THESIS

SERUM EXOSOME PROFILE AS RELATED TO EARLY PREGNANCY STATUS

IN THE MARE

Submitted by

Joanna R. Hergenreder

Department of Animal Sciences

In partial fulfillment of the requirements

For the degree of Master of Science

Colorado State University

Ft. Collins, Colorado

Summer 2011

Master's Committee:

    Advisor: Jason Bruemmer

    Gerrit Bouma
    DNRao Veeramachaneni
    Colin Clay
    Hyungchul Han

ABSTRACT

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IN THE MARE

During early pregnancy in the mare the conceptus and mare must communicate in order to establish and maintain pregnancy. This coordinate communication is most pronounced between days 12 and 16 post-ovulation, at which time the conceptus is highly mobile throughout the uterus preventing endometrial prostaglandin F2α release and subsequent luteolysis. The mechanism behind successful establishment and maintenance of pregnancy in the mare is currently unknown. Recently, cell-secreted vesicles, called exosomes, were detected in high amounts in serum of pregnant women. Exosomes are 50-100 nm vesicles containing bioactive materials such as mRNA, miRNA, and protein. Exosomes can mediate immune-responses through membrane protein interaction and delivery of bioactive products into cells. Interestingly, exosomes have been described in various body fluids, including urine, breast milk, and serum. We hypothesized that exosomes are present in serum in the mare and that their relative amount differs with pregnancy status. To test this hypothesis, we determined the presence and relative amount of exosomes in serum of pregnant and non-pregnant mares. Serum samples were obtained from mares in a cross-over design, with each mare serving as both a pregnant treatment and non-mated control (n=3/day). Blood samples were obtained by jugular

venipuncture on days 12, 14, 16, and 18 post-ovulation. Serum was removed, snap frozen, and stored at -80°C. Exosome isolation, for flow-cytometry and transmission electron microscopy (TEM), was performed using ExoQuick[TM] (System Biosciences, Inc.), a precipitation solution designed to isolate exosomes from fluids. After exosome isolation, samples were analyzed using flow cytometry with 100 nm sized beads as an internal control and a counting bead standard for relative amount determination.

Flow cytometry analysis revealed the presence of exosomes in serum of both pregnant and non-pregnant mares in variable amounts. Furthermore, analysis revealed the presence of two distinct size populations, one of smaller exosomes (< 100 nm) previously undescribed, which were more abundant in mare serum from day 12 of pregnancy, and the second of the expected 100 nm size at each day examined. TEM analysis validated the results from the flow cytometry as each population, determined by size and granularity, was visually characterized. Along with the 100 nm and slightly smaller sized vesicles, TEM also revealed the presence of vesicles slightly larger than 100 nm, with small amounts of vesicles ~200 nm in size, indicating the presence of exosomes as well as microvesicles. Therefore, we conclude that exosomes are present in mare serum and further characterization of such populations can provide clues about the intercellular mode of communication in early pregnancy.

AKNOWLEDGEMENTS

TABLE OF CONTENTS

FIGURES AND TABLES

APPENDICES

CHAPTER I

REVIEW OF THE LITERATURE

Introduction

Pregnancy in its own entirety may be considered as a more or less harmonious sequence of events that requires a balance of interests between the conceptus and mother with a common goal of continuing the species (Roberts *et al*., 1996). Communication between the mother and the conceptus is therefore vital to acquire such a balance. Perhaps one of the most critical time points in this process is in establishing the pregnancy. The mother must recognize the presence of the conceptus in order to maintain pregnancy and prevent a return to cyclicity, a physiological response termed 'maternal recognition of pregnancy' (Allen and Stewart, 2001).

Maternal recognition of pregnancy, as coined by Roger Short in 1969, refers to the mechanisms by which domestic animal species ensure continued secretion of the hormone progesterone by the corpus luteum (CL) beyond its normal cyclical lifespan, which is vital to maintain pregnancy (Allen and Stewart, 2001). In essence, the CL must be protected from the agent which causes its demise, luteolysis, in order for pregnancy to ensue. In most domestic livestock species, this luteolytic agent is endometrial

prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (McCracken *et al.,* 1970; Douglas and Ginther, 1972; Roberts *et al.,* 1996).

Early pregnancy in the mare is unique in many aspects as compared to that of various other domestic animal species. The physiological, morphological, immunological, and endocrinological changes that occur in the oviduct and uterus are vital to the success of the pregnancy. These changes in the mare are markedly different from similar processes in the other large domestic animal species, such as the cow, gilt, and ewe (reviewed by Allen, 2000). $PGF_2\alpha$ functions as the luteolytic factor in the mare (Douglas and Ginther, 1972; Douglas *et al.,* 1974; Kooistra and Ginther, 1976), as is true for other domestic animal species, however, the mechanism(s) behind maternal recognition of pregnancy in this species is currently unknown. It is known, however, that if this mechanism fails and maternal recognition does not occur successfully, luteolysis will ensue causing progesterone production to fall resulting in a subsequent loss of pregnancy, known as early embryonic loss (EEL).

EEL in the mare occurs at a rate of approximately 17% between days 12 to 20 with a majority of loss occurring during the mobility phase of early pregnancy (Villahoz *et* al., 1985; Meyers *et al*., 1991; Carnevale *et al*., 2000). Fertilization rates approach and often times exceed 90% in this species, therefore, such a loss can be considered high and equate to deleterious economic impact on the equine industry as well as necessitate the need to gain insight into the mechanism responsible for successful pregnancy establishment (Ball *et al*., 1986 & 1989). One mechanism suggested to play a role in intercellular communication involves exosomes, cell-secreted vesicles capable of

transferring bioactive material such as mRNA, miRNA, and protein from cell to cell

locally and at a distance (Valadi *et al*., 2007; Camussi et al., 2010; Lässer *et al*., 2011).

Physiology of Early Pregnancy in the Mare

Differential migration of the equine embryo in the oviduct begins the myriad of

unique physiological mechanisms employed during pregnancy in the mare. Van Niekerk

and Gerneke (1966) first described this differential migration with the finding that

unfertilized oocytes remained in the ampullary-isthmus junction of the oviduct whereas

fertilized oocytes, or embryo(s), completed passage of the oviduct to gain access to the

uterus via the utero-tubal junction at 5.5 to 6 days post ovulation (Allen, 2000). The

mechanism behind oviductal transport of the embryo in the mare was not elucidated until

the early 1990s by Weber *et al.* which led to the discovery that the equine embryo gains

the ability to secrete appreciable amounts of prostaglandin E_2 (PGE_2) as a compact

morula on day 5 post-ovulation. PGE_2 functions to induce local relaxation of the smooth

muscle fibers in the oviduct, allowing the embryo to pass and enter the uterus

approximately 24 hours later on day 6 post-ovulation (Weber *et al.,* 1995; Allen, 2000).

Upon entry into the uterus, the embryo embarks on yet another unique process of

early pregnancy as it remains mobile throughout the uterus traversing both horns and the

body several times per day, until about day 17 (Ginther, 1998; Bazer *et al.,* 2002). It is

during this period of mobility that the embryo must signal and communicate its presence

to the mother to prevent luteolysis and therefore provide for continued progesterone

secretion from the CL. Dependent on the success of this communication exchange, and

the maternal recognition of the conceptus, the embryo "fixes" or ceases mobility around

days 17 to 18. This fixation of the embryo typically occurs within the caudal portion of one of the uterine horns and is due to a combination of increasing conceptus diameter and a gradual increase in uterine tone which results in a decrease in size of the uterine lumen (Ginther, 1998).

Fixation, however, does not implicate implantation which does not occur until around day 38 to 42. At days 28 to 35, the conceptus is encircled by the chorionic girdle, a band of specialized trophoblast cells, located in the avascular region of the chorion where the yolk sac and allantoic sac join together (Ginther, 1998). This band of trophoblast cells begin to invade the endometrium and at day 40 begin formation of endometrial cups, structures capable of producing equine chorionic gonadotropin (eCG) (Allen, 2001; Allen and Wilsher, 2009). The eCG secreted by the endometrial cups has LH-like biological activity and as such causes necessary events to occur within the ovary to which it gains access through the systemic circulation. eCG causes the resurgence of the primary CL in size as well as progesterone secretion.

Also, along with continuing releases of follicle stimulating hormone (FSH) every 10 to 12 days from the anterior pituitary gland, eCG stimulates formation of secondary CLs and therefore increased/continued secretion of progesterone by luteinization of dominant follicles in the ovaries as a result of follicular waves occurring during pregnancy (Evans and Irvine, 1975; Ginther, 1998). Secondary CLs then persist in the ovaries of the mare from days 38 to 150 of gestation and as such supplement the production of progesterone needed to maintain pregnancy, that is until the allantochorionic placenta assumes this role between days 80 and 100 to term (Holtan *et*

al., 1979; Allen, 2010). Taken together, the events associated with early pregnancy in the mare are necessary for establishing and maintaining the pregnancy. Many of the underlying mechanisms of early pregnancy, however, have yet to be elucidated. The time and process of maternal recognition, a critical point in gestation, is perhaps the least understood of these mechanisms and seems to employ novel communication between mother and conceptus.

Maternal Recognition of Pregnancy

The normal reproductive cycle of the mare is approximately 21 to 22 days in length with an estrous interval of 5 to 7 days and normal luteolysis occurring around days 14 to 16 (Sharp, 1992). Around days 14 to 16 luteolysis occurs as a result of oxytocin-induced $PGF_2\alpha$ secretion by the endometrium which causes regression of the CL, a resultant decrease in progesterone secretion and ultimately a return to estrus thus allowing the mare to cycle again. The oxytocin which stimulates $PGF_2\alpha$ secretion originates from the endometrium which is in contrast to that of ruminants where the oxytocin mediating $PGF_2\alpha$ secretion originates from the CL (Behrendt-Adam *et al.,* 1999; Bae and Watson, 2003). Also in contrast to the ruminant is the pathway by which $PGF_2\alpha$ reaches the CL to mediate its luteolytic action. In the ruminant, a local route is employed through an anatomical inclusion known as the utero-ovarian pedicle in which circulation from the uterus and ovary come into close opposition and exchange signal through a countercurrent exchange. Such anatomy is not present in the mare and as such this species employs a systemic route from the uterus to the ovary (Ginther *et al.,* 1972; Ginther 1998; Allen, 2001; Gaivão and Stout 2007).

This route of transfer is pronounced by the episodic increases of PGFM, a metabolite of $PGF_2\alpha$, in systemic circulation during cyclic luteolysis in the mare (Kindahl *et al.,* 1982). In the pregnant mare, embryonic survival is dependent on preventing luteolysis to maintain progesterone production by the CL, which necessitates attenuation of the pulsatile secretion of $PGF_2\alpha$ (Goff, 2002). Such an attenuation is evidenced by a reduction in $PGF_2\alpha$ in uterine venous plasma (Douglas and Ginther 1976), peripheral plasma (Kindahl *et al.,* 1982) and the uterine lumen (Berglund *et al.,* 1982; Zavy *et al.,* 1984) in pregnant compared with cyclic mares at similar stages following ovulation. This significant decrease of $PGF_2\alpha$ concentrations in the uterine lumen of pregnant mares coupled with relatively unchanged PGFM concentrations suggests that the $PGF_2\alpha$ concentration decrease in pregnant mares is most likely due to a reduction in the synthesis or release of the fatty acid in response to the maternal recognition signal rather than an increase in its metabolism (Berglund *et al.,* 1982; Sharp *et al.,* 1984; Starbuck *et al.,* 1998).

Synthesis and secretion of $PGF_2\alpha$ is triggered by oxytocin binding to its endometrial receptors. This provides for a paracrine manner of action in which oxytocin that is released by the endometrium in the mare binds to oxytocin receptors also located in the endometrium thereby stimulating production and secretion of $PGF_2\alpha$ (Stout and Allen, 1999). Watson *et al.* (1997) demonstrated the presence of oxytocin and its carrier protein, neurophysin, in the luminal and glandular epithelium of the endometrium in the mare utilizing immunohistochemistry further supporting the paracrine manner of action of oxytocin. Interestingly, studies conducted with uterine flushings recovered from days 14 to 18 post-ovulation revealed much higher concentrations of oxytocin than that which

was detected in plasma. Also, when oxytocin was infused into the uterus a rapid and pronounced increase of PGFM was detected in plasma with no rise in systemic oxytocin suggesting a luminal secretion of oxytocin which acts back on the endometrium to trigger synthesis and release of $PGF_2\alpha$ (Starbuck *et al.,* 1998; Stout and Allen, 1999).

Equally interesting is the suppression of the normal cyclical increase in oxytocin receptor numbers and affinity seen in early pregnancy in the mare in the presence of a conceptus (Sharp *et al.,* 1997; Starbuck *et al.,* 1998) leading to an inability for oxytocin to act, and therefore $PGF_2\alpha$ synthesis and secretion is abrogated. Oxytocin receptor concentration was found to be similar at day 10 post-ovulation in pregnant and cyclic mares, however, at day 14 concentrations were approximately three-fold higher in cyclic mares when compared to days 10 and 18 with no increase detected during pregnancy (Sharp *et al.,* 1997; Starbuck *et al.,* 1998) suggesting an effect of the conceptus and the maternal recognition signal on oxytocin receptor expression in the endometrium of the pregnant mare.

It is clear that prevention of luteolysis by suppression of endometrial $PGF_2\alpha$ release as well as the absence of oxytocin receptor upregulation provide for continued secretion of progesterone by the corpus luteum, and in effect, permit maternal recognition of pregnancy to occur. The signal or mechanism behind maternal recognition in the mare remains a mystery however. It has been postulated that migration of the conceptus within the uterus involving a physical stimulus or mechanotransduction or mechanosensation signal acting on the endometrium (Merkl *et al.,* 2010).

Conceptus Structure and Migration

After passage through the oviduct and entry into the uterus, the equine embryo remains enveloped within the zona pellucida for approximately 24 hours at which time the zona is shed and a new outermost layer, the capsule, takes position (Tremoleda *et al.,* 2003). Production of the acellular glycoprotein capsule originates between the trophoblast and zona pellucida and is postulated to derive from trophoblast cells (Oriol *et al.,* 1993; Albihn *et al.,* 2003). Even though the capsule is initially a thin structure (~3 μm-thick), it is considerably elastic and resilient as it supports the developing embryo through early growth, which involves a dramatic increase in diameter from an average of 150-220 μm at day 6 to 2.5-2.8 cm after fixation at day 17 to 18(Oriol *et al.,* 1993; Ginther, 1998). Oriol *et al* (1993a) investigated the protein content of the capsule and discovered that it is composed mainly of glycoproteins that closely resemble those of the mucin glycoprotein family and harbor a remarkable resistance to chemical and enzymatic solubilization. These glycoproteins are also known to provide epithelial cell surfaces with a protective coating and play an important part in cell-cell-interactions (Oriol *et al.,* 1993b).

In combination, the capsular resilience, elasticity and mucin glycoprotein qualities provides for the successful survival of the conceptus through a time of pregnancy when it is traversing the entire uterus due to propulsive myometrial contractions. Ultrasonographic studies conducted by Ginther (1983) describes the pressure induced on the conceptus by these uterine contractions during embryo mobility, most pronounced on days 13 to 14, as actually causing periodic compressions of the conceptus. Equine

conceptus mobility occurs from the time it enters the uterus on around day 6 until the day

of fixation, around day 17, with the most active days being 11 to 14 (Leith and Ginther,

1984). Such mobility has been shown to be required for proper protection and

maintenance of the CL and pregnancy and was best described by the McDowell *et al.*

(1988) uterine ligation studies. Ligation of the pregnant mare uterus and the consequent

restricted mobility of the conceptus resulted in luteolysis, a subsequent decline in

progesterone and ultimately embryonic death. Therefore, unrestricted mobility of the

equine conceptus likely provides for necessary interaction with most of the uterine

endometrium which appears vital for maternal recognition and luteal maintenance

(McDowell *et al.,* 1988; Ginther, 1998). Interaction between the embryo and the mare

endometrium must employ a communication scheme to direct the mechanism responsible

for maternal recognition and early pregnancy. A communication scheme suggested to

play a role in intercellular cross-talk is exosomes, cell-secreted vesicles capable of

transferring bioactive material such as mRNA, miRNA, and protein from cell to cell

locally and at a distance (Valadi *et al*., 2007; Camussi et al., 2010; Lässer *et al*., 2011).

Exosomes

Exosomes are small cell-secreted vesicles of endocytotic origin ranging in size

from 50 to 100 nm that are capable of gaining access to the extracellular environment

(Johnstone *et al*., 1989). Distinct from other cell-secreted vesicles, exosomes begin as

intraluminal vesicles in multivesicular bodies (MVBs) which are released from the cell

upon fusion of MVBs with the plasma membrane (Théry *et al*., 2002). Studies have

shown a variety of cells secrete exosomes including specific antigen-presenting immune

9

cells called dendritic cells (DCs) (Théry *et al*., 1999; Lamparski *et al*., 2002), reticulocytes (Johnstone *et al*., 1987), epithelial cells (Van Niel *et al*., 2001), platelets (Miyazaki *et al*., 1996; Heijnen *et al*., 1999), B- and T-cells (Raposo *et al*., 1996; Blanchard *et al*., 2002), mast cells (Raposo *et al*., 1997), and trophoblast cells (Knight *et al*., 1998; Sabapatha *et al*., 2006; Pap *et al*., 2008). Exosomes have also been isolated from various bodily fluids including urine (Pisitkun *et al*., 2004), saliva (Ogawa *et al*., 2008), breast milk (Admyre *et al*., 2007), amniotic fluid (Keller *et* al., 2007), bronchoalveolar lavage fluid (Admyre *et al*., 2003), epididymal fluid (Gatti *et al*., 2005), plasma (Caby *et al*., 2005) and serum (Taylor *et* al., 2006). Exosome presence in bodily fluids represents a possible communication role between cells in distant areas and a possible utility as a diagnostic tool or biomarker of disease and physiological states. Also, exosome isolation from such fluids presents a less invasive mode of study or diagnosis when compared to techniques such as tissue biopsies or surgery.

MVB and exosome existence has been known for decades as electron microscopy techniques have allowed for the study of intracellular structure (Dalton, 1975). Initially, exosomes were thought to simply be either artifactual or function to remove waste from a cell, viewed as "cellular debris" (Cocucci *et al*., 2008). However, Trams *et al*. (1981), while conducting culture experiments of normal and neoplastic cells, proposed that 'exfoliated membrane vesicles' serve a physiological function and suggested that they be referred to as exosomes. Exosomes were further described by Pan and Johnstone in 1983 when studying reticulocyte maturation in sheep, in which the transferrin receptor was selectively externalized via exosomes. Harding *et al.* (1983) described small vesicle release from rat reticulocytes that same year.

A physiological function of exosomes was discovered some years later in 1996 with work done by Raposo *et al*. that demonstrated exosomes act as immune modulators. Interestingly, this group revealed that exosomes not only were secreted by B lymphocytes but also contained major histocompatibility complex class II molecules on their surface that could be transferred to CD4$^+$ T cells for antigen presentation stimulating an antigen-specific T-cell response. These studies initiated great interest in the characterization of cell-secreted vesicles and provided for the beginning of what has become an exciting area of research in many fields including immunology, cancer, and reproduction.

Exosomes can be distinguished from other classes of cell-secreted vesicles such as microvesicles (MVs) and apoptotic bodies by several classification features including size, biological content, and most markedly, by biogenesis and mode of release from the cell. Exosomes range in size from 100 nm or less while MVs are typically larger at 100 to 1000 nm in diameter (Théry *et al*., 2009). Some evidence exists, however, to show that a lower size range of MVs may exist overlapping that of exosomes (Yuana *et al*., 2010). Apoptotic bodies are much larger with diameters ranging from 1000 to 5000 nm (1 to 5 µm)(Hristov *et al*., 2004).

Biogenesis and release from the cell is perhaps the most distinctive difference describing the various classes of vesicles as well as providing for the different content within each vesicle population. For example, apoptotic vesicles are produced from cells undergoing physiological or programmed cell death. Active blebbing of the cell's plasma membrane occurs and leads to the detachment of vesicles that often contain cellular organelles and nuclear fragments of DNA, and display exposed phosphatidylserine (PS),

normally segregated in the inner leaflet of the lipid bilayer of the cell plasma membrane (Savill *et al*., 2002; Hugel *et al*., 2005). MVs are released directly from the plasma membrane of a cell in a process known as reverse budding and, depending on the population of MVs, PS externalization may also occur (Connor *et al*., 2010). Depending on cell type, MV release can be constitutive or require an activating signal such as increased intracellular calcium (Pap *et al*., 2009). Constitutive release appears to primarily occur in tumor cell release of not only MVs, but also of exosomes and resultant induction of apoptotic body release from immune cells (Fevrier and Raposo, 2004; Van Niel *et al*., 2006).

Cellular Synthesis and Trafficking of Exosomes

Exosome formation begins with initiation of the endocytotic pathway as material, such as protein, is engulfed by the cell at its surface. Endocytosis may be dependent on clathrin, as is true in the case of the transferrin receptor, or may be independent of clathrin mediation, as is the case with glycosylphosphatidylinositol (GPI)-anchored proteins, caveolae, or lipid raft mechanisms (Mayor and Riezman, 2004). Once internalized, the endocytotic vesicles are transported to early endosomes in which the mildly acidic pH (~6.2) causes an un-coupling of housekeeping receptors from their ligands to provide transport, along with other proteins and lipids, either to be recycled back to the plasma membrane or a continued commitment further through the endocytotic pathway to MVBs and late endosomes (Maxfield and McGraw, 2004).

The manner in which MVB formation occurs is not entirely clear, however, two theories have been put forth to explain the possible pathways involved. MVBs are

formed either from a maturation of early endosomes (maturation model) or by vesicular transport to MVBs from the early endosome via direct detachment (stable-compartment model) (Gruenberg and Stenmark, 2004). In either case, as MVBs are formed, proteins, lipids, mRNA, and miRNA are collected into vesicles, termed intraluminal vesicles (ILVs), formed within the MVB lumen by invagination and scission of buds from the limiting membrane via mechanisms that are not entirely clear (György *et al*., 2011). This process occurs as the MVB travels along microtubules to the late endosome, often located in the perinuclear region, both organelles tending to be more spherical in shape than the early endosome with the late endosome providing a more acidic environment at a pH of approximately 5.0 to 6.0 (Maxfield and McGraw, 2004). Late endosomes can progress through the endocytotic pathway via three main sorting routes including; trans-golgi network return, lysosomal fusion for degradation, or through a direct plasma membrane interaction in which the late endosomal contents, ILVs, are released into the extracellular environment as exosomes (van der Goot and Gruenberg, 2006).

The mechanisms underlying sorting of content into ILVs, as well as docking and fusion of the MVB and late endosome with the cellular plasma membrane for the release of exosomes, are currently unknown, however potential theories have been put forth. One theory involves the Endosomal Sorting Complex Required for Transport (ESCRT) machinery which includes four multimeric protein complexes, ESCRT – 0, I, II, and III (de Gassart *et al*., 2004). ESCRT-0 is made up of HRS (hepatocyte growth factor-regulated Tyr-kinase substrate) or Vps27 in the case of yeast, and STAM (signal transducing adaptor molecule). ESCRT-0 first recognizes cargo, typically protein, tagged

with ubiquitin, a 76 kDa protein that attaches covalently to its target protein (Reggiori and Pelham, 2001).

Clathrin is recruited and ubiquitin-tagged cargo is sequestered in clathrin-coated microdomains through ESCRT-0 association with endosomal membranes via the interaction of HRS FYVE (Fab1, YOTB, Vac1, and EEA1) domain with PtdIns3P (phosphatidylinositol-3-phosphate), a phosphoinositide found in high abundance in endosomal membranes (Williams and Urbé, 2007). Cargo tagged with ubiquitin can also be recognized by HRS itself through a ubiquitin-interacting motif (UIM). HRS also functions to recruit downstream ESCRT complexes by direct interaction with TSG101 (tumor susceptibility gene-101), a component of the ESCRT-I complex (Clague and Urbé, 2003). The ESCRT-I complex is not only composed of TSG101, which binds ubiquitin via its N-terminal UEV domain, but also of Vps28, and one of four isotypes of Vps37 (Vps37A-D) (Teo *et al* 2004). Vps (vacuolar protein sorting) proteins are encoded by the class E VPS genes originally described in yeast (Raymond *et* al., 1992). ESCRT-I recruits the next pathway complex, ESCRT-II, which is composed of Vps22, Vps25, and Vps36 and functions to bind ubiquitin via its GLUE (Gram-like ubiquitin-binding in EAP45) domain of Vps36 as well as transiently associate with endosomes and provide for ESCRT-III recruitment (Babst *et al.*, 2002).

ESCRT-III is composed of the most constituents and associated proteins, more than the other ESCRT complexes, including Vps2A,B, CHMP3 (charged MVB proteins or chromatin modifying protein-3), SNF7-1,-2,-3, and CHMP7, among others (Williams and Urbé, 2007). ESCRT-III has an integral function in the final steps of the pathway as

it removes ubiquitin via Doa4 (degradation of alpha-4), a de-ubiquitinase, as well as disassembly of the entire ESCRT complex via Vps4, an AAA+ ATPase (Babst *et al*., 1998). The physical invagination mechanism of the endosomal membrane for content uptake into the endosome into ILVs is currently unclear, however possible mediators have been described. Gruenberg and Stenmark (2004) suggest that due to the small, highly charged coiled-coil proteins of ESCRT-III in polymeric form, inward vesiculation occurs. Williams and Urbé (2007) proposed that the ESCRT machinery recruits factors that induce curvature of the limiting membrane, possibly located at the neck of the budding vesicle, or via sequestration of inverted cone-shaped components. Possible factors include LBPA (lysobisphosphatidic acid) and its putative effector Alix (ALG-2 interacting protein X) or specific transmembrane proteins (van der Goot and Gruenberg, 2006). An example of a transmembrane protein that may play a role includes the tetraspanins which have high amounts of glycosylation on the luminal side of the membrane and form microdomains, termed tetraspanin-enriched membrane microdomains (TEMs), which provide a platform for signaling (Zöller, 2009).

Another theory for the underlying mechanism behind sorting of content into ILVs involves a ubiquitin-independent pathway while utilizing the ESCRT machinery, most likely by direct interference with ESCRT-I and –III. For example, the transferrin receptor in reticulocytes involves recruitment of the ESCRT machinery by a direct interaction between Alix with Tsg101 and CHMP4 (de Gassart *et al*., 2004). Yet another proposed mechanism involves protein sorting into ILVs independently of the ESCRT complex, involving lipid raft-based microdomains and ceramide. Trajkovic *et al*. (2008)

studied the molecular content of mouse oligodendroglial cell (Oli-neu) derived exosomes with nano-electrospray ionization tandem mass spectrometry and found that the lipid content was very similar to that of lipid rafts with an enrichment in sphingolipids and ceramide. When Oli-neu cells were treated with a neutral sphingomyelinase inhibitor (GW4869), used to block the formation of ceramide, exosome formation and release was inhibited, suggesting a role for ceramide in this process (Trajkovic *et al.*, 2008). Sorting of exosomal content and ILV formation is most likely a function of several pathways evidenced by the diversity of proteins and other material found in vesicles upon release from the cell. Cargo itself may even affect vesicular membrane properties by simply recruiting different partners (van der Goot and Gruenberg, 2006).

ILV organization and formation is followed by release from the cell as exosomes which necessitates an interaction with the plasma membrane including transport, docking, and fusion of MVBs with the plasma membrane. The mechanisms underlying these processes are largely unknown; however studies suggest the involvement of proteins responsible for membrane transport and fusion events such as Rab11, a member of the Rab family of small GTPases that mediate regulation of vesicle trafficking between various cellular compartments along the endosomal and secretory pathway. Savina *et al.* (2005) studied involvement of Rab11 and calcium in MVB docking and release of ILVs as exosomes in K562 cells, a human erythroleukemic cell line that expresses a high amount of Rab11 mRNA. Rab11 was found to act in the docking of the MVB to the plasma membrane while calcium was required for the final fusion and release event.

Content of Exosomes

Exosomes secreted from various cell types share common characteristics such as lipid bilayer composition, size, density, protein composition, mRNA, and miRNA composition, as well as characteristics specific to the cell of origin, such as cell-specific proteins, mRNA, and miRNA (Valadi *et al*., 2007; Camussi et al., 2010; Lässer *et al*., 2011). Exosomal membranes are composed of a sphingomyelin and ceramide enriched lipid bilayer that displays a rapid flip-flop of lipids between the bilayer leaflets as well as an increase in rigidity upon exposure to a more neutral pH as encountered when released from the MVB with ~pH 5 to the extracellular environment with ~pH 7 (Laulagnier *et al*., 2004; Trajkovic *et al*., 2008). Exosomes range in size from 50 to 100 nm and exhibit density characteristics in a continuous sucrose gradient at 1.13 to 1.15 g/ml (Raposo *et al*., 1996; Théry *et al*., 2009).

Characteristic protein content of exosomes from various cell types reflects an endosomal origin which includes proteins from the endosomal compartment, plasma membrane, and cytosol and exclusion of proteins from other cellular compartments such as the nucleus, mitochondria, or endoplasmic reticulum (Keller *et al*., 2006). Exosomal surface proteins include: ESCRT proteins (Tsg101 and Alix), tetraspanin proteins (CD63, CD81, CD82, and CD9), integrins, and GPI-anchored molecules (CD55 and CD59) (Record *et al*., 2011). Exosomal lumen proteins include: cytoskeletal proteins (actin and tubulin), proteins involved in transport and fusion found on the luminal side of exosome membranes (clathrin, annexins, GTPases of the Rab family proteins), and chaperone proteins (Hsc70 and Hsp90) (Chaput and Théry, 2010). Proteins characteristic of specific

cell type derived exosomes include: CD11c on DC-derived exosomes, A33 antigen from intestinal epithelial cell-derived exosomes (van Niel *et al*., 2001), CD3 from T-cell-derived exosomes (Blanchard *et al*., 2002), MHCI and MHCII from antigen presenting cell-derived exosomes (Raposo *et al*., 1996), and aquaporin-2 in exosomes derived from urine (Pisitkun *et al*., 2004).

Exosomes have also been found to harbor mRNA and miRNA, which can then be transferred to a target cell and function to produce new proteins (Valadi *et al*., 2007). An mRNA or miRNA target for exosomes from multiple cell types has yet to be identified; however, various cell type specific mRNA and miRNA profiles have been recently identified. Valadi *et al*. (2007) revealed mRNAs from approximately 1,300 different genes from mouse (MC/9) and human (HMC-1) mast cell line-derived exosomes. Exosomes from glioblastoma tumor cells were also found to contain mRNA, miRNA, and protein which could be taken up and utilized by brain microvascular endothelial cells (Skog *et al*., 2008). More recently, Pegtel *et al*. (2010) described the release of exosomes from infected cells containing viral miRNA and subsequent transfer to non-infected cells that resulted in repression of target genes known to be regulated by these specific miRNAs. Also, human villous trophoblast cells were found to release exosomes containing miRNAs specific to the placenta into the circulation of the mother, suggesting a possible role in fetal-maternal communication (Luo *et al*., 2009). Exosomal cell type-specific proteins, mRNA, and miRNA possibly reflect their different functions and cells of origin, which provide for their potential use as diagnostic markers from multiple biological sources, such as urine, plasma, and serum.

Possible Modes of Exosome Interaction

Upon release from the parent cell, exosomes gain access to the extracellular environment and can interact with target cells locally or at a distance. Exosome action at distant sites is perhaps best demonstrated by tumor cell-derived and trophoblast cell-derived exosomes in circulation of patients, showing that secreted exosomes can travel far from the cells that produced them (Taylor and Gercel-Taylor, 2008; Luo *et al*., 2009). The study of exosome interaction with target cells has proven difficult due to their small size as conventional techniques, such as confocal microscopy, are unable to directly visualize them. Transmission electron microcopy is currently the only reliable method to visualize exosomes and their interactions and uptake in cells. Even so, indirect evidence and *in vitro* studies have proposed possible types of interaction between exosomes and target cells.

Interactions proposed to mediate exosome communication with a target cell include receptor-mediated adhesion, endocytosis, direct fusion, and possible fusion with endocytotic compartments upon internalization (Théry *et al*., 2009). Adhesion molecules are expressed on the surface of exosomes produced from various cell types and appear to provide for target cell capture as demonstrated by the reduced DC capture of exosomes when co-incubated with specific antibodies blocking various adhesion molecules, integrins, and tetraspanins (Morelli *et al*., 2004). A receptor-ligand interaction has also been proposed. For example, ICAM-1 (intercellular adhesion molecule 1) on the surface of DC-derived exosomes and LFA-1 (ligand lymphocyte function-associated antigen 1) on mouse DCs provide for exosome contained MHC-peptide complex presentation in CD

8[+] DCs and activated T-cells (Segura *et al*., 2007; Nolte-t' Hoen *et al*., 2009). Also,

TIM1 (T cell immunoglobulin domain and mucin domain protein 1) and TIM4 (T cell

immunoglobulin domain and mucin domain protein 4), molecules recently found to bind

phosphatidylserine (PS), are expressed on activated lymphocyte and phagocyte surfaces

which provide for uptake of exosomes, known to expose surface PS (Miyanishi *et al*.,

2007).

Events following receptor-ligand or adhesion interaction are currently

undescribed, however, studies suggest the possibility that exosomes can fuse with the

target cell membrane and either expel contents directly into the cytosol or be transported

to an endosome where fusion would then occur (Zöller, 2009). For example, exosomes

derived from mouse mast cells contained RNA that not only was transferred to human

mast cells but also induced transcription of mouse proteins in the human cell, indicating

that a form of fusion had occurred (Valadi *et al*., 2007). Another study demonstrated

fusion interaction with the transfer of an oncogenic form of EGF receptor (EGFR)

contained within vesicles to cells expressing the non-oncogenic, or wild-type, form of

EGFR where the oncogenic receptor induced abnormal intracellular signaling and

ultimate recipient cell transformation (Al-Nedawi *et al*., 2008).

Further evidence of exosome-target cell interaction is described in pregnancy and

immune system related experiments. Taylor *et al*. (2006) investigated exosomes isolated

from serum of pregnant women and their effects on the immune system, specifically T-

cell signaling. Serum exosomes from pregnant women that delivered at term with no

complications expressed FasL, a type-II transmembrane protein that belongs to the tumor

necrosis factor family (TNF) which causes apoptosis upon binding with its receptor, Fas (Taylor *et al*., 2006). When incubated with T cells (Jurkat cells), the serum-derived exosomes induced suppression of CD3-ζ and JAK3 proteins in the T cells, suggesting again that a fusion event had occurred.

The ζ-chain (zeta) of CD3 is an important intermediate for receptor signaling in T and NK cells while JAK3 is involved in the JAK/STAT pathway which is critical for regulation of T and NK cells, both of which are modified during pregnancy to provide for immune-tolerance of the fetus. Interestingly, exosomes from women with normal pregnancies that went to term exhibited higher expression of FasL and an increased amount of exosomes in the circulation when compared to exosomes isolated from pregnant women with pre-term delivery, suggesting a role in the normal immune regulatory function of pregnancy upon interaction of exosomes with T cells of the mother's immune system (Taylor *et al*., 2006).

The distinction between exosome surface fusion versus endosomal fusion within a target cell remains unclear, however, further study with systems such as pregnancy can provide worthy models to explain not only exosome functional roles in physiological states and disease, but also explain how exosomes are mediating the response. Results from such studies could provide the capabilities required to manipulate exosomes and in effect, manage the processes they are involved in, such as pregnancy and disease.

CHAPTER II

SERUM EXOSOME PROFILE AS RELATED TO EARLY PREGNANCY

STATUS IN THE MARE

Joanna R. Hergenreder

Colorado State University

Fort Collins, CO 80523

Introduction

Maternal recognition of pregnancy, as coined by Roger Short in 1969, refers to the mechanisms by which domestic animal species ensure continued secretion of the hormone progesterone by the CL beyond its normal cyclical lifespan (Allen and Stewart 2001), which is vital to maintain pregnancy. In essence, the CL must be protected from the agent which causes its demise, or luteolysis, in order for pregnancy to ensue. In many species, this luteolytic agent is endometrial prostaglandin $F_2\alpha$ ($PGF_2\alpha$) (McCracken *et al.,* 1970; Douglas and Ginther, 1972; Roberts *et al.,* 1996).

Early pregnancy in the mare is distinct in many aspects to that of various other domestic animal species. The physiological, morphological, immunological, and endocrinological changes that occur in the oviduct and uterus of the mare are vital to the success of the pregnancy yet are markedly different from equivalent processes in other

large domestic animal species, such as the cow, gilt, and ewe (reviewed by Allen, 2000).

$PGF_2\alpha$ functions as the luteolytic factor in the mare (Douglas and Ginther, 1972; Douglas

et al., 1974; Kooistra and Ginther, 1976), as is true for other domestic animal species,

however, the mechanism(s) behind maternal recognition of pregnancy in this species is

currently unknown. It is known, however, that if this mechanism fails and maternal

recognition does not occur successfully, luteolysis will ensue causing progesterone

production to fall resulting in a subsequent loss of pregnancy, known as early embryonic

loss (EEL).

EEL in the mare occurs at a rate of ~17% between days 12-20 with a majority of

loss occurring during the mobility phase of early pregnancy (Villahoz *et* al., 1985;

Meyers *et al*., 1991; Carnevale *et al*., 2000). Fertilization rates approach and often times

exceed 90% in this species, therefore, such a loss is high and has a deleterious economic

impact on the equine industry as well as necessitate the need to gain insight into the

mechanism responsible for successful pregnancy establishment (Ball *et al*., 1986 &

1989). One mechanism suggested to play a role in intercellular communication, such as

pregnancy, is exosomes, cell-secreted vesicles capable of transferring bioactive material

such as mRNA, miRNA, and protein from cell to cell locally and at a distance (Valadi *et

al*., 2007; Camussi et al., 2010; Lässer *et al*., 2011).

Various techniques have been employed to study exosomes in pregnancy and

include, among others, protein analysis, flow cytometry, and transmission electron

microscopy. Taylor *et al.* (2006) utilized protein levels in exosomes isolated from sera of

pregnant women to detect differences in exosome quantities between normal pregnancies,

abnormal pregnancies, and non-pregnant women. Redman and Sargent (2007) suggest the use of forward and side scatter in flow cytometry to detect a quantifiable difference of exosomes between pregnant and non-pregnant women. Flow cytometry characterizes exosomes by passing a fluid stream of said vesicles through a laser beam at high speed. Exosome passage through the laser creates light scattering properties detected as side scatter, indicative of texture or granularity, and forward scatter, indicative of relative size (György *et al.*, 2011). We hypothesized that exosomes would be present in mare serum and that their profile would differ with pregnancy. Specifically, we first aimed to identify exosomes in serum from pregnant and non-pregnant mares using flow cytometry. Secondly, we aimed to determine the relative amount of exosomes in pregnant and non-pregnant mare serum, also using flow cytometry. Thirdly, we aimed to determine the exosome profile in pregnant and non-pregnant mares using transmission electron microscopy, providing a validation measure with visual representation.

Experiment 1:   Exosome Protein Isolation

Materials & Methods

Care and Management of Horses

Two geldings, two stallions, and two mares were utilized in the exosome protein isolation experiment and were housed at Colorado State University Equine Reproduction Laboratory and Equine Sciences Teaching and Research Center in Ft. Collins, Colorado. Blood samples were obtained via jugular venipuncture using red-top BD Vacutainer® tubes (BD). All blood samples were allowed an hour at room temperature to clot, after

which they were centrifuged at 3000 x g for 10 minutes. Serum was removed, snap

frozen in liquid nitrogen, and stored at -80°C until further use. This experiment was

designed not to compare groups of horses (geldings, stallions, mares) but to develop an

effective exosome protein isolation protocol. Also, this experiment began as a

preliminary study with a future goal to study the exosomal protein concentration and

content in the serum of pregnant versus non pregnant mares and thus provide an answer

for aim one and two of our hypothesis. The results for this protocol therein provide for

future use in the study of protein in exosomes from serum in the mare.

Exosome Isolation from Serum

Exosomes were isolated from serum samples with ExoQuick[TM] (System

Biosciences, Inc), a proprietary precipitation solution designed to isolate exosomes from

body fluids. 400 μl of serum was transferred to a 1.5 mL sterile microcentrifuge tube and

100 μl ExoQuick[TM] was added. Gentle inversion was performed to ensure proper mixing

of serum and ExoQuick[TM] which was then incubated at 4°C overnight per manufacturer's

instructions. Exosomes were pelleted following the incubation step by centrifugation at

1500 x g for 30 minutes at room temperature. Supernatant was carefully removed, taking

care not to disturb the exosome pellet, and discarded. The resultant exosome pellet was

re-suspended in 100 μl 1X PBS and further processed for protein isolation.

Ultracentrifugation and flotation on a sucrose gradient was also performed to provide an

internal control following Théry *et al.*'s recommended protocol for isolating exosomes

from cell culture supernatants and biological fluids (Appendix I) (Théry *et al.*, 2006).

Protein Isolation

Protein was isolated from the re-suspended exosome pellet with TRI

REAGENT®BD (Molecular Research Center, Inc.) following manufacturer's

instructions. Modifications were applied to the protein wash and solubilization steps

within the protocol by using 0.3M guanidine hydrochloride in 95% ethanol and 8M urea

in Tris-HCl, pH 8.0 preceded by sonication to solubilize the protein pellet (Appendix II).

The Bradford Assay was utilized to determine protein concentration of the supernatant

(Appendix III) and Coomassie Blue staining was utilized to visualize protein on a 10%

(50 µl well) Tris-HCl SDS-PAGE gel (Bio-Rad, Hercules, CA). Cell lysate and protein

isolated from exosomes obtained from follicular fluid (Winger & Bouma Laboratories of

the Animal Reproduction and Biotechnology Laboratory, Ft. Collins, CO) were run on

the same gel to provide for a negative and positive internal control, respectively.

Results

Protein yield, indicated by the protein concentration obtained from the Bradford

Assay results (µg/µl), was highest when 8M urea in Tris-HCl (pH 8.0) along with

sonication in a BioRuptor system (Diagenode, Inc.) was utilized to solubilize the protein

pellet (Figure 1). Various other solubilization agents were utilized without success,

including; 1% SDS, BioRuptor sonication alone, a 30000K MWC Amicon Ultra

centrifugal dialysis filter (Millipore), a 1:1 ratio of 8M urea in Tris-HCl with 1% SDS,

and 8M urea in Tris-HCl. Also, M-PER® Mammalian Protein Extraction Reagent (Pierce

Biotechnology, Inc.) was utilized and provided similar protein yield as TRI-REAGENT®

BD isolated protein. Coomassie Blue stained gels indicated similar molecular weight

proteins as exosomes isolated from follicular fluid (Bouma Laboratory of the Animal

Reproduction and Biotechnology Laboratory, Ft. Collins, CO). Bradford Assay results

and Coomassie Blue stained gels indicated that protein yield from exosomes isolated by

ultracentrifugation and a sucrose gradient were much lower than from exosomes isolated

with ExoQuick™ (Figure 1 & 2). Lastly, when two different starting volumes of serum

were utilized for exosome isolation with ExoQuick™ (400 µl serum + 100 µl

ExoQuick™ and double that at 800 µl serum + 200 µl ExoQuick™), no doubling was

detected, indicated by Bradford Assay protein concentrations.

Discussion and Conclusion

In this experiment, we examined the use of TRI-REAGENT®BD to isolate protein

from exosomes isolated with ExoQuick™ from serum of the horse. Solubilization of the

protein pellet for analysis was found to be the most difficult step in this process and

required an 8M urea in Tris-HCl solution to successfully provide for solubilization and

high protein yield. This may be due to the presence of lipid rafts in exosomal membranes

which are found to be highly resistant to solubilization by non-ionic detergents as

indicated by de Gassart *et al*. (2003). Also, the lack of protein concentration doubling

when the serum sample used in exosome isolation was doubled indicates that this

technique will not provide a reliable means to identify the differences in exosome

concentrations found in serum and should therefore not be used to study the relative

exosome amount in serum of pregnant and non-pregnant mares. Results indicate the

utility of this protocol for future use for isolating protein from serum-derived exosomes

in the horse, however, further research is needed to determine the purity of the isolate as

well as validation of characteristic exosomal proteins to ensure that exosomes and their

associated proteins are indeed being isolated at high purity. Such results may be possible

with 2-dimensional SDS PAGE (polyacrylamide gel electrophoresis) and mass

spectrometry studies as well as western blots using antibodies against proteins known to

be found in exosomes, such as CD63, Hsc70, or Hsp90. Also, applications such as flow

cytometry and TEM could provide further validation based on size, shape, and

immunoreactivity. Flow cytometry is also a very good tool for accurately determining

cell counts and could possibly provide such a service for determining relative amounts of

exosomes as well.

Figure 1

Bradford Assay Results

Protocol & Modification	µg
Tri Reagent, 1% SDS	0.234
Tri Reagent, 1% SDS, Sonication	0.350
Tri Reagent, Dialysis filter	0.000
Tri Reagent, 4M Urea, 0.05% SDS	0.176
Tri Reagent, 8M Urea	7.027
Tri Reagent, 8M Urea, 0.5 mL sample	6.063
Tri Reagent, 8M Urea, 1.0 mL sample	7.587
M-PER	6.840
Ultracentrifugation	0.056

Figure 1)   Bradford assay results of exosomal protein isolated with TRI-REAGENT® BD, M-PER, and Ultracentrifugation. Protocol variants applied to samples are indicated in the table.

Figure 2

Coomassie Blue 1-D PAGE Results
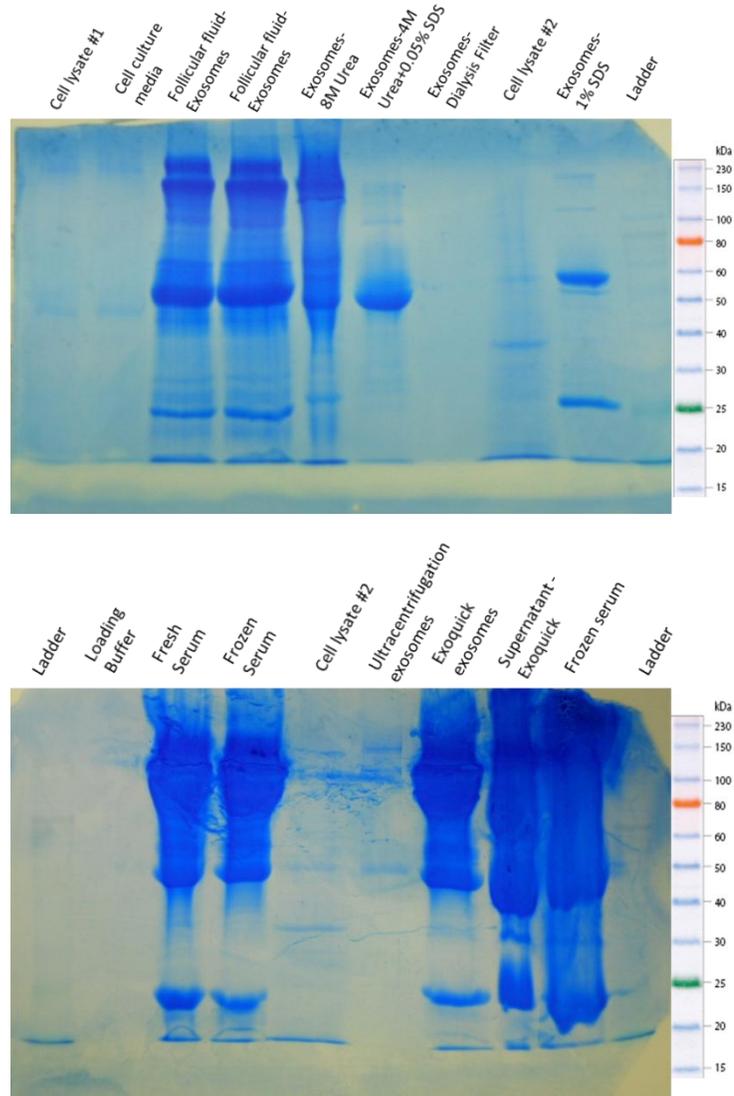
Figure 2)   Coomassie blue 1-D PAGE results, showing protein isolated from serum   exosomes using TRI-REAGENT® BD. Protocol variants applied to samples are indicated at the top of each lane.

Experiment 2:   Flow Cytometry and Transmission Electron Microscopy

Materials & Methods

Care and Management of Mares

Three Quarter Horse type mares were utilized for this study, each ranging in age from 8 to 15 years with a history of normal cyclicity. A simple cross-over design was employed with each mare serving as both a pregnant treatment and non-mated control. Ovarian follicular development was monitored daily by trans-rectal ultrasonography to determine estrous cycle status. For the pregnant treatment, follicular development was followed until a follicle of ≥ 35 mm was identified, after which mares were artificially inseminated every other day until ovulation was detected. Insemination with a minimum of 500 million progressively motile sperm from a stallion of known fertility was used and subsequent embryo presence was determined by trans-rectal ultrasonography at day 12, 14, 16 and 18 post-ovulation. Blood samples were obtained at days 12, 14, 16, and 18 via jugular venipuncture using red-top BD Vacutainer® tubes (BD). Following blood collection on day 18, embryos were collected by terminal uterine lavage to further confirm pregnancy status.

For the non-mated control, the following estrous cycle was utilized. Trans-rectal ultrasonography was performed on a daily basis to determine the day of ovulation with the mares left unmated. Blood samples were obtained at days 12, 14, 16, and 18 post-ovulation as described to serve as the non-pregnant control sample for each mare. Blood was also obtained from three additional mares including one day 7 non-pregnant mare,

31

one day 12 pregnant mare, and one day 43 pregnant mare which were all pooled to

provide for preliminary troubleshooting experiments with the flow cytometer and a

biological standard to manage flow cytometry settings within the main experiment. All

blood samples were allowed an hour at room temperature to clot, after which they were

centrifuged at 3000 x g for 10 minutes. Serum was removed, snap frozen in liquid

nitrogen, and stored at -80°C until further use.

Exosome Isolation from Serum

Exosomes were isolated from serum samples with ExoQuick[TM] (System

Biosciences, Inc.), a proprietary precipitation solution designed to isolate exosomes from

body fluids. Serum was slowly thawed on ice and 400 µl transferred to a 1.5 ml sterile

microcentrifuge tube after which 100 µl ExoQuick[TM] was added. Gentle inversion was

performed to ensure proper mixing of serum and ExoQuick[TM] which was then incubated

at 4°C overnight per manufacturer's instructions. Exosomes were pelleted following the

incubation step with centrifugation at 1500 x g for 30 minutes at room temperature.

Supernatant was carefully removed, taking care not to disturb the exosome pellet, and

discarded. The resultant exosome pellet was re-suspended and processed for flow

cytometry or transmission electron microscopy.

Flow Cytometry

Exosome pellets were re-suspended in 700 µl of 1X PBS (DBPBS HyClone

Sterile, 0.1µm filtered). The exosome pellet was relatively sticky, therefore re-

suspension was more successful when a pipet was used to gently push the PBS back and

forth at the juncture of the pellet and tube wall and continued until material in the PBS

could no longer be visualized. Preliminary troubleshooting experiments using the serum

samples intended for the first experiment with exosomal protein isolation were also

utilized here and labeled with a mouse monoclonal antibody for human CD63 (Alexa

Fluor®488-CD63; Santa Cruz Biotechnology), a tetraspanin protein found to be highly

enriched in exosomes (Escola *et al*., 1998). Re-suspended samples were then transported

on ice to the Proteomics and Metabolomics Core Facility at CSU for analysis by the

MoFlo (Dako Colorado, Inc.) Flow Cytometer and High Speed Cell Sorter. Immediately

prior to analysis by the flow cytometer, samples were pipetted through a 50 μm filter to

ensure no large clumps were present to cause a blockage in the flow cell nozzle. Each

filtered sample was then split into two 300 μl aliquots in microcentrifuge tubes for

analysis which provided for a duplicate of each exosome pellet.   Counts of exosome

events were obtained through gating on forward and side scatter along with the single

platform approach using counting beads (CountBright™ absolute counting beads;

Invitrogen) following the manufacturer's recommendations (Appendix IV). Each 300 μl

sample received 25 μl of CountBright™ absolute counting beads prior to analysis.

Multiple controls were utilized and included the following; a pooled serum

exosome sample, 1X PBS, 100 nm-sized standard beads, 1X PBS with 25 μL counting

beads, and a pooled serum exosome sample with counting beads. The pooled serum

exosome sample was used to determine the area of focus on the scatter plot and set the

gates of analysis, while the 1X PBS was used to determine the background noise of the

machine. Background was determined acceptable at ≤ 1% of events detected in the

exosome gates set for analysis. Sized beads of 100 nm (PolySciences, Inc.) provided for a size standard detectable on the scatter plot. Serial dilutions of the sized beads were performed and the 2 x 10^9/ml dilution was chosen for analysis. Summit V4.3 software was utilized to analyze all flow cytometry data (Dako Colorado, Inc.). The experimental design for the MoFlo involved analyzing the controls first followed by the pregnant and non-pregnant mare serum-derived exosome samples. Also, a wash step with sterile filtered water was employed after each sample was run to ensure that neither beads nor exosomes were sticking to the lines of the machine, which provided for prevention of subsequent sample contamination. Statistical analysis was performed on the absolute counts of exosome events detected in gates R4 and R10, two areas on the FACS scatter plot indicative of exosomes, calculated with the CountBright^{TM} bead formula and analyzed with the ProcMixed model in SAS. Paired T-test was used to compare relative exosome amounts of the R10 gate on day 12 between pregnant and non-pregnant samples.

Transmission Electron Microscopy Analysis

The pooled serum sample was utilized for transmission electron microscopy (TEM) analysis. Exosomes were isolated from serum with ExoQuick^{TM} as described above. For this TEM experiment, three samples from the pooled serum were generated yielding three exosome pellets. Exosome pellets were isolated and re-suspended in 200 μl of 1X PBS (DBPBS HyClone Sterile, 0.1μm filtered). The exosome pellet was relatively sticky, therefore re-suspension was more successful when a pipet was used to gently push the PBS back and forth at the juncture of the pellet and tube wall and

continued until material in the PBS could no longer be visualized. The re-suspended

pellets (3) were then transferred to the same 5 ml ultracentrifuge tube. After transfer, the

volume of the tube was brought to 5 ml with additional 1X PBS, a requirement to prevent

the tube from collapse when centrifuged at high speeds. Ultracentrifugation was

performed at 100,000 x g for 70 minutes to wash and remove the ExoQuick^{TM} and

sediment the exosomes.

After ultracentrifugation, the supernatant was carefully removed with a gel-

loading tip. The pellet and remaining PBS were transferred to a 1.5 ml microcentrifuge

tube with a normal pipet tip after 200 μl of 2% agar gel was added, taking care not to

place directly on the pellet. Bench-top centrifugation was performed at 17,500 x g for 15

minutes to form a more visible exosome pellet. Supernatant was carefully removed and

discarded, after which, 50 μl of 2% agar was added to the pellet. The sample was then

placed on ice for 15 minutes to allow for the agar matrix to harden. The exosome pellet

in agar matrix was removed from the tube with a spatula and excess matrix was sectioned

out. The resultant pellet was fixed in 2.5% glutaraldehyde in 5% sucrose, 0.1M sodium

cacodylate, pH 7.4 for 30 minutes and washed thrice in cacodylate buffer. The pellet was

post-fixed with 1% osmium tetroxide (OsO_4) for 90 minutes, washed thrice in cacodylate

buffer, dehydrated in a series of alcohols and propylene oxide, and embedded in Poly/bed

812 (Appendix V). Thick (~5 μm thick) sections and thin (~1 μm thick) sections were cut

on an ultramicrotome equipped with a diamond knife. Thick sections were prepared for

light microscopy and thin sections for TEM. Thin sections were placed on nickel coated

grids and stained with uranyl acetate and lead citrate for 15 minutes each. Grids were

allowed to dry and examined with a JEOL 1200EX transmission electron microscope.

Results

Flow Cytometry Analysis

Results obtained from the flow cytometry experiments indicate isolated vesicles

were exosomes as scatter profiles revealed greater than 90% of the population events fall

within the 100 nm size range. This is the characteristic size of exosomes, as indicated by

forward scatter measurements with the specific scatter profile area set by the 100 nm-

sized standard beads (Figure 3). Also, preliminary flow cytometry experiments indicated

approximately 40% of the vesicles stained positive for anti-CD63, providing further

evidence that the vesicles isolated harbor CD63, which is characteristic of exosomes

(Figure 4). All samples analyzed revealed this 100 nm size range scatter profile, while

also exhibiting varying degrees of granularity or texture, as indicated by side scatter

measurements. Counting bead recovery was determined to be 91.8% and found to be

consistent between samples. Relative exosome amounts did not significantly differ

between pregnant and non-pregnant mares at any of the days examined (p>0.05) (Figure

5), which may be due to the small sample size and variation observed between mares

(Figure 6). However, when events measured in gate R10 (a unique population of smaller

and less granular exosomes) were considered alone, a trend for higher amounts (p=0.09)

were found in pregnant samples at day 12 (Figure 7). Also, statistical analysis revealed

an effect by day, indicating a decrease in relative exosome amounts in both pregnant and

non-pregnant mares from day 12 to day 14 (p≤0.05) and an increase in exosome amounts in pregnant mares from day 14 to day 16 (p≤0.05) (Figure 8).

Transmission Electron Microscopy Analysis

TEM revealed similar profiles by size and shape to those detected by flow cytometry, showing a small variation in size and granularity of exosomes isolated, corroborating our findings with flow cytometry. A majority of the vesicles visualized were in the 100 nm size range indicative of exosomes (Figure 9). Additional vesicles were present both at size ranges larger than exosomes (~200 nm) and smaller than exosomes (~≤ 50 nm) (Figure 9), suggesting the presence of microvesicles in addition to exosomes. Interestingly, the smaller less granular exosomes detected with flow cytometry (gate R10) may indeed be the smaller less dense population visualized with TEM (Figure 9), further validating our results and the utility of these applications to study exosomes in serum of mares. Broken-down membranous profiles were also evident indicating degradation of membranes because of possible freeze damage as serum was snap-frozen in liquid nitrogen without cryoprotectants and large amounts of electron-dense material possibly containing serum constituents and agar matrix were also observed (Figure 10).

Discussion

Preliminary flow cytometry experiments indicated nearly half of the events recorded to be positively stained with Alexa Fluor®-CD63, a mouse monoclonal antibody raised against the full length of human CD63. It is possible that this CD63 specific to the

human sequence may not bind efficiently to the horse CD63 being analyzed and therefore may explain the lack of binding. Time constraints prevented further optimization using this antibody as well as the option of attempting other CD63 antibodies which could provide multiple sequence options for possible binding with more efficiency to the horse CD63 located on the serum exosomes analyzed in this experiment. As a result, antibody staining was not employed in the subsequent flow cytometry experiments with serum exosomes from pregnant and non-pregnant mares. Further investigation into antibodies specific to exosomes from horse serum could provide an additional means of characterization and validation when conducting flow cytometry experiments. These may include the use of multiple targets in addition to CD63, such as Hsp70, Hsp 90, and Tsg101, all found to be present in exosomes (Record *et al*., 2011; Chaput and Théry 2010). Even so, positive staining for CD63 suggests the presence of exosomes in pregnant and non-pregnant mare serum.

Flow cytometry analysis revealed the presence of exosomes in pregnant and non-pregnant mare serum as indicated by forward and side scatter with a size of approximately 100 nm. Flow cytometry as an application for quantifying exosomes in serum of the mare was made possible with the use of counting beads and internal controls which provided accuracy and reproducibility. The positive controls, such as the pooled exosome sample and 100 nm sized standard beads used in our experiment as well as negative controls, such as the PBS, were necessary for setting up the flow cytometer properly each time. For instance, the lower border was determined after assessing the signal versus noise with positive and negative controls. The gates were essentially set in a way to exclude possible noise while still detecting the highest number of signal events,

which is a challenge when detecting objects of such a small size. Background noise in this experiment was kept at ≤1% within established gates; however, a further improvement could be made by utilizing a smaller pore-filtered PBS to exclude any particles that may overlap in size with exosomes. A 0.01 μm pore size membrane filtered PBS from Millipore was suggested for use with flow cytometry recently by Gyorgy *et al.* (2011) to minimize noise attributed to the sample diluent and could perhaps provide an even better negative control than what was used in the present experiment which utilized 0.1 μm filtered PBS. In addition, a wider range of sized standards such as 60nm to 1000nm, could assist in setting gates and provide more parameters to judge noise versus signal events (Abusamra *et al.*, 2005).

Flow cytometry results indicated no difference of overall exosome amounts between pregnant and non-pregnant mares, however, TEM analysis revealed the presence, not only of exosomes and various other sized vesicles, but also an abundance of unidentified dense material. Interestingly, Gyorgy *et al.* (2011) revealed that protein complexes, especially those of insoluble immune complexes, overlap vesicles in biophysical properties such as size (50-250nm), light scattering, and sedimentation when analyzed by flow cytometry. This could perhaps explain the dense material viewed with TEM in the present experiment, although further investigation with specific markers is needed. Even so, if the dense material is similar in properties with insoluble immune complexes, it stands to reason that the difference of relative exosome events or amounts in this experiment was masked by these proteins. Future experiments quantifying exosomes in mare serum should employ a modified exosome isolation procedure to

ensure the exclusion of serum protein contamination. TEM analysis also indicated

multiple sites of membrane degradation which suggests freeze damage and therefore

future experiments should utilize fresh samples. Also, immunogold labeling of specific

targets characteristics of exosomes, such as CD63 or Hsc70, when analyzing with TEM

will provide further validation of our findings and delineate exosomes from microvesicles

or other cellular vesicles present in the isolate.

Collectively, our results indicate no significant difference in overall exosome

amounts in pregnant and non-pregnant mare serum during days 12 to 18 of early

pregnancy (Figure 5). It is possible that overall exosome amounts are quantitatively

different later in pregnancy when the endometrial cup reaction, or first attachment (day

~38 to 80), occurs and formation of the placenta ensues due to the presence of more

trophoblast cells (Ginther, 1998). Interestingly, human trophoblast cells have been found

to produce and secrete exosomes and can be detected in high amounts in late-term

pregnant women (Taylor *et al*., 2006; Luo *et al*., 2009). Results from the relative

amounts of the small less granular population of exosomes would suggest that even

though overall exosome amounts did not differ, specific populations (R10: small less

granular exosomes) tended to be higher in pregnant mare serum suggesting a role for this

population of exosomes at day 12 of pregnancy in the mare and warranting further study

at this time point in early pregnancy in the mare. These results do not indicate what

particular role this may be as exosomes in the serum can essentially originate from any

organ which has access to the circulation, however, additional research is needed to

indicate cell of origin and content of exosomes which will provide valuable tools and

insight into their involvement in the process of early pregnancy in the mare. For

example, an *in vivo* endometrial explant culture system with equine embryo explants/secretions, as well as *in vitro* endometrial cell culture could elucidate the profile of exosomes secreted from the endometrium and conceptus including specific markers that could be used to detect and isolate pregnancy specific exosomes from the circulation.

Conclusion

Our results demonstrate the presence of exosomes in the serum of pregnant and non-pregnant mare serum and according to our flow cytometry results, the relative amount of exosomes do not differ with pregnancy status. Also, we have shown that flow cytometry can be utilized to determine relative exosome amounts in serum. TEM analysis validated our flow cytometry findings and provided a visual profile of exosomes in mare serum, further supporting the use of such an application for the study of exosomes. Even though relative amounts did not differ with pregnancy status, exosomes were indeed present and this may suggest that exosomes are playing a role in early pregnancy not by sheer number, but possibly by cargo that they carry. Further research is therefore needed to analyze the exosomal content of pregnant and non-pregnant mare serum during early pregnancy.

Figure 3

Representative Scatter Profile of 100nm Sized Beads, PBS, and Pooled Exosome Controls
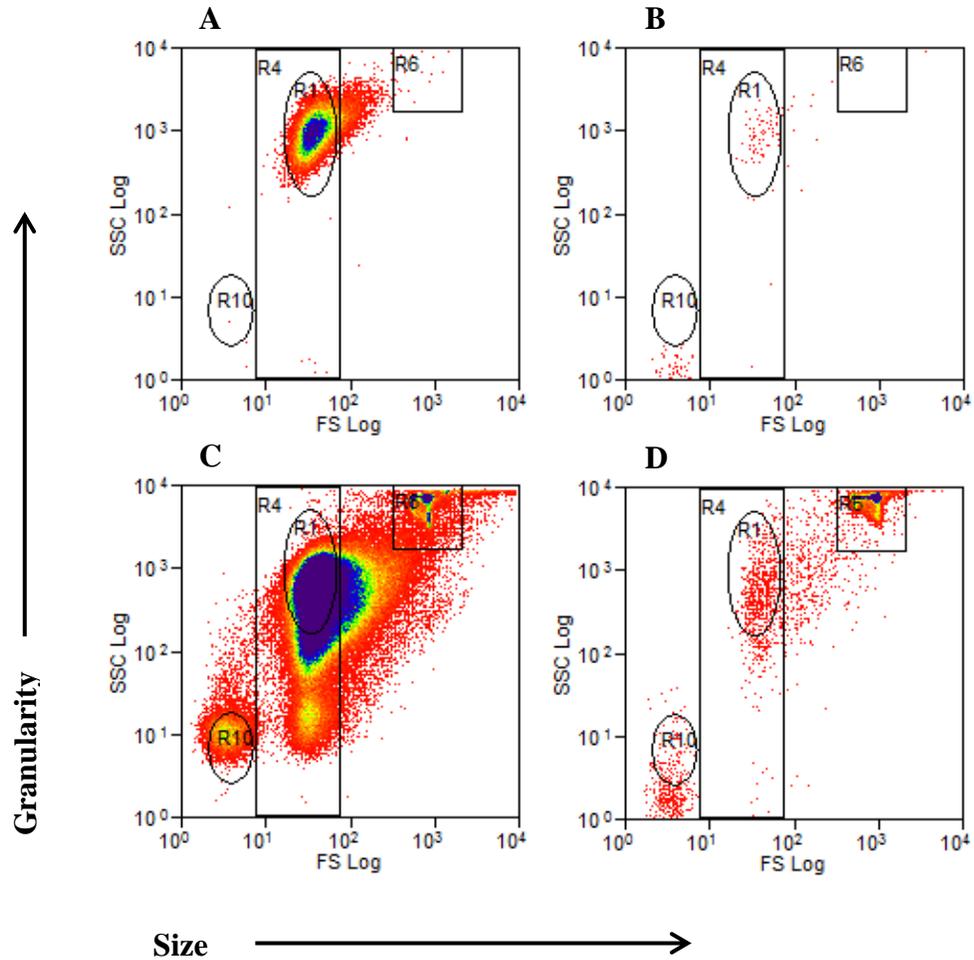
Figure 3)   Representative scatter plots of controls utilized to set gates and threshold (setting). R1: 100nm sized standard beads, R4: Events in the 100 nm size range with variable granularity, R6: CountBright^{TM} absolute counting beads, R10: Unique smaller and less granular population. **A**: 100 nm sized standard beads, **B**: PBS alone, **C**: Pooled exosome sample, **D**: PBS with counting beads.

Figure 4

CD63 Histogram

A

B
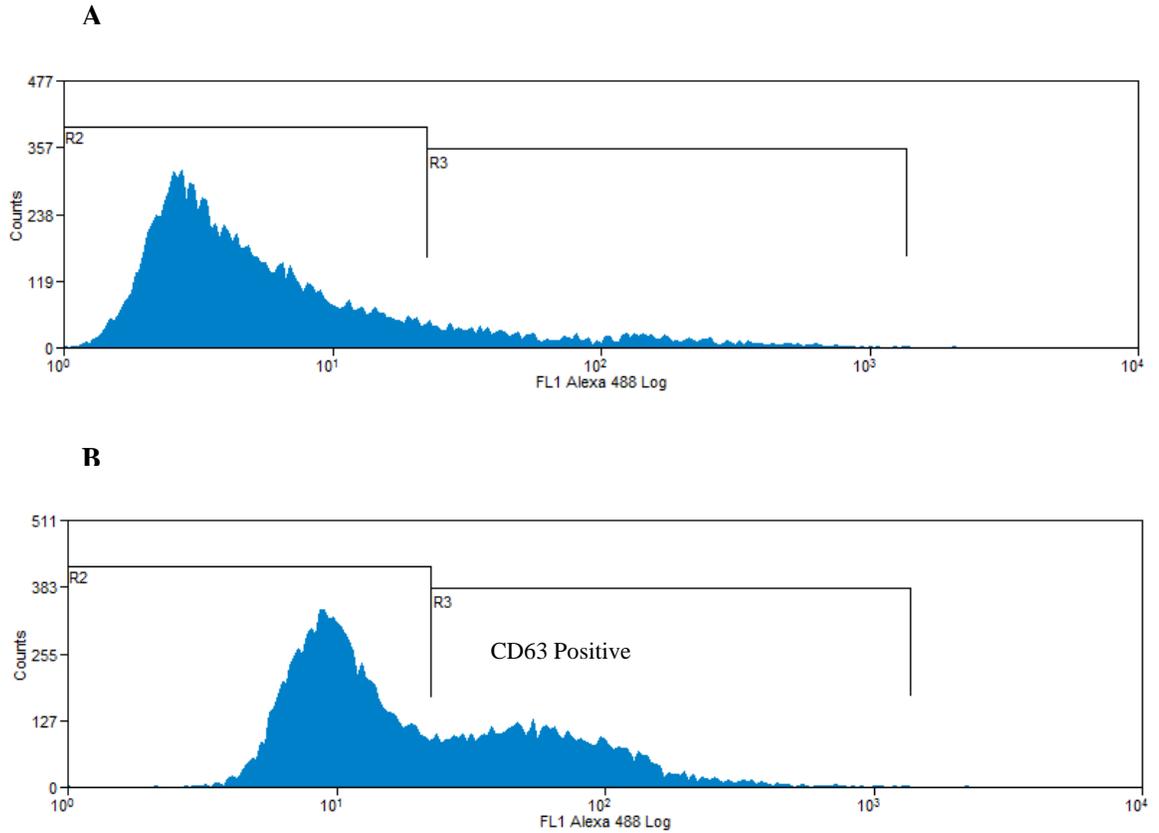
Figure 4) Representative histograms of non-pregnant mare exosomes labeled with Alexa Fluor®488-CD63. **A**: Blank, unlabeled exosomes, **B**: Labeled exosomes from a non-pregnant mare showing 39.36% of total events positive for CD63.

Figure 5

Relative Exosome Amounts by Day and Pregnancy Status
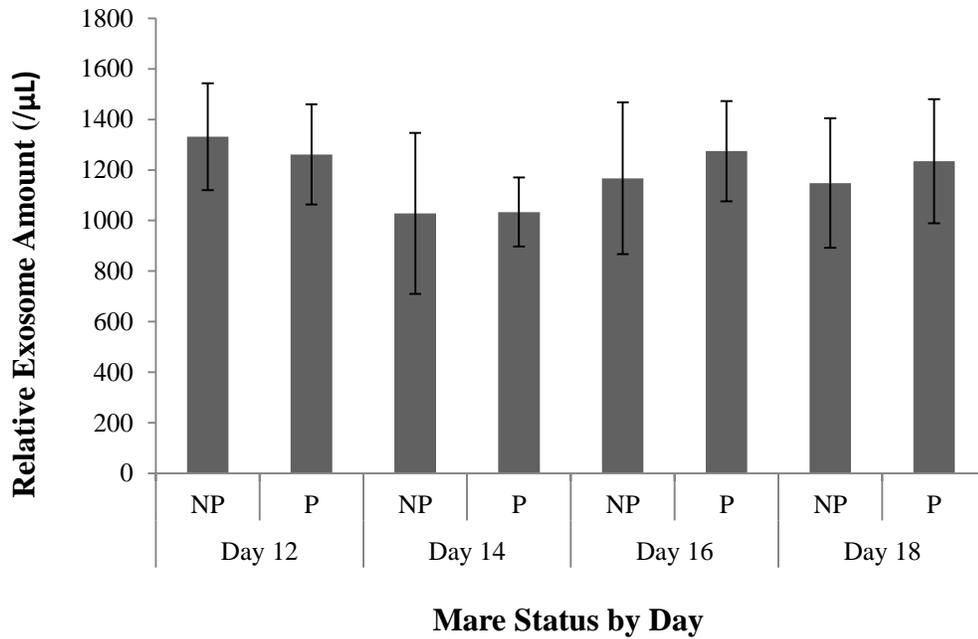
Figure 5)   Changes in relative exosome amounts per time point of serum collection and mare pregnancy status. NP denotes non-pregnant. P denotes pregnant. Error bars represent SEM values.

Figure 6

Relative Exosome Amounts by Day, Pregnancy Status, & Mare
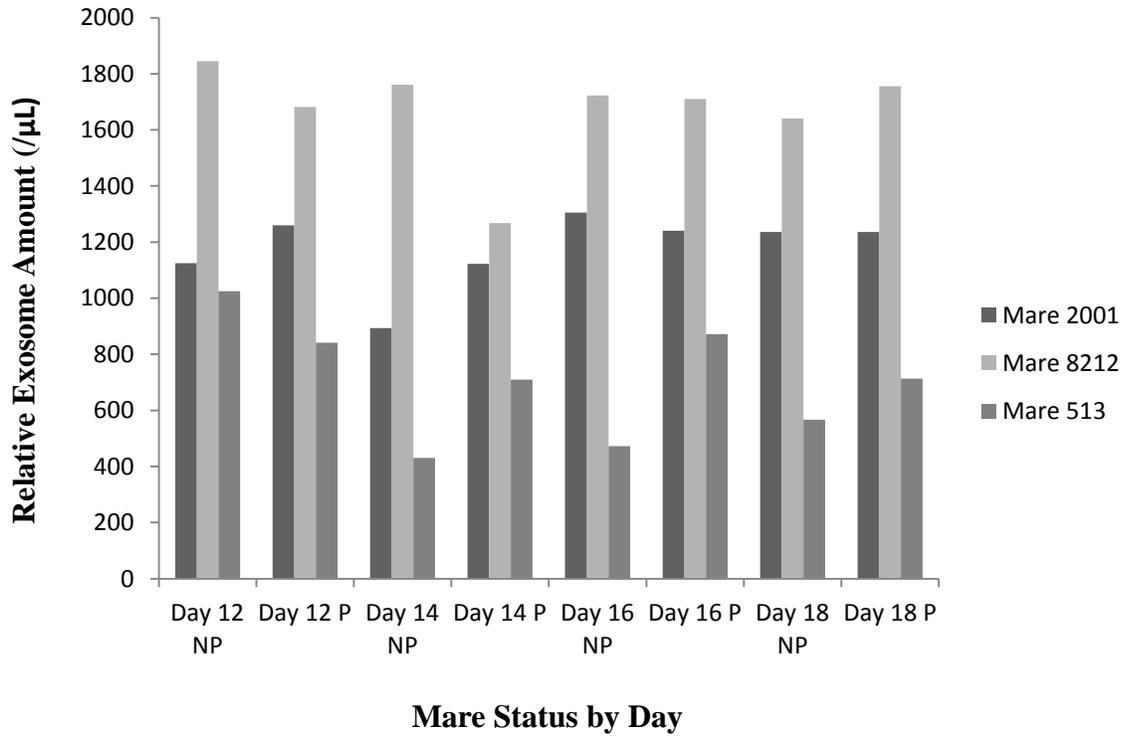
Figure 6) Changes in relative exosome amounts per time point of serum collection, mare pregnancy status, and individual mare. NP denotes non-pregnant. P denotes pregnant.

Figure 7

Changes of Smaller Exosome Population at Day 12
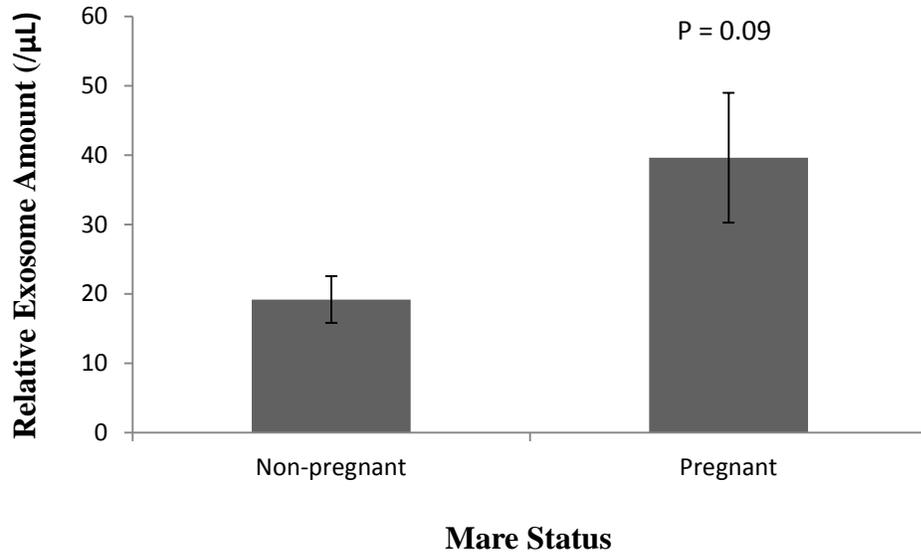
Figure 7)   Changes in relative exosome amounts of events detected in the R10 gate of the flow cytometer scatter plot at day 12 of serum collection. Error bars represent SEM values.

Figure 8

Changes of Relative Exosome Amounts by Day
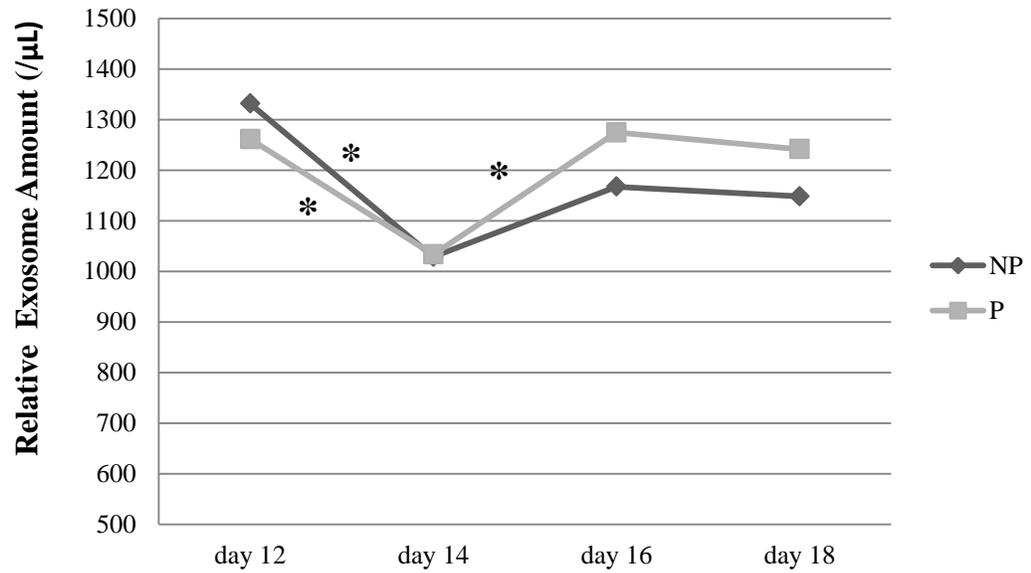
Figure 8) Changes in relative exosome amounts of events by day. NP denotes non-pregnant. P denotes pregnant. (*) indicates significant difference between days analyzed within pregnancy status at $P < 0.05$.

Figure 9) (**A**), (**B**) Two transmission electron micrographs of pooled serum exosomes (20,000 x). Open arrow denotes 50-100 nm sized exosomes. Solid arrows denote exosomes around 50 nm. Solid arrow heads denote vesicles larger than 200 nm, possible microvesicles. Scale bar equals 200nm.

Figure 9

TEM Images of Pooled Serum Exosomes
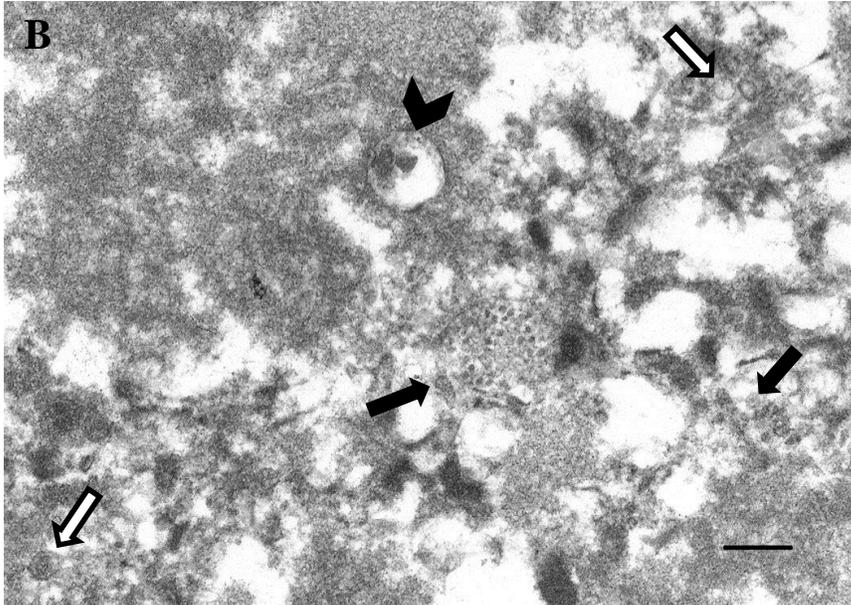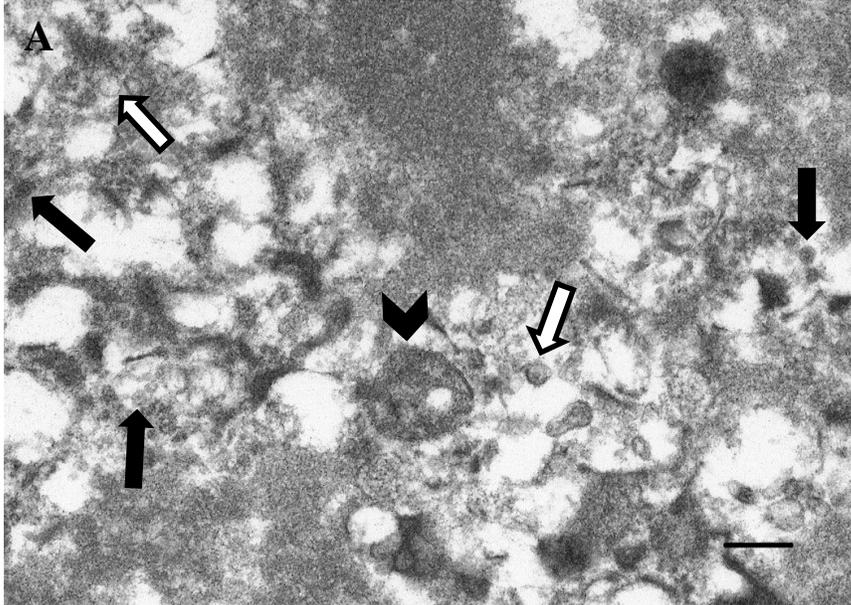
Figure 10

TEM Image of Pooled Exosomes with Freeze Damage
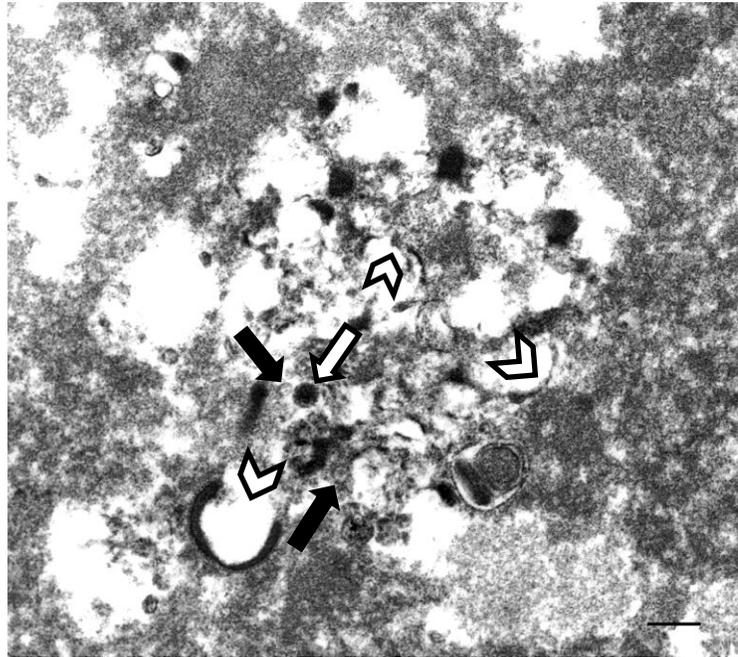
Figure 10)   Transmission electron micrograph of pooled serum exosomes (20,000 x).
Open arrow denotes 50-100 nm sized exosomes. Solid arrows denote exosomes around
50 nm. Open arrow heads denote freeze-damaged membranes. Scale bar equals 200nm.

CHAPTER III

DISCUSSION

In this study we examined the possibility that exosomes are present and detectable in serum from the mare and that early pregnancy in this species would involve a quantifiable difference in relative exosome amounts when compared to the non-pregnant state. Flow cytometry and TEM data revealed that exosomes can be isolated from mare serum using ExoQuick^TM with relative ease; however, further modifications are needed to remove or prevent possible contamination by serum proteins and RNA. Perhaps incorporating differential centrifugation before ExoQuick^TM incubation will help remove excess serum proteins and RNA and in so doing, eliminate or reduce the chance of contamination and possibly reduce the amount of dense material visible with TEM analysis.

Differential centrifugation will also remove platelets, which have been shown to be activate upon sheer stress (shaking of the tube) and release vesicles, possibly making this method a more accurate isolation procedure and representation of exosomes present in serum (Miyazaki *et al*., 1996; Shah *et al*., 2008). Along these lines, it may also be useful to analyze platelet-free plasma in addition to serum as procurement of serum requires the formation of a clot and possible exosome loss as a result. Most importantly,

if the dense material viewed with TEM in our experiments contains serum proteins, such as insoluble immune complexes, reducing or eliminating their presence in serum exosome preparations could reveal quantifiable differences between pregnant and non-pregnant mare samples when analyzed by flow cytometry, results we were unable to detect with statistical significance (Figure 5, 6). Supporting evidence for such an influence was revealed upon examination of the smaller less granular population (R10) of exosomes at day 12 with pregnant mares showing a trend (p=0.09) for higher amounts present in serum. The R10 population differences at day 12 also suggest the possibility of a role for exosomes in the mechanisms behind early pregnancy at this time point. Exosomes in serum, however, could be from a variety of regulatory events and/or sources that are not exclusively related to pregnancy. Future research should focus on exosomal cells of origin through immunolocalization using potential pregnancy-associated markers to delineate a more precise role for exosomes in the process of early pregnancy in the mare. In addition, statistical analysis determined a decrease in exosome amounts in both pregnant and non-pregnant mare serum from day 12 to day 14 and an increase in pregnant mare serum from day 14 to day 16. Further research is needed to determine what specific effects are *caused* by exosome amount differences between days or what *causes* exosome amount differences in the circulation of pregnant and non-pregnant mares from day to day in early pregnancy.

Results presented from our experiments go beyond answering our hypothesis as we have provided contributions to further study early pregnancy in the mare with successful technical methods useful in the study of exosomes, including flow cytometry and TEM. Modifications to our methods used in these experiments could greatly

improve results in future studies, as mentioned previously, however; an even more important piece of the puzzle lies in the future direction of studies involving early pregnancy in the mare and exosomes. Future experiments should include analysis of exosomal contents, including protein characterization, mRNA content, and miRNA content. Insight into the cargo of these exosomes isolated from pregnant and non-pregnant mare serum or plasma may provide valuable evidence as to what is being carried from the cell of exosome origin to its target cell. Such evidence would not only provide new clues to the mechanisms involved in early pregnancy of the mare, but also the development of a diagnostic tool. If a specific exosomal protein, mRNA, or miRNA were to be discovered as unique to pregnancy, it could be utilized to diagnose pregnancy, possibly manage pregnancy, or manage non-pregnant mares by preventing cyclicity and in effect prevent pregnancy.

CHAPTER IV

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APPENDICES

Appendix I

Ultracentrifugation Protocol

Differential Centrifugation

1. Pellet cells:
   - Fluid (Serum)
   - 300 x g for 10 minutes
   - Continue with supernatant

2. Pellet dead cells and debris
   - 2000 x g for 10 minutes
   - Continue with supernatant

3. Pellet remaining cell debris
   - 10,000 x g for 30 minutes
   - Continue with supernatant

Ultracentrifugation (step 4 to 7: extend with PBS)

4. Pellet exosomes and contaminating proteins
   - 100,000 x g for 70 minutes
   - Continue with pellet

5. Wash and pellet exosomes
   - 100,000 x g for 70 minutes
   - EXOSOME PELLET

6. Sucrose gradient
   - 30% sucrose/D_2O cushion
   - 100,000 x g for 75 minutes
   - Exosome and sucrose/D_2O pellet

7. Wash and pellet exosomes
   - 100,000 x g for 70 minutes
   - EXOSOME PELLET

Appendix II

Exosome Protein Isolation Protocol using TRI-REAGENT®BD

Exosome Isolation with ExoQuick™

Day 1:   Thaw serum
         Centrifuge: 3000 x g for 15 min
         Mix correct amt. Exoquick with serum (400 µL serum + 100 µL exoquick)
         Mix well (invert)
         Store at 4°C at least 12 hours (overnight)

Day 2:   Turn on water baths
         Centrifuge at 1500 x g for 30 min
         Carefully aspirate supernatant using pipet (do not disturb exosome pellet)
         Add 50 µl 1X PBS (use pipet to flush PBS through the top layer of
         pellet)(At least 1/10 of original vol)
         Add another 50 µl 1X PBS (Careful not to spend too much time out of
         ice!)(use pipet tip here too)

TRI-REAGENT®BD

1.   RNA Isolation Step

1. LYSIS:
         Add 8 µl Polyacryl Carrier
         Add 750 µl TRI-Reagent BD
         Homogenize
         Store at room temperature for 5 min

2. PHASE SEPARATION:
    Add 200 µl Chloroform to sample (under hood!!!!)
    - (200 µl Chloroform per 750 TRI reagent BD)
    Vortex 15 sec
    Store at room temperature for 5 min
    Centrifuge: 12,000 x g for 15 min

3. RNA PRECIPITATION:
    *****ALL SPINNING IN RNA STEPS AT MAX SPEED (16,000 g)*****
    Transfer aqueous phase to a fresh tube
    Save the interphase and organic phase at 4°C for later (DNA & protein)
    Add 500 µl Isopropanol (500 µl Isopropanol per 750 µl TRI reagent BD)
    Store at room temp for 5 min
    Centrifuge (bench-top): 16,000 x g for 8 min
    - RNA precipitate forms a gel-like/white pellet

4. RNA WASH:
    Pour off supernatant
    Add 1 ml of 75% Ethanol (1 ml 75% EtOH per 750 µl TRI Reagent BD)
    Centrifuge: 16,000 x g for 5 min & pour off supernatant
    Add 1 ml of 75% Ethanol
    Centrifuge: 16,000 x g for 5 min

5. RNA SOLUBILIZATION:
    Pour off Ethanol supernatant
    Air-dry pellet for 5 min
    Add 20 µl nuclease-free water in 55°C (flick tube 2-3 times during this
    step)for 10 min

6. RNA-DNAse Treatment (Ambion)
    Add 2 µl 10X DNAse I Buffer to 20 µl eluted RNA and 1 µl DNAse I
    Incubate for 15 min at 37 °C
    Add 3.5 µl DNA Inactivation Reagent to 23 µl sample
    - make sure Reagent is mixed well
    - (20 µl RNA + 2 µl 10X DNAse I Buffer + 1 µl DNAse I = 23 µl
      sample)
    Mix and leave at room temp. for 2 min (flick once after 1 min)
    Centrifuge: 13,000 RPM for 1 min
    Store at -80°

2. DNA Isolation Step

1. DNA PRECIPITATION:
   Use the interphase/organic phase set aside from step 1
   Add 400 µl 100% Ethanol
   Mix by inversion
   Centrifuge: 2000 g for 5 min
   Carefully remove supernatant (pipette) and put into fresh tube
   • 300 µl in each tube
   Discard old tube (should not have any DNA)

2. Protein Isolation Step

1. PROTEIN PRECIPITATION:
   Add 900 µl Isopropanol to each tube/sample
   Mix by inversion for 10-15 sec to obtain homogenous solution
   Store sample at room temperature for at least 10 min
   Centrifuge: 12,000 x g for 10 min

2. PROTEIN WASH:
   Pour off supernatant (carefully)
   Add 500 µl 0.3M guanidine hydrochloride in 95% ethanol to each tube
   Re-suspend pellet (Use pipet tip to break up pellet)
   Add remaining 500 uL 0.3M Guanidine hydrochloride in 95% EtOH
   vortex
   Store at room temp for 10 min
   Centrifuge: 8000 x g for 5 min (wash #1) and pour off supernatant
   Perform 3 X (3 total wash steps)
    Remove supernatant and add 500 µl 100% Ethanol
   Re-suspend pellet
   Add 1000 µl EtOH
   Vortex
   Store at room temp for 20 min
   Centrifuge: 8000 x g for 5 min

3. **PROTEIN SOLUBILIZATION:**

        Air-dry the protein pellet for 5-10 min

        Dissolve pellet in 8M urea in Tris-HCl, pH 8.0 (800 µl solvent per 300 µl phenol-ethanol supernatant)

        Sonicate sample for 5 min

        Place back in cold block

        Centrifuge: 10,000 x g for 10 min

        Transfer supernatant to fresh tube

        Store at -20°C or -80°C

Appendix III

Bradford Assay Protocol for Protein Analysis

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1. Make Bradford dye dilution (1:5 in H_2O)
   - Need 2 mL for each cuvette
   - Duplicates are recommended
     - Example: 90 mL (enough for 40 samples)
       $$= 18 \text{ mL dye: } 72 \text{ mL } H_2O$$
2. Make BSA dilutions from stocks:
   - For 0.1 µg/µL (100 µL of 10 mg/mL BSA stock) in 9.9 mL H_2O
   - For 0.5 µg/µL (500 µL of 10 mg/mL BSA stock) in 9.5 mL H_2O

Low Range Curve: 0.1 µg/µL

Mass	µL BSA	µL H_2O
0	0	200
2	20	180
4	40	160
10	100	100
15	150	50
20	200	0

High Range Curve: 0.5 µg/µL

Mass	µL BSA	µL H_2O
0	0	200
2	20	180
4	40	160
10	100	100
15	150	50
20	200	0

3. Turn on spectrophotometer (needs to warm up)
4. Make standard curve:
   - Pipet BSA and H_2O (from table) into standard curve cuvettes
5. .Make unknown protein samples:
   - Pipet **196 µL** H_2O and **4 µL** unknown into sample cuvettes
6. Add **2 mL** of dilute Bradford dye solution to all cuvettes
7. Allow incubation time of ~2 minutes at room temperature
8. Cover with parafilm, invert and insert into spectrophotometer (start with blank) with proper orientation and seating
9. Set absorbance at 595 nm
10. Read and record standard curve and samples
11. Dispose of cuvettes in *biohazard*


CountBright^{TM} Absolute Counting Beads Formula

$$\frac{A}{B} \ X \ \frac{C}{D} \ = \ \text{Concentration of sample as exosomes/µL}$$

A = Number of exosome events
B = Number of bead events
C = Assigned bead count of the lot (beads/50 µL)
D = Volume of sample (µL)
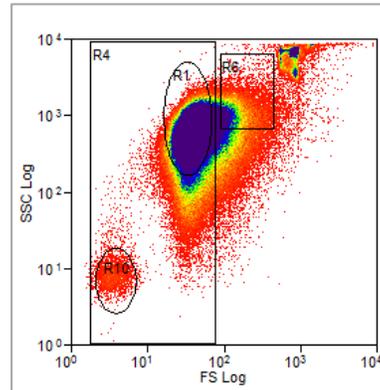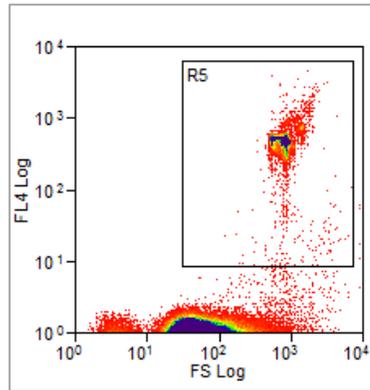
Example Calculation:

A = 60,508 exosome events (in gates R4, includes R10)
B = 20,345 bead events (in gate R5: Forward scatter plotted
     against fluorescence of beads)
C = 47,000 beads/ 50 µL (Lot # 923447)
D = 300 µL

$$\frac{60,508}{20,345} \ X \ \frac{47,000 \ \text{beads/50 µL}}{300 \ \text{µL}} \ = \ 232.97 \ \text{exosomes/µL}$$

Appendix V

TEM Exosome Prep and Embedding Protocol

Day 1.

1. Exosome isolation with ExoQuick^{TM} (described in Appendix I)

Day 2.

Exosome pellet:
1. Centrifuge: 1500 x g for 30 minutes
2. Remove supernatant with pipet tip
3. Re-suspend exosome pellet in 200 µL sterile, 0.2µm filtered 1X PBS
4. Transfer to 5 mL ultracentrifuge tube
   - make certain that the tube is clean, may need to pre-rinse with same PBS as used to re-suspend sample
5. Bring volume to 50 mL with additional PBS
   - tube must be at full volume in order to withstand ultracentrifuge speeds and force
6. Ultracentrifuge: 100,000 x g for 70 minutes
   - Rotor: SW55
   - Ultracentrifuge: Beckman model no. L8M60
7. Remove supernatant using a *gel loading pipet tip*
   - leave 200 µL supernatant on pellet
8. Transfer pellet and residual supernatant to clean microcentrifuge tube
9. Add 200 µL 2% agar
   - load with gel contacting the tube wall, not directly on the pellet
10. Centrifuge: 17,500 x g for 15 minutes
11. Remove supernatant
12. Add 50 µL 2% agar and place on ice for 15 minutes to set gel
13. Make sure exosome pellet in gel is free from the sides of the tube
    - use a small spatula to gently free the pellet in gel

Fixation:
1. Add 500 µL of 2.5% glutaraldehyde in 5% sucrose, 0.1M sodium cacodylate pH 7.4
   - incubate for 30 minutes at room temperature
2. Add 1.5 mL cacodylate buffer to wash (3 X 15 minutes)
3. Add 1% OsO_4 in buffer to cover generously and incubate for 90 minutes
4. Repeat wash in cacodylate buffer (3 X 15 minutes)
5. Store in cacodylate buffer until next day

Day 3.

Dehydration:
1. Dehydrate in labeled vials with caps
   a. 10 min in 50% EtOH (1 X)
   b. 10 min in 70% EtOH (1 X)
   c. 5 min in 95% EtOH (1 X), 10 min (2 X)
   d. 5 min in 100% EtOH (1 X), 10 min (2 X)
   e. 5 min propylene oxide (1 X), 10 min (2 X)

Embedding:
2. Add 1:1 Poly/bed 812 mixture:propylene oxide
   a. incubate overnight (make sure caps are tight)

Day 4.

Embedding in Plastic:
1. Make fresh Poly/bed 812 mix and split into two fractions
2. Transfer exosome pellet in gel to first fraction of 100% plastic
   a. incubate in vacuum at 20 lbs pressure for 4 hours
3. Transfer exosome pellet in gel to second fraction of 100% plastic
   a. incubate in vacuum at 20 lbs pressure overnight

Day 5.

1. Move pellet in 100% plastic to 45°C oven
   a. incubate for 9 hours
2. Move pellet in 100% plastic to 60°C oven
   a. incubate overnight

Day 6.

1. Remove pellet in plastic from oven and cool at room temperature
2. Pop blocks out of container and store in labeled containers

Appendix VI

## Exosome Scatter Profiles at Day 12, 14, 16, and 18

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### Day 12

Non-Pregnant
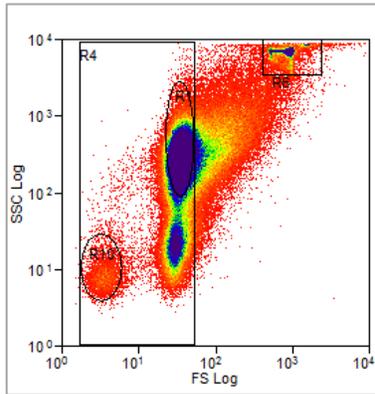
Pregnant
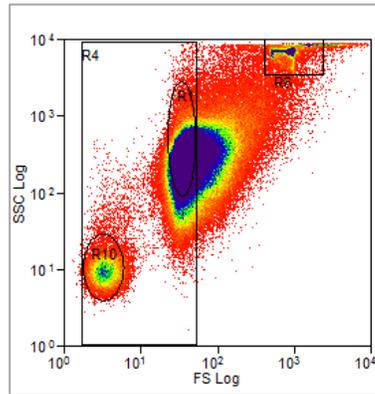
Day 14

Non-Pregnant

Pregnant

74
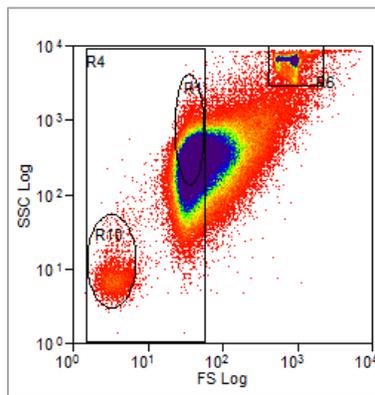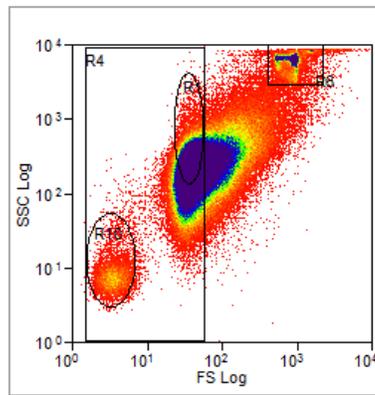
Day 16

Non-Pregnant
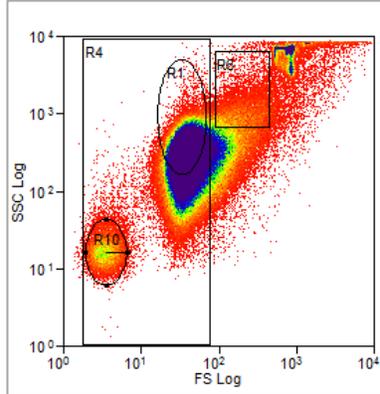
Pregnant

Day 18

Non-Pregnant

Pregnant