fertilization outcomes with equine gametes. Evaluation of additional changes relating to capacitation, namely protein tyrosine phosphorylation, intracellular PH, and *in vitro* fertilization trials would be great directions for future studies.

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