Journal of Clinical and Experimental Reproductive Medicine

Troedsson et al, J Clin Exp Reprod Med. 2024;1(1):1-7.

Commentary

The physiology of seminal plasma proteins in the equine female reproductive tract

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Received date: May 23, 2024
Accepted date: June 14, 2024

Citation: Troedsson MHT, Doty AL, Fedorka CE. The physiology of seminal plasma proteins in the equine female reproductive tract. J Clin Exp Reprod Med. 2024;1(1):1-7.

Introduction

Functional proteomics of seminal plasma has been reviewed by Samanta and co-workers, highlighting greater biologic relevancy and diverse functions of this seminal component to reproductive biology than previously thought [1]. Although not requested for successful fertilization, important biological functions are associated with seminal plasma and the authors suggested that selected proteins may serve as diagnostic biomarkers for various conditions. In most mammalian species, seminal plasma is involved in a variety of processes during sperm maturation, capacitation, acrosome reaction, and sperm-egg interactions [2-5]. Specific seminal plasma proteins have been associated with antioxidant protection of spermatozoa, heparin binding, and fibronectin binding [6-9]. More recent observations indicate that seminal plasma also play an important role in immune function of the female reproductive tract after breeding as well as the fetal development [10-19].

The total protein fraction of equine seminal plasma is approximately 10 mg/ml, and the majority of the proteins range in molecular weight from 12 kDa-30 kDa [3]. One of these proteins, cysteine-rich secretory protein 3 (CRISP3) is a major seminal plasma protein in horses that constitutes 0.3-1.3 mg/ml, and polymorphism within the CRISP3 gene has been positively correlated with fertility [20-22]. We have characterized the expression of CRISP3 in the equine ampulla of the vas deference and to a lesser degree in the seminal vesicles and implied this protein in the regulation of breeding-induced endometritis, where it interacts with polymorphonuclear neutrophils (PMN)-binding of spermatozoa [16,19]. Another seminal plasma protein that has been associated with breeding-induced endometritis is equine lactoferrin [23]. This 80kDa protein may be involved in sperm-oocyte binding and has also been shown to have iron binding properties suggesting a role for the protein in regulating free iron available for lipid peroxidation as well as in the innate uterine immune system of horses [24-26]. Multiple lactoferrin receptors have been identified in a variety of tissues and cells, suggesting widespread functions of this protein [27]. In addition to sequestering iron, lactoferrin may also interact with leukocyte function through its apparent control of neutrophil extracellular traps (NETs) [28].

Among other seminal plasma proteins, insulin-like growth factor 1 (IGF-1) is secreted by both Leydig cells and Sertoli cells, and its receptors have been found on these cells as well as developing spermatozoa, suggesting a role in spermatogenesis [29-32]. Seminal IGF-1 was found in lower concentrations in infertile men with oligospermia, and seminal plasma derived IGF-1, insulin-like growth factor-binding protein 2 (IGFBP-2), and insulin-like growth factor-binding protein-5 (IGFB-5) have also been associated with sperm function and fertility rates in mares [33]. A positive relationship was observed between levels of IGF-1 and sperm motility and morphology, and the authors concluded that stallions with high IGF-1 levels had improved overall pregnancy rates [33]. Others have examined enzymes and additional components in equine seminal plasma and found aspartate transferase (AST), glutamyl transferase (GGT), acid phosphatase (ACP), AIP, lactate dehydrogenase (LDH), Fe and Zn were associated with semen volume and sperm concentrations,

with LDH activity being most highly correlated with semen quality [34]. The authors suggested that a positive correlation between GGT and sperm motility indicated a protection against free radicals. In addition, seminal plasma DNase has been implemented as a sperm protective factor against NETs of spermatozoa [35].

Role of Seminal Plasma in Inflammation and the Innate Immune System in the Equine Female Reproductive Tract

We have focused our investigations on the role of seminal plasma proteins in the interaction between uterine inflammatory cells and spermatozoa in horses. In species with semen deposited into the uterus during ejaculation, such as the horse, most spermatozoa are eliminated from the uterus shortly after breeding, while a small portion of sperm is transported to the oviducts. Sperm transport is enabled by motility of the spermatozoa and uterine contractions. While sperm motility may not be necessary for sperm transport in the cow, pig, and rabbit [36], observations suggest that both sperm motility and myometrial contractility may be important for uterine and oviductal transport of sperm to the ampulla [37-40]. Excess sperm needs to be eliminated from the uterus in a timely fashion to provide a compatible endometrial environment when an embryo descends into the uterine lumen from the oviduct at approximately 6 days after fertilization [41]. This is accomplished through uterine contractions and sperm-induced inflammation, characterized by a balanced pro-and anti-inflammatory cytokine expression leading to a rapid influx of PMNs into the uterine lumen [42]. The mechanism involves increased expression of interleukin-8 (IL-8) as well as the activation of the complement cascade [43-46]. Complement factor C3b coats the spermatozoa and contributes to PMN-binding and phagocytosis [47]. The breeding-induced inflammation results in prostaglandin release [48], which causes additional myometrial contractions 2-6 hours after the initial wave of contractions [39]. The contractions physically clear the uterus from excess spermatozoa, contaminating bacteria, and residual inflammatory fluid/products within 24-36 hours after breeding, with recent unpublished observations suggesting that this process may be completed at a much earlier time point, already within 6 hours in some mares. Impaired uterine contractility following breeding has been associated with infertility due to persistent inflammation that is incompatible with the survival of an embryo [49].

There is accumulating evidence that seminal plasma play an important role in breeding-induced endometritis, specifically in the protection of viable spermatozoa for safe transport in the presence of an inflammatory uterine environment, while allowing dead spermatozoa and bacteria to be eliminated through PMN phagocytosis [13,16,50]. In addition, Alghamdi and Foster demonstrated that PMNs extrude DNA and histone along with their cellular contents to form NETs, similar to what has been described for bacteria, and there also appears to be an additional receptor ligand binding mechanisms between PMNs and spermatozoa that has not been identified [35,51]. We have confirmed these findings (**Figure 1**), but while Alghamdi and Foster [35] suggested that NETs were triggered by live spermatozoa, we were unable to confirm this observation. Our findings corroborate the works of Fuchs and co-workers who suggested that when fragmentation of DNA is not activated during cell death, it allows the chromatin within the neutrophil to unfold and be extruded into the extracellular space

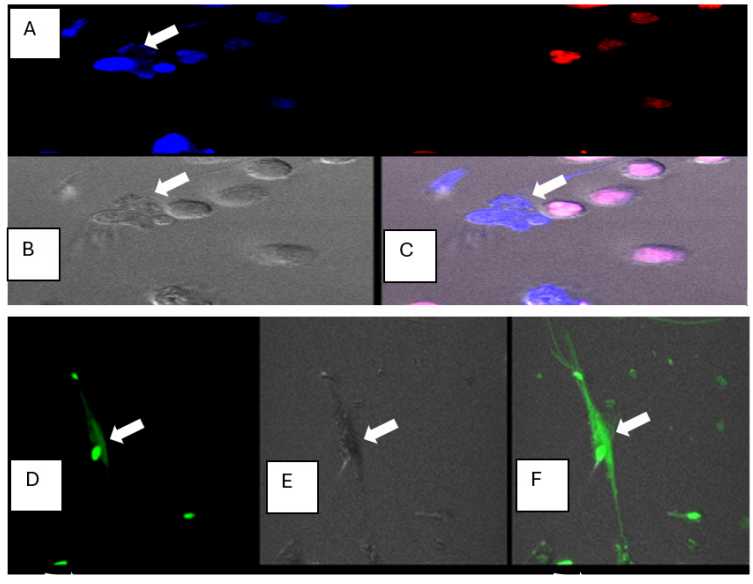


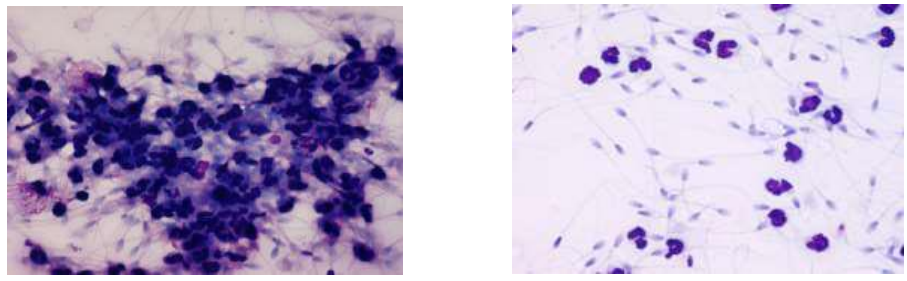
Figure 1 A-F. Fluorescent confocal microscopy images (63X) of PMN- NET formation in the presence of dead spermatozoa. PMNs and dead spermatozoa were exposed to the dicycloorange DNA stain (red color) of intact DNA in live cells, and Sytox Blue stain (blue color) of dead cells, or dicycloviolet DNA stain (green color) of dead cells. The sytox blue and dicycloviolet also stained the extruded DNA from the PMNs, which entrapped dead spermatozoa (indicated by white arrow). **A)** dead cells: both sperm and PMNs took up sytox-blue DNA stain, (white arrow denotes NET formed by PMN in contact with dead sperm), and live PMNs which are labeled with dicycloorange DNA stain (red) indicating they are viable with intact DNA; **B)** image in DIC; **C)** merged image with both fluorescent channels and DIC; **D)** PMN-NET visualized by dicylcoviolet DNA stain, showing DNA extrusion from the ruptured PMN. **E)** Image in DIC **F)** Merged image in fluorescent channel and DIC. (Adopted from Doty AL: The role of seminal plasma proteins in the interaction between polymorphonuclear neutrophils (PMNs) and spermatozoa in the horse. PhD Dissertation, University of Florida, 2012).

[52]. This is an effective mechanism of extracellular destruction of bacteria and other particles such as fungi and parasites. These authors demonstrated that NET formation was triggered by reactive oxygen species (ROS), either from the PMNs, downstream of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or in response to exposure to high concentrations of extracellular ROS. Since dead and damaged spermatozoa are releasing ROS, this mechanism appears to support our observation that dead spermatozoa triggered the formation of NETs. The formation of NETs is only partially responsible for sperm-PMN binding, since a greater suppression of PMN/sperm binding occurred when seminal plasma was added to the *in vitro* assays, compared to when DNase was added [35]. This is also supported by the finding that a seminal plasma derived protein complex of lactoferrin and superoxide dismutase (SOD-3) (LF/SOD3) specifically interacts with dead spermatozoa and promotes binding to PMNs [26].

A greater portion of morphologically normal spermatozoa are found in the oviduct, compared to the ejaculate as a whole [53]. While this may be explained by the necessity of motility and membrane integrity during sperm transport through the uterus, it suggests that the utero-tubal junction may serve as a barrier for dead and damaged sperm. In addition, spermatozoa need to be able to survive a hostile inflammatory environment, caused by a rapid influx of PMNs in response to breeding-induced inflammation [54]. The importance of this is illustrated in **Figure 2A**, showing that spermatozoa form large immobile clusters in the presence of PMNs *in vitro*. This cluster formation does not occur when the spermatozoa is extended in seminal plasma (**Figure 2B**). In subsequent experiments, breeding-induced uterine inflammation was induced by insemination with dead sperm, followed by insemination with live spermatozoa 12 hours later, with one group of mares inseminated in the absence of seminal plasma and the other group in the presence of seminal plasma in the insemination dose [12]. Only 5% of the mares became pregnant when inseminated into an inflamed environment in the absence of seminal plasma, while a normal pregnancy rate of 77% was achieved in the presence of seminal plasma. These results were somewhat contradicted by a study showing equal fertility from two individual stallions when their commercially prepared cryopreserved semen was inseminated in the same mare 6-10 hours apart respectively [55]. The difference between the reports is likely explained by the reduction, rather than absence of seminal plasma proteins in cryopreserved semen used in the study by Metcalf, further supporting a selective seminal plasma protection of viable spermatozoa. Seminal plasma derived CRISP-3 was later identified as a protein responsible for protecting spermatozoa from PMN-binding and phagocytosis, and this protective effect was subsequently shown to be selective for live spermatozoa and has no effect on dead sperm or bacteria [16,50]. This suggests a more important biological role of seminal plasma than previously recognized (**Table 1**).

Role of Seminal Plasma in Maternal Immune Tolerance

The deposition of semen into the uterus activates numerous aspects of maternal immunity, including both the innate and adaptive immune system. The compilation of seminal plasma includes various cytokines (transforming growth factor beta (TGF-β), IL-8), hormones (PG), in addition to paternal antigens. The exposure of seminal plasma to the maternal immune system is believed to activate the adaptive immune response to develop tolerance to the semi-allogeneic fetoplacental unit [15,56-57]. The impact of seminal plasma on the immune response to breeding includes activation of cytokines and chemokines from the epithelial cells, a recruitment of immune cells into the uterine lumen, and eventual activation of dendritic cells by paternal antigens [42,54,60,61]. Seminal plasma has also been found to increase the expression of various embryokines (leukemia inhibitory factor (LIF),



A B

Figure 2: Photomicrographs of equine spermatozoa mixed with PMNs in the absence (**A**) and presence (**B**) of seminal plasma. Spermatozoa formed clusters with reduced motility when seminal plasma was removed from semen (**A**), while very little binding between PMNs and spermatozoa was observed in the presence of seminal plasma (**B**), allowing the spermatozoa to maintain motility.

Table 1. Role of seminal plasma proteins in equine breeding-induced endometritis.

Seminal Plasma Protein	Biological Function	Reference
CRISP3	Selectively prevents binding and phagocytosis of live spermatozoa to PMNs	[16,50]
LF/SOD-3	Selectively promotes binding and phagocytosis of dead spermatozoa to PMNs	[26]
DNase	Modulation of NETs	[35]

interleukin 6 (IL6), colony-stimulating factor 2 (CSF2), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)) as well as transcripts relating to embryo health and metabolism (IGF-β) [58,59]. In the mouse, seminal plasma induces chemotactic factors for immune cells such as neutrophils, macrophages and dendritic cell, and mating of seminal vesicle-excised males to normal females decreased placental volume, litter size, and had a negative impact on the health of resulting offspring [17]. Others have suggested that seminal vesicle-derived CD38 is imperative in inducing the tolerogenic dendritic cells and CD4+FoxP3+ Tregs. This population of T-cells is believed to be essential for pregnancy maintenance, and depletion of these cells leads to implantation failure, impaired uterine vascular development, and fetal loss in late term gestation [62]. While this has not been extensively investigated in horses, we recently observed that seminal plasma regulated endometrial gene transcription that may have an impact on the environment for the embryo [63]. This can potentially alter embryo development through epigenetic effects and subsequently impacting the phenotype of the offspring. Recent work suggests that equine intracytoplasmic sperm injection (ICSI)-produced embryos were associated with postpartum placentas that had increased chorionic villi hyperplasia, edema, allantoic cysts, and necrosis [64]. Umbilical length of these ICSI-produced fetuses was also shorter in comparison to *in vivo* produced embryos. Additional work on *in vitro* produced embryos revealed that equine male offspring produced from embryo transfer and ICSI had higher weight gain during the first two years of age, compared to offspring produced from conventional AI at the same farm [65]. While advanced growth may be considered an advantage for early development in young athletes, it carries an enhanced risk of developmental diseases, such as osteochondrosis dissecans. These findings were preliminary and additional factors associated with Assisted reproductive technologies (ARTs) were not evaluated. Nevertheless, data from other species suggest that a potential impact of seminal plasma on fetal development needs to be studied further.

In other species, seminal plasma has been shown to enhance the endometrial and oviductal production of various embryotrophic factors, including CSF1, CSF2, CSF3, IL-6, LIF, vascular endothelial growth factor (VEGF), and TRAIL in both mice and pigs [58,66]. When females are mated with seminal vesicle-excised males, a reduced rate of zygote cleavage was noted [17], while infusion of seminal plasma at the time of insemination in these animals reinstated the appropriate inflammatory response, enhanced embryo development, and increased the number of offspring [67]. Furthermore, seminal plasma exposure of the female reproductive tract prior to *in vitro* fertilization (IVF) and ICSI, improved the success of embryo transfer in women [68,69]. However, IVF in humans does not routinely include seminal plasma and the procedure has been shown to alter placental development [70], neonatal outcomes [71], and offspring health [72]. Seminal vesicle ablation in mice altered growth trajectories in resulting offspring, with elevated adipose tissue, hypertension, and reduced glucose tolerance observed, and this was primarily found in males [17]. The alteration in offspring phenotype is hypothesized to be linked to the reduced embryokine production in the female reproductive tract following insemination with seminal plasma-voided ejaculates. Further investigations are warranted to better understand the role of seminal plasma in fertility, fetal development and the health of offspring when different forms of ART are used in horses and other species. Once these functions are characterized, specific proteins that are associated with immune tolerance and development of the fetus should be identified and possibly added to media when ART is implemented.

Conclusion

There is accumulating evidence that seminal plasma proteins have distinct biological functions associated with breeding in horses and other species (**Figure 3**). While the proteins may not be necessary for the establishment of pregnancy, they appear to facilitate the selection of viable sperm in the uterus, as well as ensuring

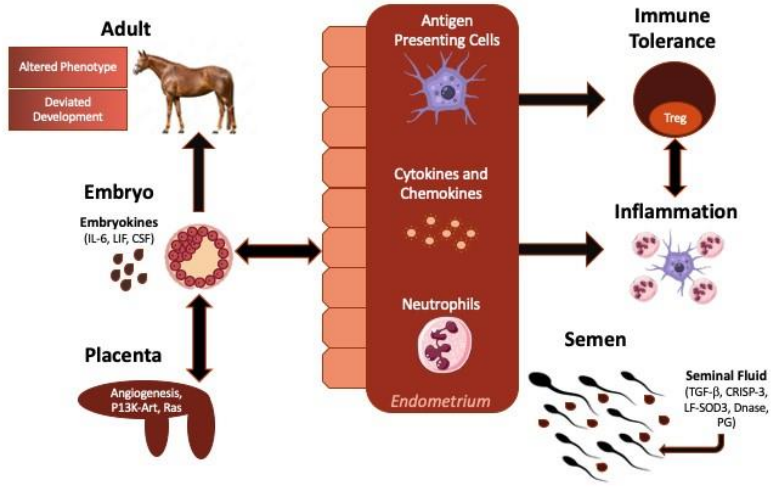


Figure 3: Schematic representation of the proposed impact of seminal plasma on the maternal immune response to breeding and pregnancy. Seminal plasma is believed to impact the maternal immune response to breeding, embryo growth and development, in addition to immunotolerance of the developing fetus, all of which are believed to improve pregnancy outcomes and optimize the growth and development of offspring. Proteins, cytokines, and paternal antigen within seminal plasma modulates the immune response to breeding, leading to the production of various cytokines which assist with the innate immune response to breeding, in addition to the cell-mediate immune response to pregnancy. Stimulated antigen presenting cells travel through the draining lymphatics of the uterus to increase the proliferation of immunotolerant lymphocyte populations, specifically Tregs. Seminal plasma is also believed to increase the production of various cytokines, deemed embryokines (IL-6, LIF, CSF), which enhance embryo growth and development. Modified from Bromfield 2016 [73].

a suitable uterine environment for optimal development of the fetus. The importance of the uterine environment extends beyond gestation and can potentially affect the health of offspring later in life. The role of seminal plasma exposure at the time of breeding should be investigated further regarding its effect on quality aspects of pregnancy and offspring.

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