DISSERTATION

EFFECTS OF MILK PROTEINS ON INTRACELLULAR Ca^{2+} CONCENTRATION AND STALLION SPERM BINDING TO ZONAE PELLUCIDAE

Submitted by

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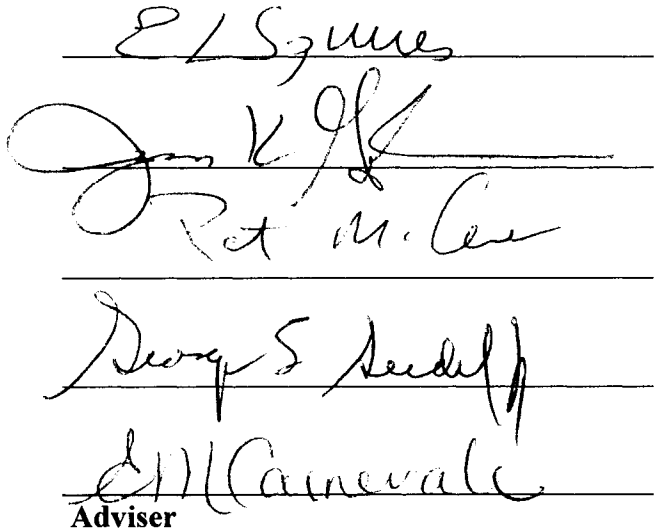
WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MARCO ANTONIO COUTINHO DA SILVA ENTITLED EFFECTS OF MILK PROTEINS ON INTRACELLULAR Ca^{2+} CONCENTRATION AND STALLION SPERM BINDING TO ZONAE PELLUCIDAE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work



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ABSTRACT OF DISSERTATION

EFFECTS OF MILK PROTEINS ON INTRACELLULAR Ca^{2+} CONCENTRATION AND STALLION SPERM BINDING TO ZONAE PELLUCIDAE

The effects of semen extender components on the ability of stallion sperm to bind to the zona pellucida (ZP) and, the suitability of using bovine ZP for a ZP-binding assay for stallion sperm were investigated in a series of experiments. In Experiment I, similar numbers of stallion sperm bound to both bovine and equine ZP. In Experiment II, a three-fold increase in the number of sperm bound to ZP was observed when sperm were diluted in a skim-milk based diluent (EZM), compared to diluents which contained no milk (TALP, Lactose-EDTA, and EmCare®). In Experiment III, centrifuging the sperm through Percoll did not increase sperm binding to the ZP but did remove any positive effect of EZM on sperm-ZP binding. In Experiment IV, exposure of either sperm or ZP to EZM prior to co-incubation did not increase sperm binding to ZP, although the number of sperm bound to the ZP was higher if EZM was present during co-incubation. In Experiment V, sperm diluted in TALP containing skim-milk, EZM or INRA96® bound more efficiently to the ZP than sperm diluted in TALP without milk proteins. In Experiment VI, sodium caseinate (SC), native phosphocaseinate (NP) and caseinoglycopeptide were added to the sperm diluent, and increased sperm binding to the ZP in a dose-dependent manner.

The objectives of subsequent experiments were to determine the effect of extracellular Ca^{2+} concentration and milk proteins (NP and SC) on intracellular Ca^{2+} concentrations of stallion sperm; and to determine the effects different caseins (α-, β-, κ-caseins) have on sperm binding to the ZP. Extracellular Ca^{2+} concentration (2 versus 4 mM) in the sperm diluents did not affect baseline intracellular Ca^{2+} concentration of sperm. However, incubating sperm in a medium containing 4 versus 2 mM Ca^{2+} resulted in higher ($P<0.05$) influx of Ca^{2+} into sperm after treatment with ionomycin. Addition of NP or SC to TALP did not affect the baseline intracellular Ca^{2+} or the influx of Ca^{2+} after ionomycin treatment. Sperm-ZP binding assays were performed by co-incubating stallion sperm and bovine ZP in TALP alone or TALP containing 1 or 3 mg/ml of individual caseins (α-, β- or κ-caseins). Co-incubating sperm and ZP in media containing single caseins resulted in similar numbers of sperm bound to the ZP compared to TALP. Higher ($P<0.05$) numbers of sperm were bound to ZP when co-incubation medium contained β- or κ-caseins, compared to α-casein.

In conclusion, bovine ZP can be used for ZP-binding assays for stallion sperm. Co-incubating sperm and ZP in diluents containing milk or milk proteins markedly enhanced the number of sperm bound to the ZP. However, intracellular Ca^{2+} concentration of the sperm was not affected by milk proteins. It appears that β- and κ-caseins are responsible for enhancing sperm binding to the ZP, however the mechanism by which they enhance sperm binding is not known.

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Chapter I

Review of Literature

Introduction and Genesis of Experiments

Advances in assisted-reproduction in the horse have helped to obtain pregnancies from subfertile animals that, otherwise, would be considered infertile. In vitro production of embryos has successfully been used in humans and in a variety of other mammalian species. However, in the horse, production of embryos after in vitro fertilization has not yet been successful. Compared to other species, reasons for the slow development of in vitro fertilization in the horse include scarcity of slaughterhouse ovaries and difficulties with in vitro systems for oocyte maturation and sperm capacitation. Only two foals have been produced after in vitro fertilization of oocytes matured in vivo (Palmer et al., 1991; Bezard, 1992). Although in vitro fertilization has not been successful in horses, different approaches for fertilization of equine oocytes in vivo and in vitro have been developed, such as oocyte transfer (OT), gamete intrafallopian transfer (GIFT), and intracytoplasmic sperm injection (ICSI).

Although the acronyms, OT and GIFT, are used interchangeably in the literature, distinctions should be made. During OT, the oocyte of a donor mare is transferred into oviduct of a recipient, and the recipient is inseminated into the uterus. GIFT involves the transfer of oocytes and low numbers of sperm (2 to 5 x 10^5 sperm) into the oviduct. Because GIFT requires a lower number of sperm and sperm are deposited near the site of

fertilization, the procedure has the potential to be used when sperm numbers are low, e.g., subfertile stallions, frozen semen and sexed sperm.

During GIFT, sperm are deposited in the infundibullar or ampullar region of the oviduct, bypassing the uterus and the utero-tubal junction. Therefore, sperm must be separated from seminal plasma, debris and contamination. In addition, morphologically normal and motile sperm must be selected from the ejaculate. Initial studies using low numbers (2 to 5 x 10^5 sperm) of fresh semen for GIFT in the horse resulted in pregnancy rates ranging from 27 to 82%, demonstrating that GIFT could be an alternative to oocyte transfer when sperm numbers are low (Carnevale et al., 1999; Coutinho da Silva et al., 2002).

However, in a commercial program, cooled or frozen semen is more likely to be available for GIFT than fresh semen. Therefore, we investigated the use of cooled and frozen semen for GIFT (Coutinho da Silva et al., 2004). In that study, oocyte recipients were inseminated within the uterus (OT) or oviduct (GIFT) with cooled semen. High embryo development rates (83%, 19/23) were obtained using cooled semen with intrauterine inseminations for OT. However, when cooled semen was placed into the oviduct with oocytes, significantly lower embryo development rates were observed (25%, 4/16). In addition, the use of frozen semen for GIFT resulted in an embryo development rate of only 8% (1/12). Although low embryo development rates obtained with cooled and frozen semen could be caused by factors associated with cooling and freezing processes that impaired fertilization after intraoviductal inseminations, we hypothesized that differences in sperm processing for GIFT, in particular the use of different semen extenders, could account for the reduction in fertilization rates. When GIFT was

performed with fresh semen, sperm were not diluted with extenders; and therefore, raw semen was used. In contrast, cooled and frozen semen were diluted in milk-based and Lactose-EDTA extenders, respectively. Potentially, components of semen extenders could interact with sperm membranes affecting sperm physiology and fertilization in the oviduct.

Results from these GIFT experiments stimulated investigation of the effects of semen extenders on the interaction between sperm and zonae pellucidae (ZP). The interaction between sperm and ZP is a critical event leading to fertilization and reflects multiple sperm functions (i.e. completion of capacitation as manifested by the ability to bind to the ZP and undergo a ligand-induced acrosome reaction; Liu and Baker, 1992b; Oehninger et al., 1992). The number of sperm bound to a ZP may reflect the functional status of both gametes and the capacity of the gametes for fertilization (Mlodawska et al., 2000). Therefore, we used a sperm-ZP binding assay as the method of choice to evaluate sperm function. The goal of the research presented in subsequent chapters is to understand the effects of semen extenders on gamete interaction and to determine the mechanism(s) by which components of semen extenders affected sperm-ZP binding. Knowledge gained with this research could be applied to: 1) improve the use of cooled and frozen semen for GIFT, 2) assist in the development of methods for IVF in the horse, and 3) develop alternative extenders for cold storage of stallion semen.

The Mammalian Egg Coat

Structure of the ZP

The ZP is a transparent, porous, extracellular matrix surrounding mammalian oocytes and preimplantation embryos (Denker, 2000; Herrler and Beier, 2000; Sinowatz et al., 2001a). The ZP of mammalian oocytes is composed of three glycoproteins, which build its typical fibrogranular structure (Wassarman and Mortillo, 1991). The porous nature of the ZP allows penetration of relatively large molecules such as immunoglobulins. On the other hand, small molecules, such as heparin, can be prevented from penetrating the ZP. The ability of molecules to pass this extracellular matrix does not primarily depend on size of the penetrating molecule, but on other biochemical and physicochemical properties, such as surface charge (Shivers and Dunbar, 1997).

In general, from mouse to human oocytes, the ZP is composed of three glycoproteins: ZP1, ZP2 and ZP3 (Wassarman, 1988). The primary structures of ZP polypeptides from different mammalian species are closely related. Much of the research on the ZP and sperm interactions has been carried out using the mouse as a model system. The similarity of the polypeptides from other species to mouse ZP1, ZP2 and ZP3 range from 40 to 90% (Wassarman, 1999). Studies in the mouse provided insight into the molecular organization of the ZP matrix (Wassarman, 1999). In mice, ZP1 (200 kDa), ZP2 (120 kDa) and ZP3 (83 kDa) are synthesized, secreted and assembled into a ZP solely by growing oocytes during a 2-3-week period (Bleil and Wassarman, 1980a,b; Wassarman, 1988). However, investigations in other species, such as human (Grootenhuis et al., 1996), monkey (Grootenhuis et al., 1996; Matinez et al., 1996), rabbit

(Dunbar et al., 1994; Grootenhuis et al., 1996), dog (Tresoriero, 1981), pig (Sinowatz et al., 1995; Kolle et al., 1996) and cow (Kolle et al., 1998), demonstrated that the oocyte and the follicle cells contribute to the synthesis of ZP glycoproteins.

The current model of the structure of the ZP was proposed by Wassarman (1988). The two glycoproteins, ZP2 and ZP3, interact to form heterodimeric units that are periodically arranged in long filaments. These filaments appear to be interconnected by ZP1, a dimer of identical polypeptides held together by disulfide bonds (Wassarman and Mortillo, 1991). The three ZP glycoproteins undergo post-translational modifications, as evidenced by the predicted molecular masses of the protein cores of ZP1 (68 kDa), ZP2 (80 kDa), and ZP3 (46 kDa), which are approximately half the actual molecular mass of the glycoproteins. The most important modification is glycosylation, which permits the matrix to maintain a high state of hydration and may be important for sperm penetration through the ZP (Rankin and Dean, 2000). In addition, some of the carbohydrate residues mediate sperm-zona interactions (Florman and Wasserman, 1985; Bleil et al., 1988; Miller et al., 1992).

Although carbohydrates of the ZP play a crucial role in gamete recognition (Sinowatz et al., 1998; Topfer-Petersen, 1999; Nixon et al., 2001; Oehninger, 2001; Wassarman and Litscher, 2001), our knowledge of the oligosaccharide structures of the mammalian zona is still limited (Nakano and Yonezawa, 2001). Each of the three glycoproteins (ZP1, ZP2 and ZP3) is heterogeneously glycosylated with both complex-type asparagine N-linked and serine/threonine O-linked oligosaccharides (Sinowatz et al., 2001a). ZP1 has six potential N-linked glycosylation sites; ZP2 has seven; and ZP3 has

six (Ringuette et al., 1988; Liang et al., 1990; Epifano et al., 1995). All three glycoproteins posses many potential O-glycosylation sites (Green, 1997).

Lectins (specific sugar-binding proteins) have been used as tools to characterize the oligosaccharide chains of the ZP proteins (Shalgi et al., 1991; Aviles et al., 1994; Maymon et al., 1994; Skutelsky et al., 1994). Comparative studies demonstrated species-dependent variations in the expression and distribution of sugar moieties throughout the ZP. Competition assays have implicated at least four monosaccharide residues as being critical for initial sperm-binding: α-galactose, β-N-acetylglucosamine, fucose and mannose (Bleil and Wassarman, 1988; Miller et al., 1992; Tulsiani et al., 1992; Johnston et al., 1998). In rodents, this pattern varies only in the expression of ligand sites for α-galactose and/or β-N-acetylgalactosamine-recognizing lectins. Interestingly, binding of these lectins is not detectable in the ZP of human, dog, cat and pig (Sinowatz et al., 2001b). In general, the increasing variation of lectin binding patterns correlates with evolutionary distance of species (Shalgi and Raz, 1997). Some carbohydrates are found in all species examined. These are predominantly sugar residues such as mannose and N-acetylglucosamine, which are usually part of the core region of N-linked oligosaccharides (Geyer and Geyer, 1998). Lectin histochemical studies demonstrate that most variations appear to affect the terminal non-reducing region of the ZP sugar chains, supporting the idea that even small structural differences may contribute to the establishment of the species-specific nature of gamete interaction (Sinowatz et al., 2001a).

Functions of the ZP

The ZP plays important roles during mammalian oogenesis, fertilization, and preimplantation development (Yanagimachi, 1994; Wassarman, 1999; Wassarman et al., 2001). The ZP provides receptors for the relatively species-specific attachment and binding of capacitated sperm and is involved in the subsequent induction of the sperm acrosome reaction (Florman and Storey, 1982; Cherr et al., 1986; Berger et al., 1989). In most species, certain exposed oligosaccharides chains of the ZP and complementary carbohydrate-binding proteins on the sperm-oocyte interface mediate, at least in part, the initial binding and recognition between sperm and ZP (Aitken, 1995; Sinowatz et al., 1997; Hoshiba and Sinowatz 1998).

Immediately after fertilization, ZP is modified by the contents of cortical granules, released upon oocyte activation, such that sperm can no longer bind to the matrix and sperm already bound can no longer penetrate it. This process provides the major block to polyspermy in most mammals. The zona matrix surrounds the developing embryo until the blastocyst stage provides protection from physical and environmental damage (Rankin and Dean, 2000; Sinowatz et al., 2001b). How the ZP accomplishes these tasks continues to be investigated, and results obtained in different species are contradictory as to the roles of individual zona glycoproteins. This review will focus on the initial sperm-ZP interactions that result in the acrosome reaction of sperm.

Physiology of Sperm-ZP Interactions

Receptors for Sperm in the ZP

Of the three glycoproteins in the ZP, only purified ZP3 binds exclusively to heads of acrosome-intact sperm, thereby preventing sperm from binding to ovulated oocytes in vitro (Bleil and Wassarman, 1980c, Wassarman, 1990b; Mortillo and Wassarman, 1991). Identification of ZP3 as the mouse sperm receptor occurred as a result of assaying individual ZP glycoproteins in vitro (Bleil and Wassarman, 1980c). These assays revealed that ZP3 from unfertilized oocytes is active as a sperm receptor, but ZP3 from fertilized oocytes or early-stage embryos is inactive. The sperm receptor activity of purified ZP3 accounted for all of the activity observed with solubilized ZP, and results from subsequent studies provided additional evidence that ZP3 behaves as expected for a sperm receptor (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991). Results of other experiments with ZP3 strongly suggested that sperm recognize and bind, not to ZP3 polypeptide, but to specific O-linked oligosaccharides attached to ZP3 (Florman et al., 1984; Florman and Wassarman, 1985; Wassarman, 1990b and 1999). Chemical or enzymatic removal of all ZP3 oligosaccharides results in complete inactivation of the glycoprotein as a sperm receptor. Furthermore, O-linked oligosaccharides removed from ZP3 inhibit binding of sperm to ZP in vitro (Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Miller et al., 1992; Litscher et al., 1995; Johnston et al., 1998). Results of these studies suggested that galactose, N-acetylglucosamine, and/or fucose are essential sugars for sperm binding. However, the identity of individual sugars that are

recognized by sperm on ZP3 remains an unresolved and controversial issue (Wassarman, 1999).

Receptors for ZP in Sperm

A number of zona receptors on sperm have been studied, but how they may function individually or collectively is not yet clear (Miller et al., 2002). Identifying zona receptors has proved to be more difficult than identifying the zona ligands, probably because of the greater complexity of the sperm surface compared to the ZP. There may be multiple receptors to account for the multiple affinities of ZP3 to sperm (Thaler and Cardullo, 1996), and there probably are differences between species.

Several receptor candidates have been isolated, based on their affinity for the ZP. Most studies on zona receptors have been performed using mouse and porcine sperm. From mouse sperm, sp56 was isolated based on its affinity for ZP3 and α-galactose residues (Cheng et al., 1994; Bookbinder et al., 1995). Sp56 is found within the acrosome; and in this location, its function may be to tether sperm onto the ZP as they are undergoing the acrosome reaction (Foster et al., 1997; Kim et al., 2001). Zonadhesin, proacrosin, sp38, P47, and a group of proteins called spermadhesins were isolated from porcine sperm based on their affinity for whole ZP (Hardy and Garbers, 1995; Ensslin et al., 1998; Jansen et al., 2001). Proacrosin and sp38 are also found within the acrosome and exposed during the acrosome reaction, where they can serve as secondary zona receptors (Jones et al., 1988; Mori et al., 1995). Spermadhesins and P47 are peripheral membrane proteins (Ensslin et al., 1998; Jansen et al., 2001), and many spermadhesins are produced by the seminal vesicles (Jansen et al., 2001). Because sperm from the cauda

epididymis that have not been exposed to secretions from the seminal vesicles have normal fertility, spermadhesins that are seminal vesicle products do not appear to be necessary for fertilization (Miller et al., 2002). Zonadhesin has been shown to be similar to von Willebrand factor in blood and posses high-affinity binding to ZP (Hardy and Garbers, 1994 and 1995). However, the localization of zonadhesin in sperm has not been determined (Hickox et al., 2001). Another acrosomal protein that has affinity for the ZP and other sulfated glycoproteins is Sp17, but its role in gamete binding remains unknown (Yamasaki et al., 1995; Wen et al., 2001). PH-20 has also been implicated in zona binding (Myles and Primakoff, 1997). PH-20 has both hyaluronidase and zona binding activity (Hunnicutt et al., 1996). PH-20 on the postacrosomal region of sperm and perhaps PH-20 released by acrosome-reacting sperm disperses the cumulus cells, allowing sperm passage. After the acrosome reaction, PH-20 is necessary for sperm binding to ZP (Hunnicutt et al., 1996). Therefore, PH-20 appears to be a secondary zona receptor, although the zona ligand is unknown.

The zona receptor candidate that has been investigated most extensively, in particular in the mouse sperm, is β1,4 – Galactosyltransferase (GalT). GalT is an integral membrane protein with a molecular weight of 60 kDa (Shur and Neeley, 1988). Presence of GalT in the plasma membrane of the sperm head has been demonstrated in several mammalian species, including guinea pig, mouse, rat, rabbit, boar, bull and stallion sperm (Larson and Miller, 1997). During spermatogenesis, GalT is synthesized and moves to the plasma membrane in the developing sperm head (Scully et al., 1987; Pratt and Shur, 1993; Charron et al., 1999). In the epididymis, glycoconjugates in the epididymal fluid bind to GalT, blocking the interaction of GalT with other ligands such as the ZP. These

glycoconjugates are lost from sperm during capacitation, enabling GalT to bind to the ZP (Shur and Hall, 1982a). During fertilization, GalT on the sperm surface binds to terminal N-acetylglucosamine residues of ZP3, but no other ZP glycoproteins (Miller et al., 1992).

The importance of GalT in sperm-zona binding has been demonstrated using mouse sperm and a series of reagents that block GalT or the GalT recognition site on the zona pellucida, all of which inhibited sperm-zona binding (Shur and Hall, 1982a and 1982b; Lopez et al., 1985; Shur and Neely, 1988). In addition, using knockout mice, Lu and Shur (1997) demonstrated that GalT-null mice have significant reduction in binding to ZP and no ZP3-induced acrosome reaction. However, sperm of GalT-null mice still binds to ZP and are fertile, demonstrating that the knockout sperm are able to acrosome react spontaneously in a situation where their normal, triggered path to acrosome react is absent. This also implies that other sperm components, either specific or nonspecific, facilitate the adhesion of sperm to ZP. Potentially, other sperm surface proteins may cooperate with GalT to increase the affinity or avidity of ZP3 binding and ensure fertilization of the oocyte.

The identification of multiple candidate molecules suggests that multiple carbohydrate ligands and sperm lectins are involved in sperm-ZP binding. Further investigation is required to determine whether sperm-ZP binding is mediated by a combination of protein and carbohydrate moieties on the sperm surface and to identify potential receptors for these sperm molecules in the ZP.

Species specificity of Sperm-ZP Binding

Although binding of mammalian sperm to ovulated oocytes is not strictly species specific, the ZP represents a significant barrier to most heterospecific crosses in vitro (Gwatkin, 1977; Gulyas and Schmell, 1981; Yanagimachi, 1994). In certain mammalian species, hybrids are viable, for example mules derived from a cross between male donkey and female horse. Nevertheless, in most instances, barriers to interspecies fertilization appear effective. These barriers are attributable to the presence of receptors for sperm in the ZP that, to a large extent, restrict binding of sperm from heterologous species (Gwatkin, 1977). Although the restrictions on binding are not absolute (e.g., mouse sperm bind to hamster ZP and hamster sperm bind to mouse ZP), they provide for a relatively high degree of species-specific fertilization in vitro (e.g., guinea pig and human sperm do not bind to mouse ZP; Wassarman, 1999).

ZP can interfere with interspecies fertilization by failing to permit initial binding of sperm to the oocyte, induction of the acrosome reaction, or penetration of bound sperm through the extracellular matrix (Wassarman, 1999). Removal of the ZP from unfertilized oocytes, thereby exposing the oocyte plasma membrane directly to sperm, eliminates the barrier to fertilization between some species in vitro (Wassarman, 1999). In vitro incubation of sperm with hamster and rabbit zona-denuded oocytes resulted in an uncontrolled uptake of sperm of different species in the oocyte (Hanada and Chang, 1976; Maleszewski et al., 1995; Wassarman, 1990a), pointing to the important role of the ZP for the species-specificity of sperm-ZP interactions.

Although O-linked oligosaccharide chains on ZP3 act as a binding epitope for sperm (Bleil and Wassarman, 1980c; Florman and Wassarman, 1985; Wassarman,

1990b), the molecular composition of the sperm-binding oligosaccharide remains obscure. There is evidence to suggest that several sugars, including galactose, fucose, and N-acetyl-glucosamine, may be involved in recognition by sperm (Miller et al., 1992; Litscher et al., 1995; Johnston et al., 1998). One observation that supports the idea of a carbohydrate-mediated mechanism for the species-specificity of gamete binding comes from the study using transgenic mice in which human ZP3 totally replaced mouse ZP3 in the ZP (Rankin et al., 1998). When human or mouse sperm were added to ovulated oocytes from such mice, mouse sperm, but not human sperm, bound to ZP. The authors suggested that human sperm did not recognize specific oligosaccharides on ZP3. Since the human ZP3 (present as a transgene) was synthesized by growing mouse oocytes, not human oocytes, it was expected that oligosaccharides linked to human ZP3 were mouse-like, not human-like. Consistent with this proposal, a recent investigation of the structure of the O-linked oligosaccharides linked to the human ZP3 synthesized by oocytes from these transgenic mice concluded that they are indistinguishable from O-linked oligosaccharides on mouse ZP3 (Dell et al., 2003). Therefore, variations in the sugar residues in ZP3 may contribute to the establishment of the species-specific nature of gamete interaction (Sinowatz et al., 2001a).

In addition to variations in the structure of ZP3, variations in the sperm plasma membrane proteins that interact with the ZP may also confer specificity. However, binding of sperm to heterologous ZP has been documented in several species (Bedford, 1977; Swenson and Dunbar, 1982; Yoshimatsu et al., 1988; Lanzendorf et al., 1992). Recently, Sinowatz et al. (2003) demonstrated that incubation of equine and porcine sperm with bovine oocytes in vitro resulted in a strong binding of these sperm to the

bovine ZP. Using electron microscopy, they showed that both equine and porcine sperm that bound to bovine ZP had undergone acrosome reaction. In addition, equine sperm were able to penetrate the ZP and fuse with the oolemma. Therefore, species-specificity of sperm-ZP interaction is not absolute but only relative.

Sperm-ZP Binding and Induction of Acrosome Reaction

Sperm that are unable to recognize and bind to the ZP, as well as sperm that are unable upon binding to respond by undergoing the acrosome reaction, fail to fertilize the oocyte (Topfer-Petersen et al., 2000). After entering the oviduct, sperm may be stored in the isthmic region of the oviduct by binding to the ciliated cells lining the oviductal epithelium (Suarez, 2001). Sperm are stored under conditions that maintain sperm viability and synchronize sperm transport and the capacitation process. This enables sperm to interact with the oocyte in the appropriate manner (Topfer-Petersen et al., 2000).

When moving to the site of fertilization, the capacitated and hyperactivated sperm must first penetrate the surrounding cumulus mass, consisting of follicular cells dispersed in a polymerized hyaluronic acid matrix (Yanagimachi, 1994). To penetrate the substantial cumulus cell barrier surrounding the ovulated oocyte, sperm use hyperactivated motility and a membrane-bound hyaluronidase named PH-20 (Yanagimachi, 1994; Lin et al., 1994; Primakoff and Myles, 2002). The motility and surface hyaluronidase are necessary, and perhaps sufficient, to digest a path through the extracellular matrix of the cumulus cells (Primakoff and Myles, 2002). Eventually, the sperm encounters and recognizes the ZP.

The initial, or primary, binding of the capacitated sperm to the egg is mediated by exposed oligosaccharide chains of the ZP matrix and complementary carbohydrate-binding proteins of the sperm surface. A multimeric receptor complex formed by the interaction with the ZP may then trigger the signaling cascade that leads to exocytosis of the acrosome (Shur, 1999). Only ZP-bound sperm that have undergone the acrosome reaction can penetrate the ZP, probably by using one or more proteases present in the acrosome together with hyperactivated motility. Furthermore, only sperm that have undergone the acrosome reaction can fuse with the oolemma (Yanagimachi, 1994).

Binding of capacitated sperm to terminal sugar residues on ZP3 starts a cascade of signaling events that lead to fusion of membranes and the acrosome reaction (Tulsiani et al., 1998). The acrosome reaction induced by the binding of sperm to ZP3 may proceed through two sperm signaling pathways. In the first, ZP3 binding to GalT and other potential receptors results in activation of a heterotrimeric GTP-binding protein and phopholipase C (PLC), thus elevating the concentration of cytoplasmic calcium. In the second pathway, ZP3 binding to the same receptor(s) stimulates a transient influx of calcium through T-type channels. In a later phase of the signaling, these initial ZP3-induced events produce additional calcium entry through Trp family calcium channels, resulting in a sustained increase in cytoplasmic calcium concentration that triggers exocytosis (O'Toole et al., 2000; Jungnickel et al., 2001). During the acrosome reaction, the sperm plasma membrane fuses at multiple sites with the outer acrosome membrane (Mattioli et al., 1996). The acrosomal contents, mainly hydrolytic enzymes, start to disperse and digest the ZP. The apical plasma membrane and the outer acrosomal membrane form "mixed" vesicles that disperse (Flesch and Gadella, 2000). Throughout

this process, the inner acrosomal membrane becomes a part of the outer barrier of the cell and will form a continuous membrane structure with the plasma membrane, which looks like a hairpin structure (Szasz et al., 2000). Consequently, the inner acrosomal membrane is exposed to and binds to the ZP (secondary ZP binding). This secondary binding of sperm to ZP is poorly understood but, in mice, ZP2 has been identified as the major ZP protein responsible for this interaction (Bleil et al., 1988; Yanagimachi, 1994). Hypermotile sperm that have acrosome-reacted properly upon binding to ZP, penetrate the ZP, enter the perivitelline space and fuse with the oolema. Thereby, the sperm induces a series of cellular responses known as oocyte activation, which are prerequisites to initial normal embryonic development (Yanagimachi, 1994).

Since fertilization is a fundamental process essential for propagation of the species, it might be expected that a number of regulatory processes would have evolved to ensure that the initial contact between the sperm and the ZP leads to fertilization. It is more likely that a combination of events takes place, some of which may not be essential, but which optimize the chances of fertilization. There might also be a degree of redundancy involved in what is a crucial process to the individual (Snell and White, 1996).

While considerable progress has been made in identifying sperm and oocyte components involved in each of the steps that lead to fertilization, several issues remain unresolved or contentious. However, it is reasonable to expect that, despite the unanswered questions that remain, it is only a matter of time until the sophisticated experimental approaches being applied to mammalian fertilization reveal its well-kept secrets. In addition to the intellectual value, this knowledge should enable development

of advanced tests and clinical treatments for infertility, as well as lead to novel approaches to contraception.

The Sperm-ZP Binding Assay

The Basis of Sperm-ZP Binding Assay

Sperm-ZP binding assays have been developed to evaluate the first, crucial step of sperm-oocyte interaction that leads to fertilization, that is, the tight binding of sperm to the ZP (Yanaguimachi, 1981). Before a spermatozoon can penetrate the ZP, there is a specific need for firm attachment between gametes. When sperm and ZP are co-incubated in vitro, some of the sperm adhere rapidly and reversibly to the ZP by a process called "attachment". Subsequently, an irreversible adhesion occurs between the two gametes, named "tight binding". Tight binding is attributed to the presence of complementary binding sites or receptors on the surface of gametes; and typically, these receptors manifest a high degree of species specificity (Yanaguimachi, 1981; Ahuja, 1985). Because the binding of gametes is a critical step for fertilization, the sperm-ZP binding assay has many potential uses in reproductive medicine including investigation of male infertility and evaluation of sperm fertilizing potential.

Assays based on the ability of sperm to bind to homologous or heterologous ZP have been developed as diagnostic tools in several animal species including the monkey (Oehninger et al., 1989b), pig (Fazeli et al., 1995a), cattle (Zhang et al., 1999), dog (Mollova et al., 1996) and horse (Fazeli et al., 1993b). The two most common sperm-ZP binding tests currently used are the hemizona assay (HZA; Burkman et al., 1988) and

intact ZP binding assay (Liu et al., 1988a). In the HZA, oocytes are microbisected into two matching hemizonas and each hemizona is incubated with sperm from different males or treatments. A disadvantage of the HZA is that it is time-consuming and technically more demanding to perform than intact ZP binding assay. In the intact ZP binding assay, sperm from each male or treatment are incubated with a different group of intact ZP. A relatively large number of oocytes and replicates should be used to reduce the variation among oocytes when performing an intact ZP binding assay (Strom Holst et al., 2001). Alternatively, a competition assay can be performed by labeling sperm from different males or treatments with different fluorochromes (FITC, green; TRITC, red). Following labeling, sperm from both groups are simultaneously incubated with the same group of oocytes. Although different in their methodologies, both assays use assessment of tight sperm binding to the ZP as the primary endpoint (Oehninger et al., 2000).

Results of ZP binding assays can be presented as the total number of sperm bound to ZP (Zhang et al., 1998), or as a ZP binding index. The index represents the binding capacity of a test semen sample compared to that of a control semen sample (Fazeli et al., 1997; Zhang et al., 1998). Outcomes of sperm-ZP binding assay have been shown to correlate with several other sperm parameters such as viability, motility, morphology and acrosomal status (Franken et al., 1989; Liu et al., 1989; Menkveld et al., 1991; Liu and Baker, 1992a). Normal sperm morphology is one of the most significant semen analysis variables related to sperm-ZP binding (Liu et al., 1989; Oehninger et al., 1989a; Menkveld et al., 1991; Liu and Baker, 1992a), which explains why normal morphology has a significant relationship with fertilization rates in vitro. Sperm motility and concentration in the insemination medium are also correlated with sperm-ZP binding (Liu

et al., 1988a and 1989). This is not surprising, since both motility and concentration directly influence the change of sperm contacting the oocyte. In addition, the proportion of sperm with intact acrosomes in the insemination medium is related to the number of sperm bound to ZP (Liu et al., 1987 and 1988b; Liu and Baker, 1992a; Menkveld et al., 1996), demonstrating that an intact acrosome is important for sperm binding. Because these semen characteristics correlate with the results of the sperm-ZP binding assay, this assay reflects multiple sperm functions and can be used to evaluate the fertilizing capacity of sperm (Oehninger et al., 1989a; Tesarik and Testart, 1989; Liu and Baker, 1992b and 1994).

In humans, sperm-ZP binding assays have been used in the clinical management of infertility. Results obtained from sperm-ZP binding assays are used to allocate patients between standard IVF or ICSI. This decision is based on assessment of sperm characteristics that are important for fertilization with standard IVF but not necessarily for ICSI. Usually, patients with subtle sperm defects that impair sperm-ZP interaction are more efficiently treated with ICSI (Liu et al., 2004). Although ICSI could be used for treatment of all patients irrespective of sperm quality, most clinics reserve ICSI treatment for patients with sperm abnormalities who are expected to be unsuccessful with standard IVF (Bhattacharya et al., 2001; Ola et al., 2001). Therefore, the sperm-ZP binding assay can be used to distinguish a population of male-factor infertility patients that will encounter low fertilization rates in IVF and, combined with information provided by other sperm parameters (e.g. morphology and motility), gives reliable and useful information in the clinical setting.

Research on sperm-ZP binding in domestic animals has been stimulated by reports from research in humans, where sperm-ZP binding is a good predictor of IVF success (Franken, 1998; Oehninger et al., 2000). However, reliance on the human sperm-ZP binding model is inadequate, because in domestic animals the purpose of the test is prediction of pregnancy rate and possibly litter size after AI with abundant sperm, pooled across many herds of females each with a different management scheme (Amann and Hammerstedt, 2002). Therefore, it is not surprising that conflicting results have been reported when sperm-ZP binding assays have been used to predict field fertility in domestic animals or when correlations have been calculated; this will be discussed in a later section.

In the horse, sperm-ZP binding assays have been used for different purposes. The ability of sperm to bind to the ZP is acquired early in the process of capacitation, and capacitation probably enhances receptor access of the sperm cell to the ZP (Florman and First, 1988). Therefore, binding of stallion sperm to homologous ZP has been used as a method to assess sperm capacitation. Ellington et al. (1993) evaluated the effects of co-incubation of sperm and oviduct epithelial cell (OEC) on sperm capacitation. Sperm-ZP binding assays were performed with sperm co-incubated with OEC for 4 h. More sperm were bound to the ZP in the co-culture group than the control. Authors concluded that co-incubation of stallion sperm with OEC stimulated sperm capacitation. Sperm-ZP binding has also been used to evaluate the effects of cryopreservation on stallion sperm function. Dobrinski et al. (1995) performed sperm-ZP binding assays with sperm from split ejaculates that were either stored at room temperature or frozen-thawed. The sperm's ability to bind to the ZP was significantly decreased in the cryopreserved sperm

compared to fresh sperm. The authors suggested that cryodamage to the plasma membrane of sperm could have impaired the ability of sperm to bind to the ZP; and this could explain, in part, the reduced fertility that is commonly observed after use of cryopreserved sperm. In addition to being used as a tool to evaluate sperm function, sperm-ZP binding assays have been used to estimate the fertility of stallions (Fazeli et al., 1993b and 1995b; Pantke et al., 1995; Meyers et al., 1996). These experiments will be detailed in a section below.

Factors Affecting the Results of Sperm-ZP Binding Assays

Most tests for gamete binding are performed to estimate the fertilization capacity of sperm. In humans, sperm-ZP binding assays using homologous oocytes are highly correlated with male fertility and can be applied to predict the outcome of IVF (Franken, 1998; Oehninger et al., 2000). Other similar tests have shown that fertility of bulls (Fazeli et al., 1993a) and stallions (Fazeli et al., 1993b, 1995b; Pantke et al., 1995; Meyers et al., 1996) is correlated with the capacity of sperm to bind the ZP of homologous oocytes. However, those tests revealed a large variability in the capacity of ZP to bind sperm (Franken et al., 1991; Fazeli et al., 1993a).

Sperm-ZP interaction is related to the functional integrity of both sperm and oocytes. Abnormalities in either sperm or oocyte can affect the sperm-ZP interaction (Liu et al., 2004). The ZP's ability to bind sperm may be influenced by several factors, including maturational stage of oocyte (Tesarik, 1990; Franken et al., 1991) and methods of oocyte preservation (Tesarik et al., 1990).

Studies performed on human and monkey oocytes indicate that the stage of oocyte maturation may affect the number of sperm that bind to the ZP (Familiari et al., 1988; Oehninger et al., 1989b, 1991; Pu et al., 1994). In monkeys, a gradual increase in the capacity of ZP to bind sperm is observed with nuclear progression from dictyate to the metaphase II stage, whereas in women the capacity of ZP to bind sperm increases after transformation from metaphase I to metaphase II. In horses, Mlodawska et al. (2000) investigated the effects of oocyte maturation on the number of sperm bound to the ZP. In that study, immature and mature oocytes were inseminated directly after collection or after 30 h of IVM. Authors demonstrated that fewer sperm were bound to immature versus mature oocytes, suggesting that mature oocytes had greater sperm-binding capacity than immature oocytes. However, after 30 h of maturation, they found no difference in the number of sperm bound to mature or immature oocytes.

The final stage of oocyte maturation is probably accompanied by changes in the ZP that increase its ability to bind sperm. These changes consist of special rearrangements of zona glycoproteins during the late stages of oocyte maturation (Oehninger et al., 1989b, 1991). Observations of human oocytes by scanning electron microscopy demonstrate that the appearance of the outer surface of the ZP may reflect oocyte maturity and sperm-binding capacity (Familiari et al., 1988). ZP with smooth surfaces, observed on immature oocytes and mature oocytes showing early signs of atresia, bind considerably fewer sperm than do ZP of spongy appearance. Such spongy, mesh-like structure of the ZP was observed on most mature (preovulatory) oocytes. Similar observations of mouse oocytes have shown that the outer surfaces of ZP become progressively flatter as oocytes age. The surfaces of ZP of oocytes recovered directly

after ovulation had fibrous network with numerous pores, whereas those of degenerate or fragmented oocytes were flat and amorphous (Nogues et al., 1988). Thus, it is inferred that the appearance of the outer surfaces of the ZP may reflect the physiological status (aging) of oocytes and its ability to bind sperm.

Oocytes used for sperm-ZP binding assays are usually obtained from offal ovaries (Fukuiet al., 1988; Pantke et al., 1995; Zhang et al., 1997; Lynham and Harrison, 1998), but they can also be retrieved after ultrasound-guided ovum-pickup in live animals (Lansbergen et al., 1997). To assure sufficient numbers of oocytes for sperm evaluation, oocytes can be stored either in salt solutions or recovered from ovaries that have been stored frozen (Wheeler and Seidel, 1987; Chian et al., 1991; Tatemoto et al., 1994; Strom Holst et al., 2000). Saline-stored oocytes have been routinely used in studies on gamete interaction in the rabbit (Fayrer-Hosken and Bracket, 1987), pig (Henderson et al., 1987), hamster (Boatman et al., 1988), human (Yoshimatsu et al., 1988), bull (McBride et al., 1988), dog (Strom Holst et al., 2000) and horse (Fazeli et al., 1993b). Yanagimachi et al. (1979) showed that the biological and physiological properties of the ZP are well preserved in concentrated salt-solutions. Salt-stored human and pig oocytes did not differ in their sperm-binding capacity compared with fresh oocytes (Yanaguimachi et al., 1979; Liu et al., 1988a; Kruger et al., 1991; Fazeli et al., 1995a). Even after 90 days of salt-storage, human zonae pellucidae retained biological and functional characteristics (Franken et al., 1991). Studies in the bovine demonstrated that salt-stored bovine oocytes were able to bind sperm similarly to fresh oocytes; however, penetration rates were lower for stored oocytes (McBride et al., 1988; Chian et al., 1991). Canine oocytes stored in salt solution had a reduced sperm-binding capacity compared with that of fresh oocytes

(Strom Holst et al., 2000). The reduced sperm-binding capacity of salt-stored canine oocytes could be related to ultrastructural changes. In the same study, scanning electron microscopy revealed that stored oocytes had a ZP that had a wider meshwork and larger fenestrations than that of fresh oocytes. In the horse, sperm-ZP binding assays have been performed using fresh (Mlodawska et al., 2000), frozen-thawed (Ellington et al., 1993; Dobrinski et al., 1995; Meyers et al., 1996) and salt-stored oocytes (Fazeli et al., 1993b and 1995b; Cheng et al., 1998). Pantke et al. (1995) investigated the sperm-binding capacity of fresh versus salt-stored equine oocytes and found that similar numbers of sperm were bound to salt-stored oocytes compared to fresh oocytes. This study clearly demonstrated the feasibility of using salt-stored oocytes for sperm-ZP binding assays in the horse.

Variation in binding capacities of oocytes can be avoided by using a large number of oocytes per batch and several batches per test (Zhang et al., 1995) or by performing HZA (Franken et al., 1993b; Fazeli et al., 1997). In species which availability of homologous oocytes is restricted, such as humans and horses, assays can also be performed using heterologous oocytes. However, human sperm-oocyte interactions are strictly species-specific, and human sperm do not bind to ZP of other mammalian species (Bedford, 1977; Liu et al., 1991). Therefore, use of heterologous oocytes for human assays is inappropriate. In domestic animals, heterologous fertilization has been documented. For example, fertilization by sperm derived from species within the genus Bos has been performed with cattle oocytes (McHugh and Rutledge, 1998). In addition, Cox et al. (1994) demonstrated that goat sperm can penetrate cattle and sheep oocytes. Horse sperm are more promiscuous than human sperm and bind to oocytes from different

species, as demonstrated by the cross of a male horse and a female donkey, resulting in a hinny. In addition, Sinowatz et al. (2003) demonstrated that stallion sperm are able to bind tightly to the ZP of bovine oocytes, undergo acrosome reaction, penetrate the ZP and fuse with the oolemma. Therefore, development of a heterologous assay using bovine oocytes to evaluate stallion sperm would be beneficial because of to the scarcity of equine oocytes for research.

Other factors that affect the outcome of sperm-ZP assays are related to quality and preparation of sperm samples. As mentioned before, sperm parameters such as motility and concentration correlate with sperm binding to the ZP (Liu et al., 1988b and 1989). Therefore, when performing sperm-ZP binding assays, sperm samples should be adjusted to contain the same concentration of motile sperm during co-incubation with the ZP.

Lastly, human error or bias can also affect the results of sperm-ZP binding assay. To avoid bias, evaluators should be blinded to the identity of males and treatments being tested. In addition, the same technician should evaluate all samples from the same batch or replicate, and the technique applied to manipulate the gametes should be consistent between samples. Finally, when removing loosely bound sperm from the ZP, the same pipette should be used for all samples in the replicate, because the diameter of the pipette will affect removal of sperm from the ZP (Yao et al., 1996).

Correlation Between Sperm-ZP Binding and Fertility

Fertility is a probability event, and the aim of semen evaluation is to establish the proportion of sperm in a semen sample that possess the traits that are necessary for fertilization, thereby estimating the potential field fertility of the sample. As a

consequence, evaluation of different aspects of sperm function, using either a combination of different tests or assays that evaluate multiple sperm parameters, usually gives a more reliable estimate of in vivo fertility than a test that evaluates only a single aspect of sperm function (Zhang et al., 1998). Among the single aspects of semen quality evaluated are sperm morphology (Barth, 1992), sperm motility (Kjaestad et al., 1993; Holt et al., 1997), biochemical compounds in semen (Hirao, 1975), presence of intact acrosomes (Correa et al., 1997), membrane integrity (Perez et al., 1997), sperm chromatin structure (Evenson et al., 1980) and ability to undergo capacitation (Januskauskas et al., 1999; Thundathil et al., 1999) and the acrosome reaction (Whitfield and Parkinson, 1995; Januskauskas et al., 2000). Because fertilization entails a complex series of events leading to early embryo development, it is unlikely that the evaluation of a single sperm characteristic or function can be predictive in an absolute manner of the fertilization potential of the male gamete. In fact, few single sperm parameters show a significant correlation with in vivo fertility for semen samples within acceptable ranges of normality (Larsson and Rodriguez-Martinez, 2000). Therefore, it seems logical to combine the outcome of different semen evaluation tests to achieve a better correlation between test results and in vivo fertility (Amann and Hammerstedt, 1993). However, more complex indices based upon a combination of routine semen parameters have proven only slightly better prediction of fertility (Hiaro, 1975; Fazeli, 1997).

Prediction of male fertility has been improved by the incorporation of parameters based on the functional characteristics of sperm in semen quality analysis (Bousquet and Bracket, 1982; Marquant-le Guienne et al., 1990; Lonergan, 1994; Henault and Killian, 1995). One of these parameters is the binding of sperm to the ZP of the oocyte. Sperm-

oocyte interaction assays evaluate multiple sperm parameters providing the most significant predictive information about sperm fertilizing ability (Strom Holst et al., 2001).

In humans, sperm-ZP binding assays constitute a useful triage to direct patient couples to IVF (good binding) or ICSI (poor binding), since defective sperm-ZP binding has been identified as a major cause of fertilization failure in human IVF (Liu and Baker, 2000; Liu et al., 2001). Consequently, results from sperm-ZP binding assays are significantly correlated with fertilization rates of standard IVF in humans (Oehninger et al., 1989a, 1997 and 2000; Liu et al., 1989; Liu and Baker, 1994; Franken, 1998). Recently, Oehninger et al. (2000) performed a meta-analysis on eight studies performed between 1988 and 1997, to investigate the relationship between sperm-ZP binding assays and human IVF outcome (Table 1.1).

Table 1.1: Sperm-ZP binding assays and IVF outcome in humans.

Study	n	r	Sensitivity	Specificity	PPV	NPV
Liu et al., 1988a	20	0.80	90	90	90	90
Oehninger et al., 1989a	28	0.79	95	83	95	83
Oehninger et al., 1992	44	0.70	100	61	79	100
Franken et al., 1993a	112	0.55	84	72	85	70
Franken et al., 1993b	48	0.50	75	68	81	68
Coddington et al., 1994	94	0.59	82	78	88	69
Gamzu et al., 1994	133	0.96	100	94	85	100
Oehninger et al., 1997	196	0.69	93	73	85	87

n = number of cases; r = Pearson correlation; PPV = positive predictive value; NPV = negative predictive value

Adapted from Oehninger et al., 2000.

Those studies prospectively evaluated a total of 675 patients using HZA or intact-zona binding assays. Authors reported a significant overall correlation of 0.641 between the outcome of ZP binding assay and IVF, associated with a PPV $\geq$ 80% and a NPV > 70% in general. Importantly, the false negative rate was consistently low (2 to 25%), demonstrating that sperm-ZP binding assay could be applied to the clinical management of infertile patients for assisted reproduction.

In domestic animals, the importance of determining the exact level of fertility that a male can achieve is most pronounced in cattle, due to the fact that semen from a single male is used to inseminate many females. In addition, due to the high degree of refinement of bovine IVF and its frequent use, most studies have been carried out on the correlation between IVF outcome and in vivo fertility (Larsson and Rodriguez-Martinez, 2000). Studies on the correlation between sperm-ZP binding and in vivo fertility, defined as a nonreturn to estrus rate (NRR) for 56 days, have provided controversial results. Fazeli et al. (1997) demonstrated a significant correlation between NRR after insemination with frozen semen and HZA results ($r = 0.46$). In contrast, neither Zhang et al. (1999) nor Braundmeier et al. (2002) found a significant correlation between NRR and results from intact ZP binding assay. Zhang et al. (1999) suggested that the use of bulls with very similar NRR (61 to 66%) could account for the absence of relationship between the number of sperm bound to ZP and fertility.

Similar to cattle, studies in pigs have provided conflicting results when investigating the relationship between sperm binding to ZP in vitro and fertility in vivo. Ivanova and Mollova (1993) and Gadea et al. (1998) observed that fewer sperm from subfertile than fertile boars bound to the ZP of oocytes. However, Berger et al. (1996)

could not demonstrate a correlation between in vivo fertility and sperm-ZP binding capacity. Interestingly, a recent report from Braundmeier et al. (2004) reported that results from HZA were positively correlated with litter size but not with farrowing rates. The authors suggested that, perhaps in litter-bearing species, litter size may be a more accurate or sensitive measurement of fertility than farrowing rate.

In the horse, four studies have been performed to evaluate the ability of sperm-ZP binding assays to predict field fertility. In the first study, Fazeli et al. (1993b) evaluated sperm from three fertile and three subfertile stallions using HZA. Stallions were paired (one fertile and one subfertile), and HZA was performed using fresh semen. Despite the low variation in fertility between fertile and subfertile stallions used in the same pair, only 10 to 15%, a significant correlation was observed between number of sperm bound to hemizonae and stallion fertility. In a subsequent study, the same group (Fazeli et al., 1995b) performed a similar experiment comparing ten pairs of stallions with varying fertility. Stallions in each pair were from the same stud farm; therefore, fertility indexes were obtained by mating stallions with mares under the same management regimen. Authors also found a significant relationship between the number of sperm bound to ZP and the probability of pregnancy after AI. In the same year, Pantke et al. (1995) performed sperm-ZP binding using semen from seven fertile and five subfertile stallions. Sperm from the fertile stallions bound in higher numbers to the ZP than sperm from the subfertile animals, supporting the use of ZP binding assays as a technique to evaluate stallion fertility. In the last study by Meyers et al. (1996), semen from three fertile and three subfertile stallions was used in an intact ZP binding assay. Fertile stallions had histories of > 70% pregnancy rates over several breeding seasons. In contrast, subfertile

stallions either had no record of live foals or had pregnancy rates < 10% from breeding trials. A significantly higher number of sperm from fertile stallions were bound to the ZP, compared to subfertile animals. In addition, a higher proportion of bound sperm from fertile than subfertile stallions were acrosome-reacted. Therefore, studies in the horse demonstrated that the outcome of sperm-ZP assays have a predictive value for in vivo fertility, when variables such as semen handling, frequency of inseminations, and mare management were standardized. In practice, sperm-ZP binding assays can be used to evaluate the semen quality of stallions of unknown fertility by comparing their sperm-binding capacity with that of a control stallion of proven fertility under similar management conditions.

In view of the inherent variation among ejaculates and the tendency for male fertility to vary with time, it seems unlikely that male fertility could be accurately predicted using sporadic in vitro tests. We still do not have any reliable method that mimics the complicated and important interactions between the sperm and the female genital tract that occur during the transport of sperm to the site of fertilization. In addition, results of studies depend upon how in vivo fertility is defined and how field fertility data are obtained and presented. In most species, the number of females bred by a single male is too small to allow adequate data to be obtained for comparisons. One exception is the bovine. A bull's fertility in vivo is most often expressed as non-return rates (NRRs) after artificial insemination of a large number of females. However, both maternal and environmental factors influence field fertility. Furthermore, almost all data for artificial insemination of cattle concern frozen semen, which may or may not correlate with fertility of fresh semen. Another factor that has to be considered in the in vitro

assessment of fertility is the variation between ejaculates. There are differences in both in vivo and in vitro fertility among ejaculates collected within a narrow time span from the same bull (Zhang et al., 1997). Therefore, a representative number of ejaculates has to be tested (Otoi et al., 1993; Zhang et al., 1997).

Despite difficulties correlating laboratory data with fertility, laboratory evaluation of semen quality remains important. Although laboratory semen analyses may not predict actual fertilizing potential of a semen sample, the analysis may predict samples of low fertility and allow exclusion of those samples from an artificial insemination program (Graham, 1996; Colenbrander et al., 2003; Amann, 2005). Therefore, sperm-ZP binding assays should be used primarily to evaluate gamete interactions under different conditions in vitro and to gain information on the physiology of sperm, oocyte and the fertilization process.

Semen extenders used for cold-storage of equine sperm

Several extender media for processing and storage of equine sperm have been developed. Base ingredients of most extenders are egg yolk or milk. These extenders are suitable for cold-storage of semen for a period of 24 to 48 h. Positive effects of extenders are based on dilution of seminal plasma, control of the pH and osmolarity, and supply of energy substrate (Aurich, 2005). The nature of the positive effect of egg yolk or milk on sperm longevity and fertility is still a point of speculation. In bovine semen, it has been demonstrated that a low density lipoprotein fraction from egg yolk interacts with seminal plasma proteins (BSP A1/A2, A3, BSP-30) known to induce cholesterol and phospholipids efflux from the plasma membrane (Bergeron et al., 2004). Therefore, egg

yolk stabilizes the plasma membrane by neutralizing detrimental components of seminal plasma. A comparable mechanism of egg yolk for equine semen can be expected because similar seminal plasma proteins exist in the horse (Calvete et al., 1994).

Milk or milk-based extenders are commonly used for the dilution, centrifugation and cold-storage of equine semen. However, the mechanisms of action of milk proteins remain unknown. Recently, a positive effect of milk-based extenders on the antioxidative activity of semen has been demonstrated (Kankofer et al., 2005). In this study, dilution of semen with a milk-based extender resulted in a significant increase in the activity of glutathione peroxidase, superoxide dismutase and catalase. Lipid peroxidation of sperm membrane lipids has been reported to be the major cause for the loss of sperm motility and fertilizing ability of human sperm incubated for long periods of time during IVF procedures (Aitken et al., 1989). A comparable situation can be expected for equine semen; therefore, the protective effects of milk-based extenders may be related to the increase in antioxidative capacity after dilution of semen. This hypothesis is supported by the fact that addition of antioxidants to semen resulted in a better maintenance of membrane integrity, motility and fertility of equine sperm (Aurich et al., 1997; Bruemmer et al., 2002).

A major problem in semen extenders based on milk or egg yolk exists in the fact that these biological substances are composed of a variety of different molecules and may differ between batches. Therefore, a number of extenders with defined compositions have been developed. For example, egg yolk has been successfully replaced by soybean lecithin in a commercial extender used to cryopreserved bovine semen (Aires et al., 2003; AndroMed®, Minitube, Verona, WI). A modification of this extender is available for the

horse and shows promising results for cold-storage (Aurich, 2005; AndroMed E®, Minitube). In the case of milk-based extenders, fractioning of milk by different methods (e.g. microfiltration, ultrafiltration or freeze-drying) has allowed the preparation of different purified fractions. Among these, native phosphocaseinate and β-lactoglobuline appear to be the major components with positive influence on equine sperm survival and quality. Therefore, a defined extender containing native phosphocaseinate was developed, and it is currently used for cold-storage of equine semen (Batellier et al., 1998; INRA96®, IMV, L'Aigle Cedex, France).

Development of extenders and methods of semen processing have led to an improvement of semen quality during storage; however, longevity of stallion sperm is still limited when compared to boars or ruminants. Therefore, studies on the effects of components of semen extenders on sperm function could assist in the development of more efficient methods to preserve equine sperm.

Concluding Remaks

From this review it is evident that, despite a few decades of investigation, the physiology of gamete interaction is still not completely understood. Several important factors involved in sperm and ZP interaction remain unknown, such as the molecules involved in gamete binding. In addition, presence and function of such molecules vary among species; and therefore, findings in one species can not always be applied to another.

Development of new assisted reproductive technologies has helped investigators gain knowledge on gamete interactions and fertilization. To date, IVF in horses has not

resulted in repeatable, successful results. The major barrier is probably penetration of the ZP by a spermatozoon (Hinrichs, 1998). However, it is not known whether such failures are related directly to the ZP or to inadequate in vitro preparation of sperm for penetration of the ZP (Mlodawska et al., 2000). In addition, little information is available on the properties and possible changes occurring in equine ZP during the processes of oocyte maturation and fertilization. More studies on sperm interaction with the ZP in vitro should be performed to increase our knowledge on equine gamete interactions, leading to the development of successful protocols for in vitro fertilization of equine oocytes.

The studies presented in subsequent chapters were conducted to develop a heterologous ZP binding assay for stallion sperm and to investigate the effects of semen extenders used in assisted reproductive technologies on the binding of stallion sperm to the ZP. Initial findings revealed a significant effect of milk-based extenders on gamete interaction in vitro. Therefore, subsequent studies were performed to identify the particular component of the milk-based extender that affected sperm binding to the ZP. Lastly, a series of studies were performed to investigate the mechanism by which milk proteins affected sperm interactions with ZP.

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Chapter II

Effects of components of semen extenders on the binding of stallion spermatozoa to bovine or equine zonae pellucidae

Introduction

Binding of sperm to the zona pellucida (ZP) plays a crucial role in the process of fertilization. In the oviduct, sperm penetrate the mass of cumulus cells surrounding the oocyte and bind to the ZP, the last physical barrier that sperm must overcome before fertilization. Inability of sperm to penetrate the ZP inevitably results in infertility (Green, 1997). The initial binding of sperm to the ZP is loose, but leads to tight linkage of the gametes through specific receptor-ligand pairs on the surfaces of the sperm head and the ZP (Yanagimachi, 1994). However, despite intense investigation, the molecular mechanism of sperm binding to ZP has yet to be determined (He et al., 2003; Dean, 2004; Hoodbhoy and Dean, 2004).

ZP-binding assays have been used to estimate the fertilizing capacity of sperm in several species. Human sperm binding to homologous ZP correlates highly with male fertility and outcomes of *in vitro* fertilization (Liu et al., 1989; Oehninger et al., 1989; Gamzu et al., 1994). In similar experiments, binding of sperm to the ZP correlated with fertility of bulls (Fazeli et al., 1993a), boars (Ivanova and Mollova, 1993; Braundmeier et al., 2004), and stallions (Fazeli et al., 1993b; Fazeli et al., 1995; Pantke et al., 1995;

Meyers et al., 1996). However, in the horse, use of ZP-binding assays has been limited because of scarcity of equine oocytes. Therefore, development of a ZP-binding assay for the horse using heterologous ZP would be useful.

During natural mating or artificial insemination of mares, semen is deposited within the uterine body, and sperm are transported to the site of fertilization in the oviduct. For some assisted reproductive technologies in the horse, such as gamete intrafallopian transfer (GIFT), sperm are deposited directly into the oviduct, and components of semen extenders may be present during gamete interaction. In the mare, GIFT has been performed in our laboratory using fresh, cooled and frozen stallion semen (Coutinho da Silva et al., 2002 and 2004). Pregnancy rates obtained with cooled and frozen semen were significantly lower than with fresh semen (25%, 8% and 82%, respectively). During GIFT with fresh, cooled or frozen semen, sperm were used raw (no extender), or diluted with skim milk-based extender and Lactose-EDTA extender (LAC; Cochran et al., 1984), respectively. Potentially, components of semen extenders were responsible for the reduced pregnancy rates.

Therefore, objectives of this study were: 1) to determine the suitability of bovine ZP for a ZP-binding assay with stallion sperm; and 2) to investigate the effects of components commonly used in semen extenders on sperm binding to ZP *in vitro*.

Materials and methods

Bovine ZP

Ovaries were recovered from cows at a slaughterhouse and transported to our laboratory in 0.15 M NaCl at 25 to 30°C within approximately 5 h of collection. Oocytes were aspirated from follicles with a 20-gauge needle, isolated under a stereomicroscope, and placed in modified Tyrode's medium (TALP; Table 1). All oocytes, with or without attached cumulus cells, were used. Oocytes were vortexed at maximum speed for 1 to 2 min to remove cumulus cells. Denuded oocytes were washed in TALP and stored in hyperosmotic salt solution (1.5 M $MgCl_2$, 40 mM Hepes, 0.1% (w/v) PVP; Yanagimachi et al., 1979) at 5°C for up to 3 months.

Equine ZP

Ovaries were recovered from mares at an abattoir and transported to a laboratory in saline within approximately 4 h of collection. Antra of follicles were scraped with a bone curette into Hepes-buffered TCM-199 with Hank's salts (Gibco Life Technologies, Inc; Grand Island, NY) into a Petri dish. Cumulus-oocyte complexes were identified with a stereomicroscope and placed in culture medium [TCM-199 with Earle's salts (Gibco), 5 μU/ml FSH (Sioux Biochemical Inc, Sioux Center, IA), 10% (v/v) fetal bovine serum (FBS) and 25 μg/ml gentamicin sulfate]. Oocytes were cultured for 24 to 26 h at 38.5°C in an atmosphere of 5% CO_2 in air. After maturation, oocytes were denuded of cumulus cells by pipetting in a solution of 0.5% (w/v) hyaluronidase in TCM-199 with 5% (v/v) FBS. Only oocytes without a polar body were used for this experiment.

Denuded oocytes were placed in hyperosmotic salt solution (Yanagimachi et al., 1979) and stored at 5°C for up to 4 months.

Stallions and sperm preparation

Semen was obtained from six fertile stallions (A - F) individually housed at the Equine Reproduction Laboratory located at Colorado State University, Fort Collins. Stallions were maintained on a diet of mixed grass and alfalfa hay, vitamin and mineral supplement, and fresh water ad libitum according to Institutional Animal Care and Use Committee protocols at Colorado State University. For all experiments, each replicate was performed with sperm from a single stallion. After collection, semen was evaluated for motility and sperm concentration. Semen was diluted 1:3 with TALP and centrifuged at 400 x g for 5 min. Sperm were suspended with 1 ml of the respective extender containing 35 μg/ml of Hoechst 33342 (ICN Biomedical, Inc., Aurora, OH), according to each experiment, and incubated for 15 min at 37°C. After incubation, sperm were centrifuged and suspended to 2 x 10^6 sperm/ml in respective extenders. Five μl of sperm suspension were added to 45-μl droplets of medium containing the ZP, resulting in a final concentration of 2 x 10^5 sperm/ml.

Binding assays

Prior to each experiment, bovine or equine ZP were washed twice and incubated in TALP for at least 1 h at 38.5°C in an atmosphere of 5% CO_2 in air, except for Experiment IV-B. After incubation, ZP (n=10 per droplet) were randomly placed in

droplets of 45 μl of medium (1 droplet per treatment) to which 5 μl of sperm suspension were added.

ZP and sperm were incubated for 2 h at 38.5°C in an atmosphere of 5% CO_2 in air. After incubation, ZP were washed vigorously in four droplets of TALP using a small-bore, fire-polished, glass pipette to remove loosely bound sperm. ZP were placed on a glass slide and covered with a cover slip supported by a mix of paraffin and petroleum jelly. ZP were observed under an epifluorescence microscope (Eclipse E800, Nikon Instruments, Inc., Melville, NY) equipped with a 360/40 nm bandpass excitation filter and a 460/50 nm bandpass emission filter. ZP were observed under 400x magnification, and the number of sperm bound to ZP was recorded by a single technician, who was blinded as to treatments.

Experimental designs

Experiment I: Use of bovine ZP for ZP-binding assay with stallion sperm

Experiment I was designed to investigate the use of bovine ZP for a ZP-binding assay with stallion sperm. Bovine and equine ZP were inseminated in parallel with semen diluted in TALP (Table 1) or in a skim milk-based extender (EZM; EZ-Mixin CST, Animal Reproduction Systems, Chino, CA). Two ejaculates from each of two stallions (A and D) were used. On each day of the experiment, semen from a single stallion was centrifuged and suspended in TALP or EZM containing Hoechst 33342 as described above. After 15-min incubation at 37°C, sperm were centrifuged and suspended in the same extender. Bovine and equine ZP were inseminated with 5 μl of

sperm suspended in TALP (BOV-TALP, EQ-TALP) or EZM (BOV-EZM and EQ-EZM) and incubated for 2 h at 38.5°C in an atmosphere of 5% CO_2 in air. After incubation, ZP were washed and evaluated as described above to determine the number of sperm bound to ZP.

Table 1. Compositions of media used (mM).

Ingredient	TALP	TSM	TGLU	THGLU	EZM	INRA**
Sanalac®	--	2.4*	--	--	24*	--
$NaHCO_3$	35	35	35	35	18	4
Hepes	10	10	10	10	--	20
Glucose	5.5	5.5	89.5	163.5	272	67
Fructose	84	84	--	--	--	--
Lactose	--	--	--	--	--	126
NaCl	37	37	37	--	--	21.5
KCl	10	10	10	10	--	5.4
KH_2PO_4	1	1	1	1	--	0.5
$MgSO_4$	2.4	2.4	2.4	2.4	--	1
$CaCl_2$	1.7	1.7	1.7	1.7	--	1.2
Na Pyruvate	0.18	0.18	0.18	0.18	--	--
Na Lactate	25.8	25.8	25.8	25.8	--	--
BSA	3*	3*	3*	3*	--	--
Na_2HPO_4	--	--	--	--	--	0.8
Native Phosphocaseinate	--	--	--	--	--	27*

* g/L
** INRA 96: Batellier *et al.* 1998.
For all media, pH was adjusted between 7.3 and 7.4; and osmolality was adjusted to 300 mOsm/kg with NaCl.

Experiment II: Effect of semen extenders on sperm binding to ZP

Experiment II was designed to investigate the effects of semen extenders used for GIFT on binding of stallion sperm to bovine ZP. Two ejaculates from three stallions (A,

B, and C) were used on separate days. Semen was diluted with TALP, centrifuged, and suspended with one of the following extenders: Lactose-EDTA, TALP, Emcare® Holding Solution (EHS; ICP, Auckland, New Zealand) and EZM. Sperm were stained with Hoechst 33342 as described, centrifuged and suspended to 2 x 10^6 sperm/ml with the same extender. Sperm (5 µl) were added to the droplets of TALP containing bovine ZP, and the binding assay was performed as described above.

Experiment III: Effect of centrifugation through Percoll on sperm binding to ZP

Experiment III was designed to investigate effects of sperm centrifugation through a Percoll gradient on sperm binding to ZP. Two ejaculates from each of two stallions (A and B) were used on separate days. After the initial centrifugation, sperm were suspended in 1 ml of TALP or EZM containing Hoechst 33342 and incubated for 15 min at 37 °C. After incubation, sperm diluted in TALP or EZM were centrifuged for 10 min at 400 x g through Percoll (TALP+P and EZM+P) or not (TALP, EZM). Percoll gradients [90 and 45% (v/v)] were prepared with TALP and 1 ml of each gradient was used. Sperm were resuspended to 2 x 10^6 sperm/ml with TALP for groups TALP, TALP+P and EZM+P. In the EZM group, sperm were resuspended with EZM to the same concentration. Sperm (5 µl) were added to the bovine ZP, and the binding assay was performed as described above.

Experiment IV: Effect of EZM during sperm and ZP interaction

Experiment IV was designed to investigate if exposure of sperm or ZP to EZM prior to co-incubation would result in an increase in sperm binding to ZP. Two

experiments (IV-A and IV-B) were performed. Two ejaculates from two stallions were used for each experiment on separate days. In Experiment IV-A, after the initial centrifugation, sperm were diluted with TALP or EZM and stained with Hoechst 33342. Sperm diluted in TALP were centrifuged and resuspended in TALP. Sperm diluted in EZM were diluted and resuspended in TALP (SP-EZM) or EZM. Sperm (5 μl) were added to the droplets containing bovine ZP, and the binding assay was performed as described above.

In Experiment IV-B, bovine ZP were incubated in TALP alone (TALP, EZM) or TALP containing 10% EZM (ZP-EZM) for approximately 1 h. Prior to the assay, all ZP were washed twice in TALP and placed in 45 μl of TALP. Sperm were diluted with TALP or EZM, stained, centrifuged and suspended in the same extender. For groups TALP and ZP-EZM, ZP were inseminated with sperm diluted with TALP. Sperm diluted with EZM were used for group EZM. Sperm (5 μl) were added to the droplets containing ZP, and the binding assay was performed as described above.

Experiment V: Effect of milk extenders and glucose on sperm binding to ZP

Experiment V was designed to investigate effects of milk extenders and glucose on sperm binding to ZP. TALP was modified (Table 1) with two concentrations of glucose (TGLU = 89.5 mM and THGLU = 163.5 mM) and with addition of skim-milk [TSM = 2.4 mg/ml of Sanalac® (Hunt-Wesson, Inc., Fullerton, CA)]. The concentration of skim-milk used in TSM corresponded to 10% of the concentration of skim-milk found in EZM. Also, INRA96® (IMV International Corp., Maple Grove, MN) and EZM were used (Table 1).

Semen from one ejaculate from each of four stallions (A - D) was centrifuged, suspended in TALP containing Hoechst 33342, and incubated for 15 min at 37°C. After centrifugation, sperm were suspended with one of the six extenders (TALP, TGLU, THGLU, TSM, INRA and EZM). Bovine ZP (n=10/trt) were placed in 45-µl droplets of TALP, except for the TSM group in which ZP were placed in a 45-µl droplet of TSM. Sperm (5 µl) from a single stallion were added to the ZP, and the binding assay was performed as described.

Experiment VI: Effect of milk proteins on sperm binding to ZP

Experiment VI was designed to investigate effects of sodium caseinate (SC), native phosphocaseinate (NP) and caseinoglycopeptide (CG) on sperm binding to ZP. Native phosphocaseinate was provided by Drs. Phil Kelly and Brendan O'Kennedy (Teagasc, Dairy Products Research Centre, Moorepark, Ireland). Sodium caseinate and caseinoglycopeptide were purchased from Sigma Chemical Co. (St. Louis, MO). One ejaculate from each of six stallions (A - F) was diluted with TALP, centrifuged, stained and suspended in TALP to 2 x 10^6 sperm/ml. Bovine ZP were washed and incubated in TALP for 1 h at 38.5°C. After incubation, ZP (n=10/trt) were placed in droplets of 45 µL of TALP containing: no additions (TALP); 1 or 3 mg/ml of SC (SC1, SC3); 1 or 3 mg/ml of NP (NP1, NP3); and 1 or 3 mg/ml of CG (CG1, CG3). Sperm (5 µL) were added to ZP, and binding assays were performed as described previously.

Statistical analysis

Data were analyzed using the Statistical Analysis System software (SAS Institute, Inc., Cary, NC). The droplet containing the ZP for each treatment was considered the experimental unit. The average number of sperm bound to ZP was calculated for each droplet and compared between groups. Data from each experiment were evaluated for homogeneity of variances and transformation was not necessary. Experiments I through V were analyzed by analysis of variance (ANOVA); and pair-wise comparisons were separated by Tukey's hsd test. Experiment V was analyzed by ANOVA; and pre-planned comparisons were compared by least significant difference (LSD).

Results

In Experiment I, the number of sperm that bound to bovine or equine ZP was similar (Table 2). However, considerably more sperm bound to bovine and equine ZP when sperm were diluted with EZM compared to TALP (Figure 1).

In Experiment II, similar numbers of sperm bound to ZP when LAC, TALP and EHS were used (Table 3). However, sperm binding to ZP was significantly higher when sperm were diluted with EZM.

In Experiment III, centrifugation through Percoll gradient did not affect the number of sperm bound to ZP when sperm were diluted with TALP (Table 4). Higher numbers of sperm were bound to the ZP when sperm in EZM were centrifuged and suspended in EZM compared to TALP. However, this effect was negated when sperm in EZM were centrifuged through Percoll and suspended in TALP (EZM+P).

Figure 1. Light microscopy (A & B) and fluorescence microscopy (A' & B') of bovine ZP co-incubated with equine sperm for 2 h. More sperm bound to ZP when sperm were diluted with EZM (B & B') compared to TALP (A & A'). Sperm were stained with Hoechst 33342 and observed at 400x magnification.

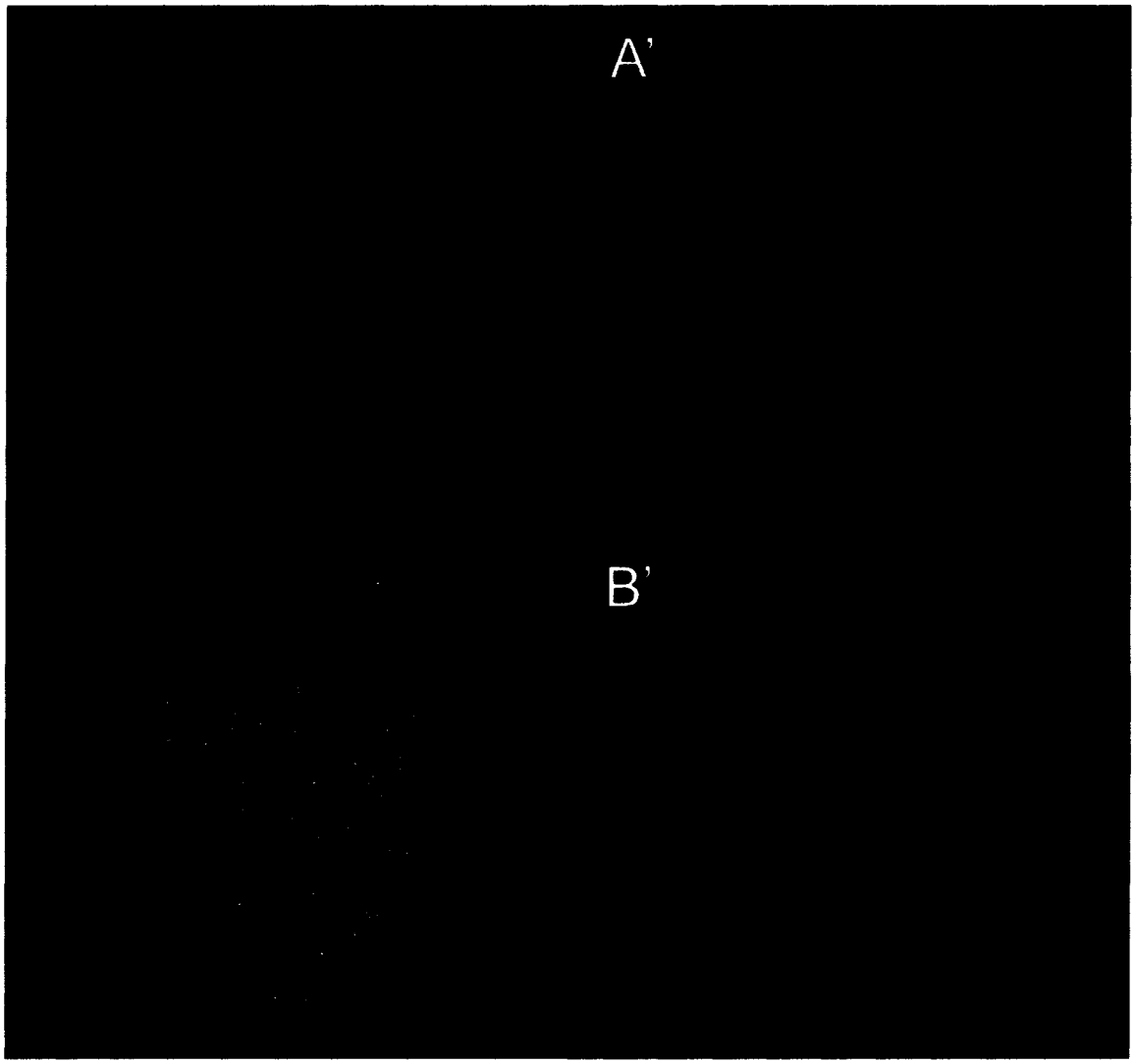

Table 2. Mean ± SEM numbers of sperm bound to bovine or equine ZP in Experiment I.

Treatment	ZP (n)	Mean ± SEM
BOV-TALP	40	29 ± 5^{a}
EQ-TALP	37	38 ± 7^{a}
BOV-EZM	40	149 ± 9^{b}
EQ-EZM	40	151 ± 17^{b}

[a,b] Values within columns with different superscripts differ ($P<0.05$).

Table 3. Mean ± SEM number of sperm bound per bovine ZP for various semen extenders in Experiment II.

Treatment	ZP (n)	Mean ± SEM
LAC	53	29 ± 7^{a}
TALP	56	32 ± 8^{a}
EHS	59	38 ± 6^{a}
EZM	60	96 ± 10^{b}

[a,b] Values within columns with different superscripts differ ($P<0.05$).

Table 4. Effect of centrifugation through Percoll on sperm binding to ZP. Mean ± SEM number of sperm bound per bovine ZP in Experiment III.

Treatment	ZP (n)	Mean ± SEM
TALP	38	20 ± 6^{a}
TALP+P	35	25 ± 4^{a}
EZM	39	140 ± 23^{b}
EZM+P	38	32 ± 17^{a}

[a,b] Values within columns with different superscripts differ ($P<0.05$).

In Experiments IV-A and IV-B, sperm binding to the ZP was significantly higher when sperm were suspended in EZM (Table 5). In Experiment IV-A, centrifugation of sperm diluted with EZM with subsequent suspension in TALP eliminated the positive effect on sperm binding to the ZP. In Experiment IV-B, pre-incubation of ZP in a

solution of 10% EZM in TALP (ZP-EZM) did not increase the number of sperm bound to the ZP. Therefore, numbers of sperm bound to the ZP were only increased when sperm and ZP are co-incubated in the presence of 10% EZM.

Table 5. Effect of the presence of EZM during sperm-ZP interaction. Mean ± SEM numbers of sperm bound per bovine ZP in Experiment IV.

Treatment	ZP (n)	Mean ± SEM
Exp. IV-A		
TALP	38	20 ± 6[a]
EZM	39	140 ± 23[b]
SP-EZM[c]	37	29 ± 9[a]
Exp. IV-B		
TALP	40	29 ± 5[a]
EZM	40	149 ± 9[b]
ZP-EZM[d]	40	28 ± 5[a]

[a,b] Values within experiments with different superscripts differ ($P<0.05$).
[c] Sperm preincubated in EZM and resuspended in TALP.
[d] ZP preincubated in EZM and washed in TALP prior to insemination with sperm diluted in TALP.

In Experiment V, glucose had no significant effect on the number of sperm bound to ZP (Table 6). However, extenders containing milk or milk protein (INRA, EZM, TSM) caused a significant increase in the number of sperm bound to ZP compared to TALP.

In Experiment VI, milk proteins enhanced sperm binding to ZP in a dose-dependent manner (Table 7). The number of sperm bound to CG1 was similar to TALP. SC and NP were more effective in increasing sperm binding than CG. The specificity of the positive effect of milk proteins on sperm binding, in particular SC, has been

determined in a separate experiment by incubating sperm and ZP with similar molar concentrations of three different caseins (unpublished data).

There was a significant ($P<0.05$) ejaculate effect for Experiments I, III, IV-A, V and VI.

Table 6. Effect of milk extenders and glucose on sperm binding to ZP. Mean ± SEM numbers of sperm bound per bovine ZP in Experiment V.

Treatment	ZP (n)	Mean ± SEM
TALP	40	53 ± 11^{a}
TGLU	40	55 ± 9^{a}
THGLU	39	82 ± 13^{a}
INRA	40	151 ± 10^{b}
EZM	40	160 ± 9^{b}
TSM	38	167 ± 14^{b}

^{a,b,c} Values within columns with different superscripts differ ($P<0.05$).

Table 7. Effect of milk proteins on sperm binding to ZP. Mean ± SEM numbers of sperm bound per bovine ZP in Experiment VI.

Treatment	Milk Protein* (mg/ml)	ZP (n)	Mean ± SEM
TALP	0	60	27 ± 4
CG1	1	60	$41 \pm 9^{a,c}$
CG3	3	60	$61 \pm 8^{e,g}$
SC1	1	60	78 ± 9^{b}
SC3	3	60	102 ± 16^{f}
NP1	1	60	83 ± 9^{d}
NP3	3	60	97 ± 21^{h}

* Milk proteins: CG, caseinoglycopeptide; SC, sodium caseinate; NP, native phosphocaseinate.

^{a,b,c,d,e,f,g,h} The superscripted pre-planned comparisons differed as follow: a,b ($P<0.05$); c,d ($P<0.01$); e,f ($P<0.01$); g,h ($P<0.01$).

Discussion

With the exception of some cross-species hybrids, the process of fertilization with normal embryo development is highly species specific. However, early events of gamete recognition and binding appear to be less restrictive. Recently, it was demonstrated that equine sperm were able to bind firmly to ZP of bovine oocytes, undergo the acrosome reaction, penetrate the ZP, and fuse with the ooplasm (Sinowatz et al., 2003). Therefore, in Experiment I, we investigated use of bovine ZP for a ZP-binding assay using stallion sperm. We observed similar numbers of stallion sperm bound to bovine and equine ZP, demonstrating the suitability of bovine ZP for a ZP-binding assay with stallion sperm. The number of sperm bound to ZP was approximately four-fold higher when sperm were diluted with skim milk-based extender (EZM) than TALP, and this effect was similar for bovine and equine ZP. TALP was used in all subsequent experiments as a control extender, because TALP is a chemically-defined medium and optically clear.

In this study, salt-stored bovine and equine ZP were used. Yanagimachi *et al.* (1979) demonstrated that salt-storage of oocytes from several species, including humans, preserved various physical and chemical characteristics of the ZP. In addition, Fazeli *et al.* (1995) demonstrated a relationship between sperm binding to ZP of salt-stored oocytes and stallion fertility. Therefore, storage of oocytes in a salt solution should not have affected binding of sperm to ZP.

Experiment II investigated effects of semen extenders previously used for equine GIFT (Coutinho da Silva et al., 2002 and 2004) on sperm binding to ZP. During GIFT, sperm are deposited directly into the oviduct; therefore, seminal extenders could affect gamete interactions. Numbers of sperm bound to the ZP were three-fold higher when

sperm were diluted with EZM; LAC and EHS did not affect sperm binding to ZP. This is in contrast with results obtained after GIFT using sperm diluted with EZM and cooled for 24 h. Coutinho da Silva *et al.* (2004) obtained similar embryo development rates when cooled sperm diluted in EZM (non-centrifuged) or EHS (centrifuged) were used for GIFT (1/6, 17% vs. 3/10, 30%), suggesting no effects of extenders on the outcome of GIFT. In the same study, overall pregnancy rates obtained with GIFT using cooled semen (4/16, 25%) were lower than when fresh semen was used (9/11, 82%). The reduction in pregnancy rates observed could be attributed solely to differences in the semen used (cooled vs. fresh), and not to differences in extenders (EZM vs. EHS). In the present experiment, only fresh semen was used, and potentially, changes in sperm during the cooling process could have negated the positive effect of EZM on sperm binding to ZP. Further studies are necessary to determine the effects of cooling on sperm binding to ZP in the presence of milk proteins.

Sperm preparation for assisted-reproductive technologies usually involves selection of highly motile sperm and removal of seminal plasma, debris and contamination. In the horse, Percoll density gradients have been used to select populations of highly motile sperm (Del Campo et al., 1990; Carnevale et al., 1999 and 2000; Alvarenga and Leao, 2002; Coutinho da Silva et al., 2002 and 2004; Galli et al., 2002; Lazzari et al., 2002; Nie et al., 2003). Arns and Shepherd (1994) demonstrated that a higher number of sperm bound to equine ZP after centrifugation through a Percoll gradient. The authors concluded that increased sperm binding to ZP was caused by initiation of capacitation during centrifugation through the Percoll gradient. These results are in disagreement with our findings in Experiment III; we observed that centrifugation

through Percoll had no effect on sperm binding to ZP. In addition, when sperm diluted in EZM were centrifuged through Percoll and resuspended in TALP (EZM + P), the positive effect of EZM on sperm binding was negated. The increase in sperm binding observed by Arns and Shepherd (1994) could have been caused by the use of a different medium (Hams F-10) containing 3% (w/v) bovine serum albumin (BSA), which is ten-fold higher than the concentration of BSA used in the present study. It has been postulated that BSA serves as a cholesterol acceptor, stimulating efflux of cholesterol from the sperm plasma membrane, resulting in changes in plasma membrane fluidity and leading to capacitation (Go and Wolf, 1985; Hoshi et al., 1990; Gamzu et al., 1997; Osheroff et al., 1999; Visconti et al., 1999a, 1999b and 1999c). Therefore, the increase in sperm binding observed by Arns and Shepherd (1994) could be attributed to initiation of sperm capacitation by BSA and not by Percoll.

In Experiments I, II and III, sperm binding to ZP increased when sperm and ZP were incubated in TALP containing 10% EZM (i.e., 5 µl of sperm diluted in EZM were added to 45 µl of TALP containing the ZP). When sperm diluted with EZM were centrifuged through Percoll and suspended in TALP (Experiment III), the increase in sperm binding was not observed. Therefore, Experiment IV was designed to determine if 10% EZM in medium during sperm and ZP interaction was necessary to enhance sperm binding to ZP. In Experiment IV-A, consistent with the previous experiments, sperm binding to ZP was enhanced when sperm and ZP were co-incubated in the presence of 10% EZM. However, the positive effect of EZM on sperm binding was negated when sperm pre-exposed to EZM were suspended in TALP, suggesting that components of EZM were directly affecting sperm-ZP interactions or ZP. In Experiment IV-B, no

increase in sperm binding was observed when ZP were pre-exposed to EZM and washed in TALP prior to assay. Therefore, we concluded that EZM needed to be present during sperm and ZP co-incubation to cause an increase in sperm binding, and components of EZM directly affected the interaction of sperm and ZP.

Formulation of EZM was based on Kenney *et al.* (1975) and consisted of dried skim-milk (24 g/L), a high concentration of glucose (272 mM) and antibiotics. Sperm binding to ZP was increased in the presence of 10% EZM. Therefore, in Experiment V, TALP was modified to contain similar concentration of skim-milk and increasing concentrations of glucose. In addition, INRA96®, an extender that contains only one milk protein, was used. Glucose did not increase sperm binding to ZP. In contrast, a three-fold increase in sperm binding to ZP was observed with skim milk-based extenders and INRA. A similar increase in sperm binding to the ZP was observed with EZM and INRA. INRA contains one milk protein, native phosphocaseinate (NP); therefore, effects of NP on sperm binding to ZP were investigated.

In Experiment VI, in addition to NP, other milk proteins such as sodium caseinate (SC) and caseinoglycopeptide (CG) were used to modify TALP medium. Incubation of sperm and ZP with TALP containing two concentrations (1 and 3 mg/ml) of milk proteins resulted in higher numbers of sperm bound to ZP compared to controls, except for CG1. SC and NP were more effective in enhancing sperm binding to ZP. NP has protective effects on stallion sperm motility and fertility after 24 to 48 h of storage; however, the mechanism of action remains unknown (Batellier et al., 1997 and 1998). According to Batellier *et al.* (2000), NP does not bind to the sperm plasma membrane as demonstrated by immunocytochemistry and electron microscopy. In the same study, the authors

demonstrated that NP must be in direct contact with sperm to exert its protective effects. This could explain why the positive effect of milk on sperm binding was negated after centrifugation and resuspension in TALP and why milk was necessary during sperm-ZP interaction.

NP is obtained from bovine milk though a process of microfiltration (Pierre et al., 1992). The structure of NP is a complex casein micelle with calcium phosphate incorporated in the micelle. Sodium caseinate is obtained by precipitation of NP and solubilisation of the casein curd. The micellar calcium phosphate is depleted during casein precipitation. CG is a peptide cleaved from κ-casein and is also in non-micellar form. Batellier *et al.* (2000) demonstrated the importance of the micelle structure on preservation of stallion sperm motility during storage. Semen was diluted with media containing NP or SC and stored for 7 days at 4 or 15°C. At 15°C storage, sperm motility was higher when semen was diluted in media containing NP compared to SC. Sperm motility was similar when sperm were stored at 4 °C in media containing NP or SC. The authors concluded that the micellar structure of casein was necessary for preservation of sperm motility during storage at 15°C, but not at 4 °C. Apparently, micelles were partially destroyed at 4°C (Rollema, 1992), which would explain the lack of protective effect of NP at this temperature. However, in our study, both the micellar and non-micellar forms of casein had a positive effect on sperm binding to ZP. Further studies should be performed to investigate the effects of SC in preserving stallion sperm motility and fertility after long-term storage. Potentially, SC could be used as a substitute for NP in semen extenders used to preserve stallion semen.

The mechanism by which milk proteins enhance sperm binding to ZP needs further investigation. A possible mechanism would involve initiation of sperm capacitation by milk proteins. Pommer *et al.* (2002) demonstrated that sperm incubated for 3 h in skim milk-based extender versus TALP had higher intracellular calcium concentration and a higher percentage of cells undergoing the acrosome reaction after treatment with calcium ionophore, A23187, indicating that sperm were undergoing capacitation during incubation in skim milk-based extender. In contrast, Batellier *et al.* (2000) observed no increase in intracellular calcium concentrations when sperm were incubated in medium containing native phosphocaseinate for 24 h. Because calcium evaluation was performed only after 24 h of incubation, intracellular calcium concentration could have been elevated and returned to basal levels by 24 h, as described by Nagai *et al.* (1994). In our experiment, sperm and ZP were incubated for 2 h in medium containing native phosphocaseinate; and an increase in intracellular calcium in sperm could have occurred. In Experiments III and IV, sperm was exposed to native phosphocaseinate for approximately 30 min, before being washed and placed in TALP. Short exposure time of sperm to native phosphocaseinate could have been insufficient to result in an increase in intracellular calcium in sperm; therefore, no increase in sperm binding to ZP was observed. The effects of milk proteins on equine sperm capacitation and intracellular Ca^{2+} concentration need further investigation.

In conclusion, similar numbers of stallion sperm bound to bovine and equine ZP, demonstrating suitability of bovine ZP for a binding assay with stallion sperm. Further studies on the relationship between stallion sperm binding to bovine ZP and in vivo fertility should be performed. Potentially, sperm-ZP binding assays using bovine ZP

could be applied to assess sperm function in cases of subfertility, evaluate fertility of frozen semen, and determine sperm capacitation. Presence of milk and milk proteins during sperm-ZP interactions resulted in a three- to seven-fold increase in number of sperm bound to ZP. The mechanisms by which milk proteins enhance sperm binding to ZP remain to be investigated. These findings could contribute to further the development of assisted reproduction techniques for the horse, such as GIFT and IVF, as well as to increase our understanding of interactions between sperm and ZP.

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Chapter III

Effects of milk proteins on sperm binding to the zona pellucida and intracellular Ca^{2+} concentration in the horse.

Introduction

The effects of components of semen extenders on the binding of sperm to the zona pellucida (ZP) have been previously investigated (Coutinho da Silva et al., 2005). Co-incubation of stallion sperm and ZP in the presence of skim milk-based extenders resulted in an approximate three-fold increase in the numbers of sperm bound to the ZP compared to TALP medium alone. In the same study, the presence of milk proteins (native phosphocaseinate, NP; and sodium caseinate, SC) in the medium during co-incubation resulted in a similar increase in sperm binding to the ZP compared to skim milk-based extenders. The positive effect of milk proteins on sperm binding to ZP was dose dependent. Mechanisms by which milk proteins enhanced sperm binding to ZP were not investigated.

The ability of sperm to bind to the ZP is acquired early in the process of capacitation, and capacitation probably enhances exposure of sperm receptors to ZP (Florman and First, 1988). Potentially, milk proteins could stimulate sperm capacitation; and therefore, more sperm would bind to ZP. Pommer et al. (2002) demonstrated that incubation of stallion sperm in a skim milk-based extender resulted in a higher proportion

of sperm undergoing the acrosome reaction after ionophore treatment, compared to TALP medium alone. In the same study, sperm incubated in skim milk-based extender showed a higher increase in intracellular calcium (Ca^{2+}) following ionophore treatment compared to sperm incubated in TALP. The authors hypothesized that incubating sperm in the skim milk-based extender stimulated capacitation, and that the mechanism for stimulating sperm capacitation involved increased intracellular Ca^{2+}.

Objectives of this research were to: 1) determine effects of extracellular Ca^{2+} concentration and milk proteins (NP and SC) on intracellular Ca^{2+} concentrations in stallion sperm and 2) determine the effects of different caseins (α-, β-, κ-caseins) on sperm binding to ZP.

Materials and Methods

Measurement of Intracellular Ca^{2+} Concentration in Sperm

Intracellular Ca^{2+} concentrations in sperm were determined by spectrofluorometry using Fura-2/AM (Zhou et al., 1990). For each experiment, semen from one ejaculate from each of four stallions was used. Semen was collected using an artificial vagina and evaluated for volume, sperm concentration and total and progressive motility. Semen was diluted 1:3 with Tyrode's medium (TALP; Parrish et al., 1988) modified as previously described (Coutinho da Silva et al., 2005), and centrifuged at 400 x g for 5 min. Sperm were resuspended in TALP to 50 x 10^6 sperm/ml, and stained with Fura-2/AM (1 μM) for 30 min at 37 °C. After incubation, sperm were washed twice (400 x g for 5 min each), resuspended in TALP to 25 x 10^6 sperm/ml, and incubated at 37 °C for

30 min before Ca^{2+} determination. Sperm suspension (2.7 ml) was placed in a polystyrene cuvette (VersaFluor™; Bio-Rad Laboratories, Hercules, CA) containing a micro stir bar, and Ca^{2+}-dependent fluorescence was measured at room temperature (22 to 25 °C) with a spectrofluorometer (Photon Technology International, Lawrenceville, NJ). Samples were alternately excited at 340 and 380 nm, and fluorescence emission was monitored at 510 nm using computer software (Felix32, Photon Technology International). The 340/380 nm ratio was calculated every 2 sec and used for comparisons between times and groups. Sperm motility was assessed visually before and at the end of each assay.

Effects of extracellular Ca^{2+} concentrations

The effects of extracellular Ca^{2+} concentrations on intracellular Ca^{2+} concentrations were investigated. Sperm stained with Fura-2AM were diluted in TALP containing 2 mM $CaCl_2$, placed in the cuvette, and equilibrated for 150 sec. After the initial equilibration period, samples were treated with TALP (2 mM; control) or 4 mM $CaCl_2$, and fluorescence was recorded for an additional 150 sec (baseline). Samples were then evaluated every 15 min for 60 sec during a 3 h period. Changes in the 340/380 nm emission ratio over the 3 h incubation period were compared between groups.

Ionomycin challenges were performed on separate samples before (0 h) and after (3 h) incubation in TALP containing 2 or 4 mM $CaCl_2$ for 3 h. Samples were treated with TALP (2 mM) or 4 mM $CaCl_2$ as described, and fluorescence was recorded for 150 sec (baseline). Immediately after baseline, one sample (0 h) received 500 nM ionomycin, and fluorescence was recorded for 300 sec. A second sample (3 h) was incubated for 3 h

at room temperature and then treated with ionomycin as described. The percentage increase in the 340/380 nm emission ratio after ionomycin treatment was compared between groups.

Effects of milk proteins

The effects of milk proteins (NP and SC) on intracellular Ca^{2+} concentration were investigated. Sperm stained with Fura-2AM were diluted in TALP, placed in a cuvette, and equilibrated for 150 sec. After the equilibration period, samples were treated with TALP (Control), 1 mg/ml NP (NP), or 1 mg/ml SC (SC); fluorescence was recorded for an additional 150 sec (baseline). Samples were then evaluated every 15 min for 60 sec during a 3 h period. Changes in the 340/380 nm emission ratio over the 3 h incubation period were compared between groups.

Ionomycin challenges were performed on separate samples before (0 h) and after (3 h) incubation in TALP alone or TALP containing NP or SC for 3 h. Samples were treated with TALP, NP or SC as described, and fluorescence was recorded for 150 sec (baseline). Immediately after baseline, one sample (0 h) received 500 nM ionomycin, and fluorescence was recorded for 300 sec. The other sample (3 h) was incubated for 3 h at room temperature and then treated with ionomycin as described. The percentage increase in the 340/380 nm emission ratio after ionomycin treatment was compared between groups.

Sperm-ZP Binding Assay

ZP binding assays were performed to investigate the effects of caseins (α-, β- and κ-casein) on sperm binding to ZP. Semen was obtained from one ejaculate from each of four stallions. For each assay, semen was collected from one stallion and evaluated for sperm concentration and total and progressive motility. Semen was diluted 1:3 with TALP and centrifuged at 400 x g for 5 min. Sperm were resuspended with 1 ml of TALP containing 35 μg/ml of Hoechst 33342 (ICN Biomedical, Inc., Aurora, OH) and incubated for 15 min at 37 °C. Sperm were then centrifuged and resuspended to 2 x 10^6 sperm/ml in TALP, before being co-incubated with ZP.

Immature bovine oocytes were collected from ovaries obtained from a slaughterhouse. Cumulus cells were removed by vortexing oocytes for 1 to 2 min at maximum speed. Denuded oocytes were washed in TALP and stored in hyperosmotic salt solution [1.5 M $MgCl_2$, 40 mM Hepes, 0.1% (w/v) PVP] (Yanagimachi et al., 1979) at 5 °C for up to one month. Prior to use, ZP were washed twice and incubated in TALP for at least 1 h. After incubation, ZP (n=10 per droplet) were placed in droplets of 45 μl of TALP containing: no additions (TALP); 1 mg/ml SC (SC); 1 or 3 mg/ml of α-casein (Alpha1, Alpha3); 1 or 3 mg/ml of β-casein (Beta1, Beta3); and 1 or 3 mg/ml of κ-casein (Kappa1, Kappa3). These caseins were obtained from Sigma Chemical Co., Saint Louis, MO. Five μl of sperm suspension were added to the 45-μl droplets of medium containing the ZP, resulting in a final concentration of 2 x 10^5 sperm/ml.

ZP and sperm were incubated for 2 h at 38.5 °C in an atmosphere of 5% CO_2 in air. After incubation, ZP were washed vigorously in four droplets of TALP using a small-bore, fire-polished, glass pipette to remove loosely bound sperm. ZP were placed

on a glass slide, and a cover slip was placed on top and supported by a mix of paraffin and petroleum jelly. ZP were observed under an epifluorescence microscope (Eclipse E800; Nikon Instruments, Inc., Melville, NY) equipped with a 360/40 nm bandpass excitation filter and a 460/50 nm bandpass emission filter. ZP were observed under 400x magnification, and the numbers of sperm bound to ZP were recorded.

Statistical Analyses

Data were analyzed using the Statistical Analysis System software (SAS Institute, Inc., Cary, NC). Changes in intracellular Ca^{2+} concentrations over a 3 h-period were analyzed by linear regression. Changes in intracellular Ca^{2+} concentrations after ionomycin treatment were analyzed as percentage increase from baseline. Data for intracellular Ca^{2+} and motility were analyzed by ANOVA with treatments and stallions in the model, and means were separated by Tukey's hsd test.

For the ZP binding assay, the droplet containing the ZP for each treatment was considered the experimental unit. The average number of sperm bound to ZP was calculated for each droplet and compared between groups. Data were analyzed by ANOVA. Pre-planned comparisons were evaluated by least significant difference (LSD).

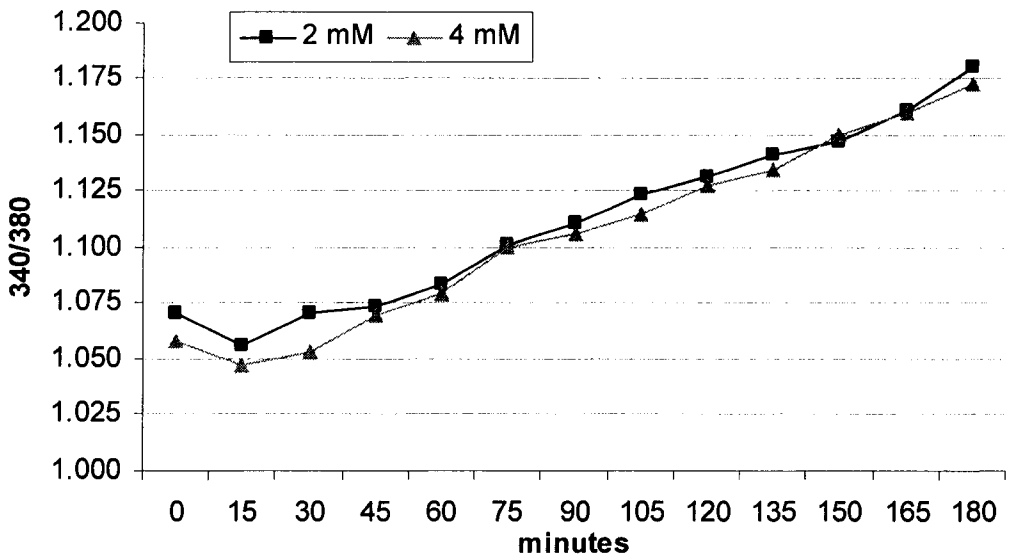
Results

Intracellular Ca^{2+} Concentration in Sperm

A slow increase in intracellular calcium occurred during a 3 h-period ($P<0.05$); however, this increase in calcium was similar between medium with 2 or 4 mM Ca^{2+}

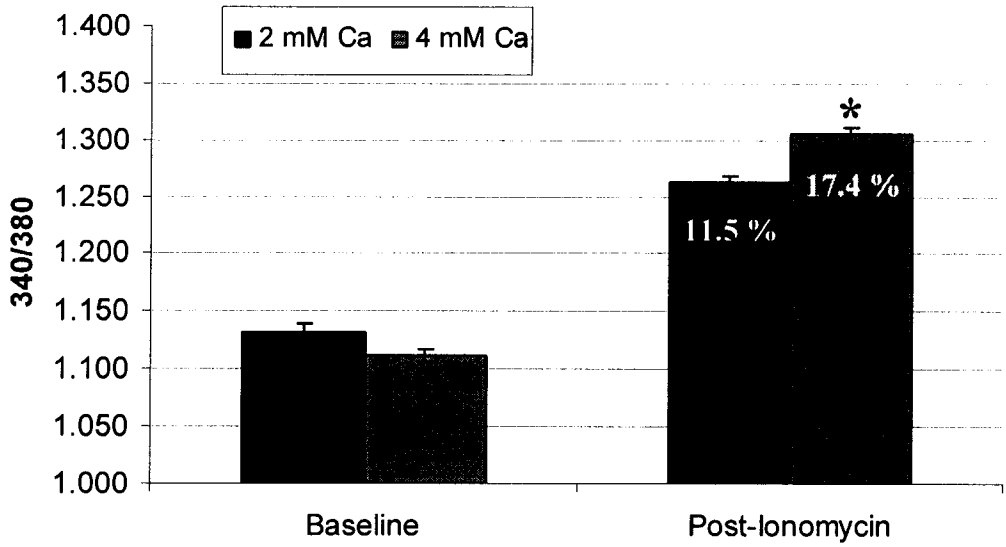
(Figure 1). Extracellular Ca^{2+} concentrations did not affect baseline intracellular Ca^{2+} in sperm at times 0 or 3 h ($P>0.05$; Figures 2 and 3). However, a greater ($P<0.05$) increase in intracellular Ca^{2+} concentration was observed after ionomycin treatment in TALP containing 4 mM versus 2 mM Ca^{2+} at both 0 h (Figure 2) and 3 h (Figure 3).

Figure 1. Effect of extracellular Ca^{2+} concentration on intracellular Ca^{2+} in sperm during a 3 h incubation in TALP containing 2 or 4 mM Ca^{2+}.



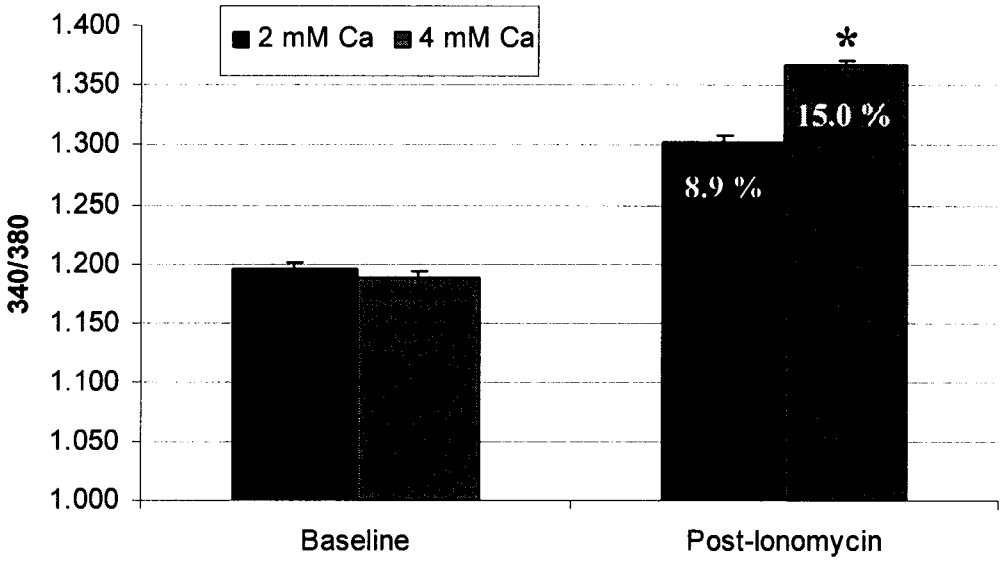
Mean ± SEM progressive motility of samples before the assay and after incubation for 3.7 ± 0.1 h in TALP medium containing 2 or 4 mM Ca^{2+} were 73 ± 4, 50 ± 4 and 46 ± 4 %, respectively. There was a significant ($P<0.05$) decrease in progressive motility during incubation; however, progressive motility was similar after incubation in TALP containing 2 or 4 mM Ca^{2+}.

Figure 2. Effect of extracellular Ca^{2+} concentration on intracellular Ca^{2+} in sperm after ionomycin treatment, at 0 h of incubation (Mean ± SEM).



* Percentage changes in 340/380 nm ratio after ionomycin treatment differed ($P<0.05$) between sperm diluted in TALP with 2 versus 4 mM Ca^{2+}.

Figure 3. Effect of extracellular Ca^{2+} concentration on intracellular Ca^{2+} in sperm after ionomycin treatment, at 3 h of incubation (Mean ± SEM).



* Percentage changes in 340/380 nm ratio after ionomycin treatment differed ($P<0.05$) between sperm diluted in TALP with 2 versus 4 mM Ca^{2+}.

Intracellular Ca^{2+} slowly increased during a 3 h-period ($P<0.05$); however, calcium increased at similar rates for all groups (Figure 4). Addition of milk proteins to TALP did not affect intracellular Ca^{2+} in sperm ($P>0.05$; Figures 5 and 6). The increase in intracellular calcium observed after ionomycin treatment was similar for TALP, NP and SC groups (0 h = 10.5%, 7.8% and 9.2%; 3 h = 8.9%, 8.3% and 8.6%, respectively). Progressive motility for samples before evaluation and after incubation for 3.8 ± 0.1 h in TALP medium alone or containing NP and SC were 73 ± 1, 50 ± 8, 49 ± 8 and 54 ± 6 %, respectively. There was a significant ($P<0.05$) decrease in progressive motility during incubation; however, progressive motility was similar after incubation in TALP alone or with NP or SC.

Figure 4. Effect of milk proteins on intracellular Ca^{2+} in sperm during a 3 h incubation in TALP alone or TALP containing native phosphocaseinate (NP) or sodium caseinate (SC).

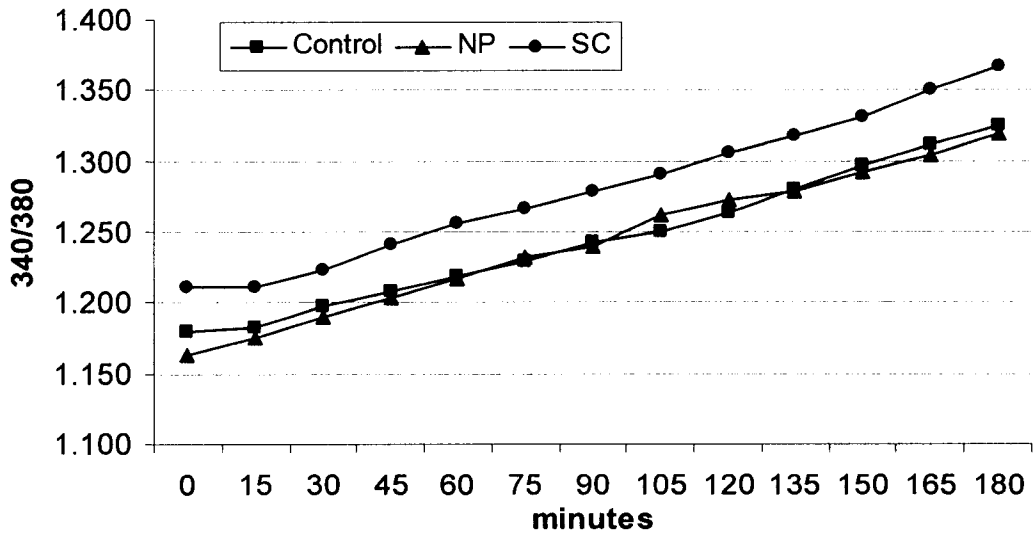
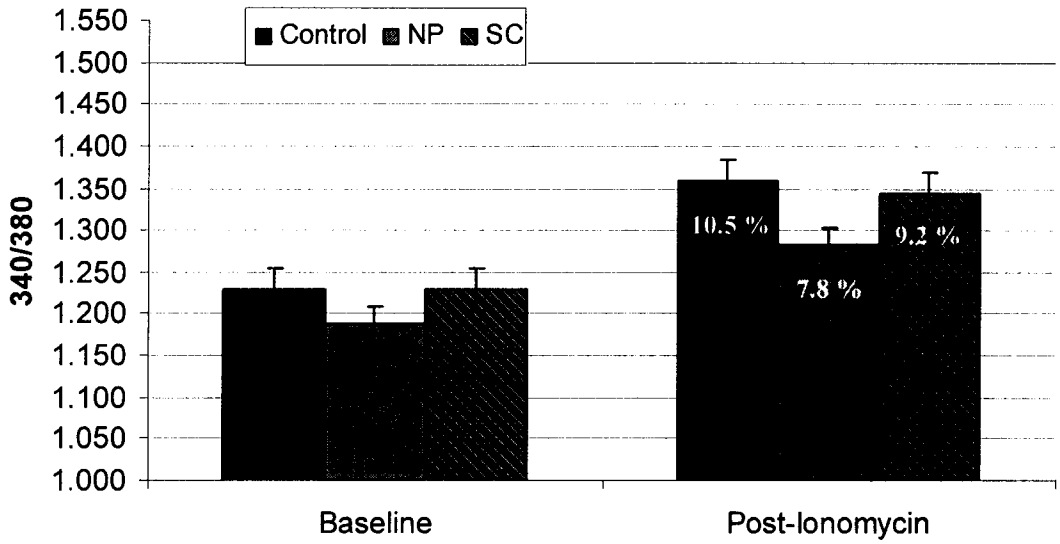
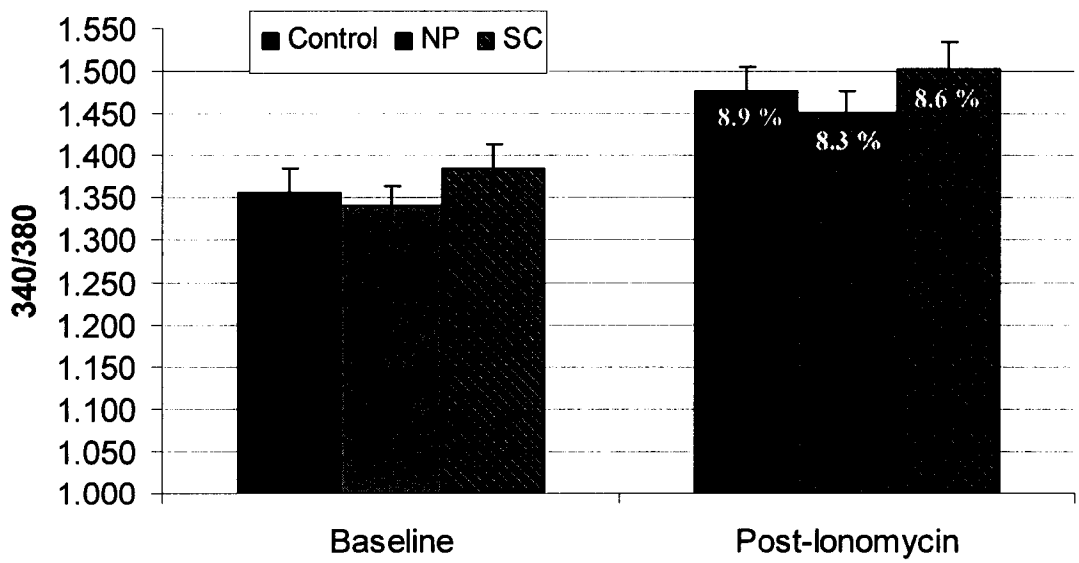


Figure 5. Effect of milk proteins on intracellular Ca^{2+} in sperm after ionomycin treatment, at 0 h of incubation (Mean ± SEM).



Percentage changes in 340/380 nm ratio after ionomycin treatment were not different between groups (P>0.05).

Figure 6. Effect of milk proteins on intracellular Ca^{2+} in sperm after ionomycin treatment, at 3 h of incubation (Mean ± SEM).



Percentage changes in 340/380 nm ratio after ionomycin treatment were not different between groups (P>0.05).

Sperm-ZP Binding Assay

Sperm binding to ZP was similar for gametes incubated in TALP alone or TALP supplemented with α-, β-, and κ-caseins. However, more sperm bound to ZP when gametes were co-incubated in TALP containing β- and κ-caseins compared to α-casein. There was a significant stallion effect ($P<0.05$).

Table 1. Effect of caseins on sperm binding to ZP. Mean ± SEM numbers of sperm bound to ZP.

Treatment	Casein (mg/ml)	ZP (n)	Mean ± SEM
TALP	0	60	68 ± 4
Alpha1	1	60	53 ± 6[a]
Alpha3	3	60	53 ± 7[c,e]
Beta1	1	60	69 ± 9[b]
Beta3	3	60	81 ± 8[d]
Kappa1	1	60	60 ± 5
Kappa3	3	60	71 ± 11[f]

[a,b,c,d,e,f]The superscripted pre-planned comparisons differed as follow: a,b ($P<0.1$); c,d ($P<0.05$); e,f ($P<0.1$).

Discussion

The main hypothesis of the present experiment was that extracellular Ca^{2+} would not affect homeostasis of Ca^{2+} in sperm, resulting in no changes in baseline Ca^{2+} concentrations; however, addition of NP or SC to the medium would cause an increase in Ca^{2+} uptake by sperm. In previous experiments, we demonstrated that milk proteins (NP and SC) had a positive effect on stallion sperm binding to the ZP (Coutinho da Silva et al., 2005). Casein has also been shown to have a high Ca^{2+}-binding capacity (Mykkanen and Wasserman, 1980; Sato et al., 1986) and promote Ca^{2+} uptake in somatic cells (Hira

et al., 2003; Ferraretto et al., 2003). Nagai et al. (1994) and Mori et al. (1996) demonstrated that casein phosphopeptides promoted Ca^{2+} uptake by porcine sperm and induced sperm capacitation and the acrosome reaction, leading to sperm penetration of oocytes. Potentially, presence of NP and SC in the medium could increase Ca^{2+} uptake by equine sperm, initiating sperm capacitation and leading to increased sperm binding to the ZP.

NP is composed of milk caseins (α-, β-, κ-), and the method used to purify the NP maintains its three-dimensional micelle structure (Leboeuf et al., 2003). In the micelle, α- and β-casein form a core that is surrounded by κ-casein on the surface; calcium phosphate is associated with the micelle (Schmidt, 1982; Murray and Maga, 1999). In contrast, SC is obtained by isoelectric precipitation of NP followed by solubilizing the casein curd with sodium hydroxide, resulting in depletion of micellar calcium phosphate (Batellier et al., 2000). Despite different purification methods, both NP and SC are thought to be chemically identical (Batellier et al., 2000). However, differences in the calcium content between the two proteins resulted in changes in the total Ca^{2+} concentration of the medium. We observed an increase of approximately 0.4 mM in the total Ca^{2+} concentration when NP was added to TALP medium at 1 mg/ml (data not shown). This increase was expected due to the presence of calcium phosphate associated with the micelle. Micellar calcium phosphate did not dissociate in TALP, as no changes in free Ca^{2+} were observed. Because SC does not have Ca^{2+} associated with it, addition of SC to TALP did not result in changes in total and free Ca^{2+} concentrations.

The effects of extracellular Ca^{2+} concentration and milk proteins (NP and SC) on intracellular Ca^{2+} concentrations of equine sperm were also investigated. Results from

our experiments demonstrated that extracellular Ca^{2+} concentration, at the levels tested, did not affect the baseline intracellular Ca^{2+}. Despite the two-fold increase in extracellular Ca^{2+} concentration in the medium, equine sperm were able to maintain a Ca^{2+} homeostasis, with no significant increase in intracellular Ca^{2+}. However, treating cells with ionomycin, a calcium ionophore, resulted in a greater influx of Ca^{2+} when sperm were incubated in TALP with 4 mM Ca^{2+} versus 2 mM Ca^{2+}. This was expected since ionomycin causes calcium channels in the membrane to open, thereby allowing Ca^{2+} to enter the cells at a rate dependent on the Ca^{2+} gradient between the two compartments. Addition of NP and SC to TALP did not affect Ca^{2+} uptake by stallion sperm, either during long-term incubation or after ionomycin treatment. These results are in agreement with those by Battelier et al. (2000), who found no significant increase in intracellular Ca^{2+} concentration after incubation of stallion sperm for 24 h in medium with or without NP. In contrast, Pommer et al. (2002) observed a higher increase in intracellular Ca^{2+} concentration after ionophore treatment of stallion sperm incubated in skim milk-based extender compared to TALP. In the same study, sperm incubated in skim milk-based extender showed a higher percentage of acrosome reactions after ionophore treatment compared to TALP, suggesting that skim milk-based extender facilitated sperm capacitation. However, the concentration of Ca^{2+} in the skim milk-based extender used by Pommer et al. (2002) was nearly six-fold higher than TALP; and this could have been the cause for the higher increase in intracellular Ca^{2+} concentration observed after ionomycin treatment. Therefore, based on results from our experiments, we conclude that the mechanism by which NP and SC enhanced sperm binding to the ZP does not involve increased intracellular Ca^{2+}.

A slow increase in intracellular Ca^{2+} was observed in all groups, including the TALP alone. Potentially, sperm capacitation was initiated during the incubation in TALP medium, resulting in elevation of intracellular Ca^{2+}. Long-term incubation of sperm in TALP medium has been used to capacitate sperm from several species, including horses (Gadella and Harrison, 1998; Flesch et al., 2001; Rathi et al., 2001; Pommer et al., 2003). Components of TALP medium, particularly BSA and bicarbonate, cause changes in membrane fluidity (Harrison et al., 1996; Flesch et al., 2001), protein tyrosine phosphorylation (Visconti et al., 1995 and 1997) and cAMP metabolism (Visconti et al., 1995a, 1999; Osheroff et al., 1999), leading to sperm capacitation. The bicarbonate-mediated increase in membrane fluidity allows a subsequent increase in the permeability of that membrane to Ca^{2+} (Rathi et al., 2001), and could be responsible for the slow elevation in Ca^{2+} observed in the present study.

Because NP and SC contain α-, β- and κ-caseins, we investigated the effects of these individual caseins on sperm binding to ZP. Although no significant increase in sperm binding to ZP was observed with any individual casein compared to TALP, higher numbers of sperm were bound to ZP when β- and κ-caseins were used. We suggest that increasing the concentration of these proteins in the medium would result in a significant increase in sperm binding to ZP. The lack of effect of α-casein on sperm binding demonstrated that the positive effect of NP and SC on sperm binding is specific and could involve only β- and κ-caseins.

Findings from our experiments indicate that milk proteins, in particular caseins, do not stimulate sperm capacitation by increasing Ca^{2+} uptake. Little is know about the conditions needed to capacitate stallion sperm in vitro, even though the area has been

investigated for many years (Graham, 1996). The lack of standard protocols for capacitating stallion sperm in vitro appears to be the major cause for the failure of IVF in the horse (Hinrichs et al., 1998). Therefore, additional studies are necessary to determine the mechanisms by which milk proteins enhance sperm binding to ZP. Potentially, milk proteins could be added to the fertilization medium to increase sperm binding to the ZP and, perhaps, fertilization of equine oocytes.

In conclusion, increased extracellular Ca^{2+} concentration in the medium did not affect baseline intracellular Ca^{2+} concentration in the stallion sperm. Milk proteins (NP and SC) did not affect the uptake of Ca^{2+} by stallion sperm. The beneficial effect of NP and SC on sperm binding to ZP does not appear to involve increasing intracellular Ca^{2+}. Further studies are necessary to determine the mechanism(s) by which milk proteins enhance sperm binding. In addition, studies on the effects of milk proteins on in vitro penetration and fertilization of homologous and heterologous oocytes may be beneficial for the development of a successful IVF protocol for the horse.

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Chapter IV

Summary

Despite over a decade since the birth of the two foals produced by IVF (Palmer et al., 1991; Bezard, 1992), very little progress has been achieved in the development of a successful IVF system for the horse. Compared to other species, reasons for the slow development of equine IVF include the scarcity of offal ovaries to provide adequate numbers of oocytes for studies and difficulties with in vitro systems for oocyte maturation, sperm capacitation and embryo culture. Therefore, development of a model using bovine oocytes to study gamete interaction in vitro would be beneficial. We demonstrated that similar numbers of stallion sperm were bound to bovine and equine ZP. In addition, previous experiments have demonstrated that stallion sperm bound to bovine ZP were able to undergo the acrosome reaction, penetrate the ZP and fuse with the oolemma (Sinowatz et al., 2003). Therefore, there is good evidence that bovine oocytes are suitable as a model for gamete binding assays using stallion sperm. The ability to use bovine oocytes for ZP binding assays with stallion sperm makes feasible the use of this technique to evaluate sperm function, capacitation status and field fertility and to develop IVF in the horse.

Our initial hypothesis was that components of skim milk-based extender negatively affected gamete interaction during GIFT, resulting in the low (25%)

pregnancy rates observed (Coutinho da Silva et al., 2004). However, we demonstrated that presence of milk proteins during gamete co-incubation enhanced sperm binding to ZP. Results from sperm-ZP binding assays have been correlated with in vivo fertility after insemination of mares within the uterus (Fazeli et al., 1993 and 1995; Pantke et al., 1995; Meyers et al., 1996). During GIFT, sperm are deposited within the oviduct, and therefore results from ZP binding assay may not correlate with fertility. In addition, oocytes used for GIFT are surrounded by cumulus cells, and potentially, the positive effect of milk proteins on sperm binding could be removed during sperm passage through the cumulus matrix, as seen after sperm centrifugation.

Milk-based extenders are generally considered to be the best diluents to ensure survival of stallion sperm during storage before artificial insemination (Kenney et al., 1975; Palmer, 1984; Varner et al., 1988). However, milk is a complex and variable medium. In our studies, we were able to identify components of milk that were responsible for an increase in sperm binding to ZP. Based on the observations that the increase in sperm binding to ZP was similar for EZM and INRA96®, and that INRA96® contains only one milk protein, we investigated the effects of NP on sperm binding. As expected, co-incubation of sperm and ZP with NP resulted in increased sperm binding to ZP. Interestingly, SC, the non-micellar form of casein caused a similar response. Potentially, in addition to its effects on sperm binding, SC could also have protective effects on stallion sperm, similarly to NP. Further studies on the effects of SC on sperm motility and viability could lead to the development of new extenders for stallion semen transport and storage.

Native phosphocaseinate represents approximately 80% of the total protein of cow's milk and is composed of α-, β- and κ-caseins (Murray and Maga, 1999). Thus, the effect of individual caseins (α, β or κ) on sperm binding was also investigated. A dose-response increase in sperm binding to ZP was observed only for β- and κ-caseins, but not α-casein, demonstrating that the effect was specific.

The mechanisms by which caseins enhance sperm binding to ZP are unknown. We hypothesized that milk proteins could stimulate sperm capacitation by increasing intracellular calcium; and therefore, more sperm would bind to the ZP. However, we did not observe an increase in intracellular calcium in sperm incubated in media containing milk proteins, indicating that the mechanism for enhanced sperm binding does not involve increased intracellular calcium. Perhaps, milk proteins stimulate capacitation by a different pathway, or directly affect sugar residues in the ZP. Further research could investigate the effects of milk proteins on different aspects of sperm capacitation, such as cAMP metabolism, protein tyrosine phosphorylation, changes in plasma membrane architecture and induction of the acrosome reaction.

Findings from these studies can be used to increase our understanding about sperm and ZP interactions in the horse. In addition, the suitability of bovine oocytes for studies on gamete interaction may be beneficial to further the development of IVF in the horse.

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