FACULTY OF VETERINARY MEDICINE

Department of Reproduction, Obstetrics and Herd Health

NEW INSIGHTS AND ADVANCES IN BOVINE FERTILIZATION *IN VITRO*

Mirjan Thys

Proefschrift ter verkrijging van de graad van Doctor in de Diergeneeskundige Wetenschappen (PhD) aan de Faculteit Diergeneeskunde, Universiteit Gent.

2009

Promotor: Prof. Dr. A. Van Soom
Co-promotor: Prof. Dr. H. Nauwynck

ISBN: 9789058641908

Cover: designed by An Van Loo.

Picture cover: kindly granted by Ferring NV (Aalst, Belgium).

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADAM	family of proteins containing a disintegrin and a metalloprotease domain
AI	artificial insemination
ALH	amplitude of lateral head deviation
APMP	p-aminophenyl derivative of D-mannose
AR	acrosome reacted
BCF	beat cross frequency
BSA	bovine serum albumin
BSP	bovine seminal plasma
CAP	capacitated
CASA	computer-assisted sperm analysis
CD	cumulus-denuded
CE	cumulus-enclosed
COC	cumulus-oocyte complex
CRISP-1	cysteine-rich secretory protein
DABCO	1,4-diazabicyclo (2.2.2) octane
DIC	differential interference contrast
ECM	extracellular matrix
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fn	fibronectin
GalNAc	N-acetylgalactosamine
GlyCAM-1	glycosylated cell adhesion molecule 1
GPMS	glycidoxypropyltrimethoxysilane
GPI	glycosyl phosphatidylinositol
GS	glycerolpropylsilane
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HYA	hyaluronic acid
hpi	hours post insemination
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization

LIN	linearity
LPC	lysophosphatidyl choline
MUC1	mucin glycoprotein 1
NT	non-treated
PBS	phosphate-buffered saline
PI	propidium iodide
PSA-FITC	fluorescein isothiocyanate-conjugated pisum sativum agglutinin
PVP	polyvinyl pyrrolidone
RGD	arginine (R) - glycine (G) - aspartic acid (D)
ROS	reactive oxygen species
RT	room temperature
SD	standard deviation
SEM	standard error of the mean
SPP1	secreted phosphoprotein 1 (also known as osteopontin)
STR	straightness
TALP	Tyrode's albumin lactate pyruvate
UTJ	uterotubal junction
VAP	velocity average pathway
VCL	velocity curvilinear
VSL	velocity straight line
Vn	vitronectin
ZP	zona pellucida

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE ROLE OF GLYCOMOLECULES (CARBOHYDRATES AND GLYCOPROTEINS) IN BOVINE REPRODUCTION

Involvement of various carbohydrates and glycoproteins in adhesion and binding events during several reproductive processes has been described in ruminants, ranging from roles in sperm-oviduct adhesion (Revah et al., 2000; Suarez, 2001; Talevi and Gualtieri, 2001; Sostaric et al., 2005), sperm-oocyte interactions (Gougoulidis et al., 1999; Amari et al., 2001; Tanghe et al., 2004a, 2004b) to embryo implantation (Spencer et al., 2004).

1.1.1 GLYCOMOLECULES AND SPERM-OVIDUCT ADHESION

In order to successfully fertilize an oocyte, sperm cells must overcome several barriers in the female genital tract. Besides escaping mechanical entrapment in the cervical folds, spermatozoa have to reversibly bind the oviductal epithelium (resulting in the formation of a functional sperm reservoir) ensuring that fit sperm cells reach the oocyte at the right time.
Sperm-oviduct binding has been demonstrated to involve carbohydrate recognition (Suarez, 2001). In cattle, the reservoir is formed by the binding of sperm to fucose-containing glycoconjugates (particularly within trisaccharide Lewis-a) on the surface of oviductal epithelial cells (Gwathmey et al., 2003; Suarez, 2008; Fig.1). Explants of oviductal mucosa were evaluated for carbohydrate specificity of sperm binding. Binding of sperm to these explants of oviductal epithelium *in vitro* was established to be specifically blocked by fucoidan and its component fucose (Lefebvre et al., 1997). By means of lectin histochemistry, Danguy et al. (1998) confirmed that the mucosal surface of the bovine oviduct was covered with fucose-containing molecules. Furthermore, pretreatment of oviductal epithelium with fucosidase (but not galactosidase) appeared to reduce sperm binding (Lefebvre et al., 1997) and fluorescein-labeled fucosylated bovine serum albumin specifically labeled the rostral head regions of motile bovine spermatozoa (Revah et al., 2000). A member of the bovine seminal plasma (BSP) family of proteins, PDC-109, has been identified as a protein responsible for binding bull sperm to oviductal epithelium (Gwathmey et al., 2003; Ignotz et al., 2001). This bovine protein was initially named PDC-109 according to its N- and C-terminal amino acids followed by the total number of amino acid residues (Manjunath et al., 2009). However, this BSP protein actually appeared to be composed of two proteins differing only in their degree of glycosylation, still displaying an identical amino acid composition Seidah et al., 1987). Hence, the term PDC-109 was replaced by its components BSP-A1 and BSP-A2 (with ‘A’ referring to their acidic nature). Two other BSP proteins (BSP-30-kDa and BSP-A3) have also been shown to significantly enhance binding of epididymal bull sperm to epithelium (Gwathmey et al., 2006). Both proteins are present in seminal plasma at merely

one-tenth the level of PDC-109 (Nauc and Manjunath, 2000). However, they could assist in increasing the adaptability of spermatozoa to variations in the female tract (Suarez, 2007). Such redundancy of oviduct binding proteins suggests that formation of the sperm reservoir is crucial for successful reproduction (Suarez, 2008).

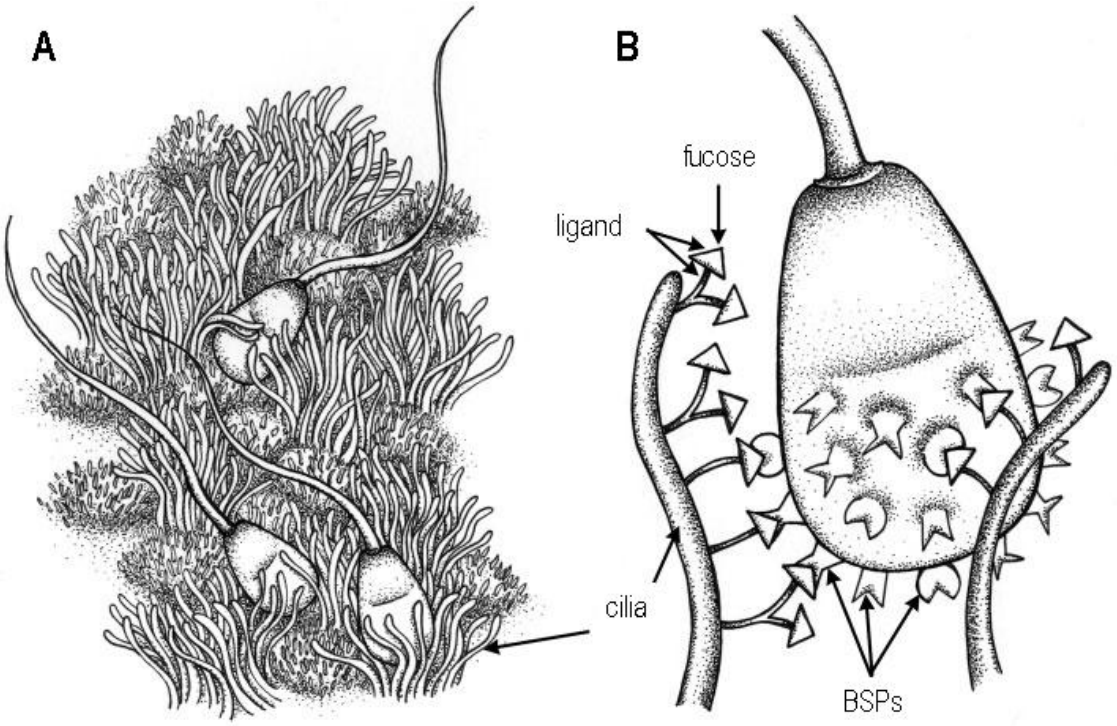


Figure 1: Formation of the sperm reservoir through binding of bull spermatozoa to the oviducal epithelium. (A): sperm cells primarily bind to cilia via the acrosomal region of the head; (B): binding of bovine seminal plasma (BSP) proteins (PDC-109, BSP-A3 and BSP-30-kDa) on the plasma membrane overlying the sperm acrosome to fucose-containing ligands on the surface of cilia (Illustration by C. Rose Gottlieb; from Suarez, 2007).

The underlying mechanism of sperm release from the oviductal epithelium at the time of ovulation is not yet fully understood. Hypothetically, either loss of binding sites on the oviductal epithelium or modifications of the sperm cell surface could be responsible for release of sperm from the reservoir (Suarez, 2008). Nevertheless, oviductal epithelium does not seem to induce sperm release through reduction of expression of the carbohydrate ligand. The epithelium may – on the other hand – secrete initiators of capacitation and/or hyperactivation instigating sperm release (Suarez, 2001). However, changes in the hormonal state of oviductal epithelium (related to imminent ovulation) appeared not to affect the

expression of sperm binding sites in a number of species (Suarez, 2008). There is strong evidence that modifications of the sperm head surface (occurring during capacitation and hyperactivation) include loss or inactivation of the corresponding carbohydrate-binding molecule on the sperm head (Suarez, 2001). The observed reduction of binding affinity of capacitated spermatozoa for oviductal epithelium can be attributed to a loss of carbohydrate-binding affinity. After capacitation, bovine spermatozoa exhibit reduced binding to oviductal epithelium as well as to the carbohydrate ligand involved in sperm binding (Revah et al., 2000; Ignotz et al., 2001). Since binding can be restored in capacitated sperm by supplementing purified PDC-109, the loss in binding affinity is likely due to shedding of the adsorbed seminal plasma protein from the sperm head during capacitation (Gwathmey et al., 2003). Accordingly, incubation of sperm with heparin appeared to remove PDC-109 from the sperm cell surface (Gwathmey et al., 2003), whereas addition of heparin to bovine sperm (bound to cultured oviductal epithelium) enhanced their release (Bosch et al., 2001). Talevi and Gualtieri (2001) demonstrated that heparin, fucoidan and other sulfated glycoconjugates – but not their unsulfated counterparts – significantly inhibited sperm binding to oviductal cells *in vitro* and that they are able to instigate the mechanism of sperm release, probably through early capacitation-associated effects. They assume that sulfated glycosaminoglycans secreted by the oviduct at estrus mediate the release of fertilization-competent sperm from the oviductal reservoir, allowing their ascension towards the ampulla (Talevi and Gualtieri, 2001). Recently, Gualtieri et al. (2009) discovered that redox modulation of sperm-surface protein sulphhydryl is involved in the release of spermatozoa adhering to the oviduct *in vitro*. They suspect that the reversible action of disulphide-reductants might be responsible for intermittent phases of adhesions and releases, and that the irreversible action of heparin may represent a terminal releasing signal. To effectively free sperm from the oviductal reservoir, the combination of capacitation-induced changes in the sperm head plasma membrane (responsible for reducing binding affinity) and hyperactivation (providing the force essential to overcome the attraction between sperm cell and epithelium) is likely required (Suarez, 2008).

Carbohydrate recognition has been established in binding of sperm to oviductal epithelium in different species (hamster, horse, cow and boar; Suarez and Pacey, 2006). In each of the species studied so far, a different monosaccharide seemed to be most effective at inhibiting binding *in vitro*. Such species differences can be expected bearing in mind that one amino acid residue in a lectin can determine its carbohydrate ligand-binding specificity, and that closely related animal lectins display different carbohydrate specificities. The role of

glycomolecules in mammalian reproduction should – hence – be separately investigated in each species.

1.1.2 GLYCOMOLECULES AND SPERM-EGG INTERACTION

Fertilization is the unification of a single sperm cell and an oocyte, and involves a complex – highly coordinated – sequence of molecular interactions between male and female gametes in order to be successful (Benoff, 1997). Although a substantial amount of knowledge has been gained especially in human and mouse (Almeida et al. 1995, Bronson and Fusi 1996, Fusi et al. 1996a, 1996b, Nixon et al. 2007, Vjugina and Evans 2008), a clear identification of the underlying mechanisms and molecules involved in bovine fertilization is still required.

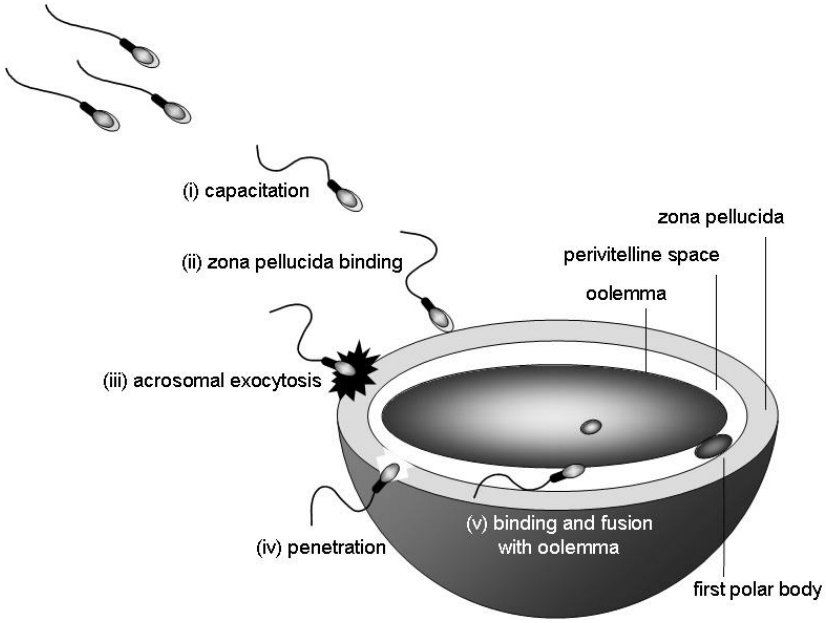


Figure 2: Mammalian fertilization. Within the female reproductive tract (i) spermatozoa undergo a sequence of surface and intracellular modifications, collectively referred to as capacitation, which allows them (ii) to bind to the zona pellucida (ZP) and (iii) undergo the acrosome reaction. (iv) The release of hydrolytic enzymes from the acrosome facilitates sperm passage through the ZP and (v) fusion with the oolemma (from Nixon et al., 2007).

After capacitation (and release from the oviductal reservoir), mammalian spermatozoa 1) respond to chemoattractants; 2) penetrate the cumulus complex; 3) bind to the zona pellucida (ZP); 4) undergo an acrosome reaction; 5) enter the perivitelline space; 6) bind to the oolemma; 7) fuse with oolemma, 8) enter the ooplasm and undergo nuclear decondensation and activate zygote development (Fig.2; Benoff, 1997).

Glycomolecules and sperm-cumulus interaction

One of the final hurdles for the sperm cell to conquer is the cumulus oophorus surrounding the mammalian oocyte. This egg coat is composed of about 3000 to 5000 cumulus cells and an extracellular matrix (ECM) secreted by those cells (Tanghe et al., 2002b). The extracellular cumulus matrix comprises a variety of molecules, such as hyaluronic acid, dermatan sulphate and adhesive glycoproteins among which laminin, fibronectin (Fn) and type IV collagen (Camaioni et al., 1996). In 2007, Diaz et al. found evidence that Fn is able to stimulate the acrosome reaction and increase the intracellular Ca^{2+} concentration in human sperm. They postulated that Fn at the extracellular cumulus matrix activates intracellular signal transduction in human sperm upon binding to the corresponding integrin $\alpha_5\beta_1$ receptor on the sperm cell surface. These intracellular signals are assumed to culminate in proteasome activation (through phosphorylation of particular proteasome subunits) and subsequent acrosomal exocytosis. The proteasome is a multi-enzymatic threonine protease complex (present in sperm of several species) which is believed to play a role in different steps of the fertilization process in marine invertebrates (Mykles, 1998; Diaz et al., 2007). This hypothesize is additionally supported by the discovery that epoxomicin (a potent and specific inhibitor of the proteasome) considerably inhibited the Fn-induced acrosome reaction (Voorhees and Orlowski, 2006). In boar sperm, both 500 nM Fn and 500 nM laminin appeared to stimulate acrosomal exocytosis, however, the latter being far more effective and inducing saturating levels of acrosome reaction (Mattioli et al., 1998). Laminin (also an extracellular cumulus matrix glycoprotein) has been shown to induce Ca^{2+} influx in boar sperm (Barboni et al., 2001). Based on their results these researchers put forward the hypothesis that the hyaluronic acid matrix of the expanded cumulus oophorus could serve as a first sperm-egg recognition mechanism preceding the one involving sperm-ZP interaction (Barboni et al., 2001). Previously, it has already been recognized that particular cumulus components and/or secretions (such as progesterone and hyaluronic acid) enhance capacitation or acrosome reaction in human sperm (Sabeur et al., 1998). Cumulus-induced

acrosome reaction might be used to eliminate supernumerary 'overcapacitated' sperm that – after reacting with the cumulus – stick on it, and hence cannot reach the ZP (Mattioli et al., 1998; Diaz et al. 2007). By inducing premature acrosome reaction it may serve as a mechanism preventing polyspermy. Spermatozoa still in the process of capacitation will be able to traverse the cumulus matrix and appropriately interact with the ZP (Mattioli et al., 1998). Alternatively, sperm contact with the cumulus matrix might initiate the cascade of events leading to acrosome reaction resulting in actual acrosomal exocytosis after interaction with the ZP, which provides the reinforcing stimulus (Mattioli et al., 1998). Recently, a novel human sperm glycosylphosphatidylinositol-anchored membrane protein (NYD-SP8) has been identified which appears to be released during sperm-cumulus interaction. Binding to the cumulus seemed to result in a calcium dependent release of progesterone, eventually ending in acrosome reaction (Yin et al., 2009). The potential ability and biological relevance of extracellular cumulus matrix glycoproteins to induce the acrosome reaction in bovine spermatozoa – however – needs further investigation. In cattle, the expanded mucified cumulus oophorus is after all eliminated shortly after ovulation leaving the secondary oocyte only surrounded by the ZP when reaching the fertilization site (Lorton and First, 1979). Nevertheless, biochemical cumulus contributions in the immediate vicinity of the oocyte may possibly generate a microenvironment supportive for fertilization (Tanghe et al., 2003).

Glycomolecules and sperm-ZP interaction

The mammalian ZP is a porous cell type-specific extracellular matrix or coat basically composed of three (or four) sulphated glycoproteins, namely ZP1, ZP2, ZP3 and ZP4 secreted by either the developing oocyte (rodents) or a combination of the oocyte and adjacent granulosa cells (pig, cattle, human), depending on the species (Wassarman, 1988; Töpfer-Petersen 1999a, Primakoff and Myles, 2002; Nixon et al., 2007; Kanai et al., 2008). Sperm appear not willing to bind to the ZP of foreign species (Oehninger et al., 1993). The extracellular egg vestment – hence – is assumed to provide some kind of barrier to cross fertilization, possibly due to the observed difference in patterns of expression, distribution and structure of O-linked oligosaccharides (Benoff, 1997; Nixon et al., 2007). However, cross-binding has been observed: porcine and equine sperm seem to bind tightly to the ZP of bovine oocytes, undergo the acrosome reaction and (in the case of equine spermatozoa) penetrate the ZP and finally enter the oocyte (Sinowatz et al., 2003). Even though the sperm cell surface actively takes part in the processes of binding to ZP, acrosome reaction and actual passage

through the ZP, the carbohydrates of the ZP barrier are assumed to substantially contribute as well (Benoff, 1997). Sperm-ZP binding is a biphasic event starting with a – non-specific – loose attachment followed by species-specific tight binding. It is nowadays generally accepted that an O-linked carbohydrate on mouse ZP3 serves as the primary sperm receptor regulating tight binding to the ZP (Benoff, 1997; Primakoff and Myles, 2002). Purified mouse ZP3 seemed to competitively inhibit sperm-ZP binding *in vitro* (Bleil and Wassarman, 1980; Florman and Wassarman, 1985), whereas ZP1 and ZP2 lacked this inhibitory activity. In addition, mouse ZP3 has been demonstrated to preferentially bind the plasma membrane region overlying the acrosome of acrosome-intact spermatozoa (Bleil and Wassarman, 1986). Furthermore, binding of sperm to ZP3 triggers a cascade of intracellular signals resulting in fusion of the plasma membrane with the outer acrosomal membrane (Talbot et al., 2003). During acrosome reaction, spermatozoa are required to remain reversibly attached to the ZP prior to actual ZP penetration. In mice, secondary ZP-binding of the inner acrosomal membrane of acrosome-reacted sperm cells could be involving ZP2, since acrosome-reacted spermatozoa appear to lose affinity for ZP3 and attain affinity for ZP2 (Mortillo and Wassarman, 1991; Nixon et al., 2007). Following fertilization, the ZP is being modified in a way that mouse sperm receptor activity is lost: embryonic mouse ZP3 fails to bind to mouse spermatozoa or inhibit sperm–ZP binding *in vitro* (Benoff, 1997). However, species-differences in ZP proteins and their corresponding functions should be kept in mind (Kanai et al., 2008): human and rat ZP appear to consist of four glycoproteins (ZP1, ZP2, ZP3 and ZP4), whereas the pig ZP is composed of three glycoproteins (ZP2 or ZPA, ZP3 or ZPC and ZP4 or ZPB). In the mouse, the ZP is comprised of three glycoproteins as well, but in this case the respective glycoproteins are ZP1, ZP2 and ZP3 (Wassarman, 1988). While in the mouse and hamster the recognition signals and the ability to induce the acrosome reaction have indeed been assigned to ZP3 (ZPC), sperm-binding activity in the pig, rabbit and cow has been linked to ZPB (Töpfer-Petersen, 1999b; Yonezawa et al., 2001). In 2001, Amari et al. demonstrated that – in the bovine species – the nonreducing terminal alpha-mannosyl residues of the N-linked carbohydrate chain of the ZP glycoproteins are essential for sperm-egg binding. Furthermore, recognition and binding between spermatozoa and eggs should be regarded as the result of the joint actions of a multimeric receptor system (Shur, 1998). To accomplish high-affinity binding between sperm and egg, an essential density of the different biologically active carbohydrates must be expressed within the supramolecular architecture of the ZP matrix (Töpfer-Petersen, 1999b). Mouse scientists have found evidence to support the hypothesis that the three-dimensional structure of the ZP matrix, rather than a single

carbohydrate, is essential for sperm binding (Nixon et al., 2007). Recently, it has been postulated that sperm-ZP recognition actually involves at least two separate binding events. Sperm-ZP3-dependent binding is in fact preceded by sperm adhesion to a ZP3-independent ligand peripherally associated with the ZP. Preliminary identification of this presumed ligand has pointed towards a 250-kDa glycoprotein (Shur et al., 2006). Since the ZP matrix is altered after ovulation by adsorption of oviduct-secreted glycoproteins (Malette et al., 1995), it should be considered that the sperm-binding glycans exposed on the ZP surface may well be different from those assessed when the ZP is obtained from ovarian homogenates (Talbot et al., 2003). Extrapolation of data, gathered using *in vitro* matured oocytes, to the *in vivo* situation should always urge caution.

Glycomolecules and sperm-oocyte interaction

After ZP binding and acrosome reaction, sperm cells penetrate the ZP and enter the perivitelline space (the extracellular region between ZP and oolemma). Subsequently, the final adhesion of the sperm plasma membrane to the oolemma occurs, prior to actual sperm-oocyte fusion (Talbot et al., 2003). The initial contact and binding between sperm cell and oolemma may be initiated by any region of the sperm head. Actual fusion – though – appears to be confined to the membrane at the equatorial segment and to the postacrosomal region gaining fusing ability during the acrosome reaction (Töpfer-Petersen, 1999a).

Contrasting the well-characterized sperm receptor on the ZP, the molecular basis by which acrosome-reacted sperm cells bind to receptors on the oolemma and initiate sperm-oocyte fusion is still unresolved. Whereas the careful use of genomic and proteomic techniques has revealed a plethora of molecules assumed to play a role in sperm-oocyte interaction, the picture is still far from complete (Nixon et al., 2007).

Gradually, a model for sperm-oolemma binding has been put forward suggesting that sperm-egg binding results from the adhesion between an integrin on the oolemma and an integrin ligand (the disintegrin domain of a member of the ADAM family of proteins, an acronym for proteins containing a disintegrin and a metalloprotease domain) on the sperm cell surface (Primakoff and Myles, 2002; Talbot et al., 2003). With respect to the male gamete, the most suitable candidates for a role in sperm binding to the egg plasma membrane are fertilin β (ADAM2) and cyritestin (ADAM3). Sperm plasma membrane binding (and fusion) appeared to be inhibited by peptides representing the active site of the disintegrin domain from either fertilin β or cyritestin (Yuan et al., 1997). However, these two sperm ADAMs should be

redundant with other molecules, since spermatozoa null for both fertilin and cyritestin seem still capable of fusion with eggs (Nishimura et al., 2001). Very recently, involvement of ADAM4 and ADAM6 in a reproductive ADAM system that functions in fertilization in mice has been demonstrated (Han et al., 2009). On the female side, the preliminary indication that a disintegrin could be responsible for sperm-oolemma adhesion suggested that the oocyte adhesion molecule might be an integrin. Fertilin β has – indeed – been found to bind to the integrin $\alpha_6\beta_1$ on the egg surface and a monoclonal antibody to α_6 has been shown to block sperm adhesion (and fusion) with ZP-free eggs (Almeida et al., 1995; Chen and Sampson, 1999), pointing towards $\alpha_6\beta_1$ as a potential sperm receptor on the oolemma. However, oocytes from mice null for the α_6 integrin subunit were demonstrated to have no reduction in sperm-egg binding (or fusion), indicating that if α_6 integrins do participate in sperm-egg adhesion, they are not absolutely essential (Miller et al., 2000).

Carbohydrates and glycoproteins have been generally acknowledged to mediate communication between cells in many biological systems (Gabius, 1997), including modulation of adhesion and binding events during sperm–oocyte interactions (Tulsiani et al. 1997, Gougoulidis et al. 1999, Amari et al. 2001, Tanghe et al. 2004a, 2004b). It could be hypothesized that large and flexible sugar chains may initiate the first contact between the sperm and the egg oolemma, preparing the next steps of tight binding and fusion (Töpfer-Petersen, 1999a). The exploration of potential adhesion molecules on male and female gamete began with the observation that peptides containing the integrin-binding Arg-Gly-Asp (RGD) amino acid sequence were able to inhibit binding of human and hamster sperm to ZP-free hamster oocytes (Bronson and Fusi, 1990). Subsequently, a variety of adhesion molecules of the integrin family and ECM glycoproteins (such as fibronectin, vitronectin and laminin) have been recognized on the sperm cell surface, displaying a dynamic distribution pattern during capacitation and acrosome reaction (Fusi and Bronson, 1992; Bronson and Fusi, 1996; Glander et al., 1998). Integrin subunits have been detected on the oolemma in several species (Bronson and Fusi, 1996; Zuccotti et al., 1998), supporting the hypothesis that egg integrins and corresponding integrin-binding sperm glycoproteins could play a role in sperm-egg interaction possibly by tightly connecting the sperm cell to the oolemma (Töpfer-Petersen, 1999a).

Membrane fusion necessitates transformation of two lipid bilayers (originating from two different barriers) into one single bilayer. In contrast to intracellular membrane fusion and fusion of a virus with its host cell, the molecular basis of membrane fusion between two eukaryotic cells (sperm and egg) is poorly understood (Talbot et al., 2003). Recent results

point away from the involvement of sperm ADAMs and egg integrins. Alternatively, oocyte CD9 appears to play a fundamental role (Talbot et al., 2003). Female mice carrying a gene knockout for CD9 appear to be infertile, producing normally maturing oocytes with proper sperm binding ability, but defective in sperm-egg fusion (Primakoff and Myles, 2002; Nixon et al., 2007). Notwithstanding this lack of fusibility, the use of intracytoplasmic sperm injection (ICSI) resulted in viable embryos developing to term (Miyado et al., 2000). Additionally, fertility in CD81 null mice has been demonstrated to be compromised as well due to a deficiency in sperm-oocyte fusion (Rubinstein et al., 2006). Accordingly, double knockout mice ($CD9^{-/-}$ / $CD81^{-/-}$) appeared totally infertile and so CD9 and CD81 seemed to play complementary roles in sperm-oocyte fusion. Only recently, a complementary role for tetraspanins in conjunction with integrins in fertilization has been put forward by Ziyyat et al. (2006). These authors claim that CD9 mediates the configuration of integrin $\alpha_6\beta_1$-clusters on the oolemma (in human and mouse) and that both $\alpha_6\beta_1$ and CD9 appear to play a role in human sperm-oocyte fusion. Despite the unequivocal role for CD9 established in murine sperm egg interaction, further research to elucidate the protein interactions mediated by this tetraspanin is essential (Nixon et al., 2007). Additionally, a glycosyl phosphatidylinositol (GPI)-anchored protein could be of some importance as well (Talbot et al., 2003): GPI knockout females were found to be infertile, with a minority of oocytes fertilized *in vivo* despite the presence of multiple spermatozoa in the perivitelline space (Alfieri et al., 2003). Since, *in vitro* fertilization of ZP-free oocytes (from these females) did not result in a significant reduction in sperm-oolemma binding, infertility appeared to be completely due to defects in sperm-oocyte fusion. Further research is required to identify the proposed 35-45 kDa GPI-anchored protein or to discover previously undetected GPI-molecules on the egg surface (Nixon et al., 2007). A particular epididymal-derived cysteine-rich secretory protein (CRISP-1, also referred to as DE) has also been suggested as a potential mediator of sperm-oocyte fusion as a result of a series of observations mainly obtained in rats (Ellerman et al., 1998; Nixon et al., 2007). Both *in vitro* antibody inhibition experiments and *in vivo* knockout studies indicated a vital role in sperm-oocyte interaction for Izumo, a novel protein member of the immunoglobulin superfamily located on the inner acrosomal membrane and equatorial segment (Okabe et al., 1988; Inoue et al., 2005). $Izumo^{-/-}$ males seemed sterile despite normal mating behaviour, ejaculation and sperm migration to the site of fertilization. Furthermore, spermatozoa from $Izumo^{-/-}$ males were able to penetrate the ZP and subsequently bind to but not fuse with the oolemma. Sterility in these null mice – hence – appears to be particularly due to defective sperm-oolemma fusion (Nixon et al., 2007).

In bovine, involvement of the TEC-2 epitope (a carbohydrate located on the oolemma) in sperm-oolemma fusion has been suggested (Gougoulidis et al., 1999). Oocytes exposed to N-acetylgalactosamine (GalNAc), part of the TEC-2 epitope, prior to sperm insemination experienced no effect on fertilization, however, sperm pretreatment with the carbohydrate resulted in inhibition of fertilization, with a reduction in cleavage rates as the GalNAc concentration increased. A significant decrease in sperm-oolemma fusion and a significant increase in sperm-oolemma binding were also observed. The carbohydrate GalNAc, associated with the TEC-2 epitope, hence, seems to play a specific role during bovine sperm-oolemma fusion through interaction with a carbohydrate-binding molecule on the sperm cell surface. The authors proposed that the epitope and its sperm complement could be acting as a secondary binding system involved in bringing into close apposition the sperm and oolemma for fusion to take place (Gougoulidis et al., 1999). Furthermore, the p-aminophenyl derivative of D-mannose (APMP) has been verified to have a blocking effect on bovine fertilization mainly by inhibiting sperm–oocyte fusion (Tanghe et al., 2004a). Based on the results of this study, D-mannosyl residues seemed to be localized on the oolemma and APMP probably was exerting its inhibitory effect on spermatozoa by specifically obstructing the receptor-ligand system between the male and female gametes.

Previous research at our department was focussed on a screening to identify which carbohydrates and glycoproteins may be involved in bovine *in vitro* fertilization (Tanghe et al., 2004b). *In vitro* matured cumulus–oocyte complexes (COCs) were fertilized in the presence of a selection of glycomolecules to determine which glycoconjugates could act as competitive inhibitors of oocyte penetration. Among the glycomolecules tested, D-mannose, fucoidan, dextran sulfate and fibronectin appeared the most effective inhibitors of oocyte penetration (≥ 90% inhibition), whereas L-fucose and vitronectin inhibited the penetration rate to a lesser extent (about 50% inhibition). Other carbohydrates reduced sperm penetration by less than 40% (i.e., D-galactose, N-acetyl-D-galactosamine, D-fucose and sialic acid) or were not successful at all as inhibitors of oocyte penetration (i.e., mannan, N-acetyl-D-glucosamine, dextran and heparan sulfate). Heparin was found to be the only glycomolecule significantly increasing the penetration rate, which is not unexpected since this molecule is promoting bovine sperm capacitation (Parrish et al., 1988). To eliminate a possible toxic effect on spermatozoa, sperm motility was evaluated over time in the presence of the most potent carbohydrates and/or glycoproteins. L-fucose, dextran sulfate and vitronectin did not affect total and progressive sperm motility, while D-mannose, fucoidan and fibronectin

slightly, but significantly reduced both motility parameters. These results suggested involvement of D-mannose, L-fucose, fucoidan, dextran sulfate, fibronectin and vitronectin in bovine IVF (Tanghe et al., 2004b). Accordingly, these glycoconjugates represent intriguing candidates for further research to explore which fertilization step(s) are exactly affected by their presence and to locate the carbohydrate receptor sites and corresponding ligands on male and female gametes.

1.1.3 GLYCOMOLECULES AND EMBRYO IMPLANTATION

Successful implantation of the mammalian embryo involves a highly organized series of events requiring developmental synchrony between the conceptus and uterine endometrium (Burghardt et al., 2002). At the blastocyst stage, the embryonic trophectoderm has gained competence to attach to the uterine luminal epithelium (the site of hormonally regulated receptive sensitivity). If synchronization has properly occurred, an implantation adhesion cascade is initiated upon engagement of cell adhesion molecules on the maternal and embryonic side. The uterine luminal surface appears to contain a variety of ECM glycoproteins, such as mucin glycoprotein 1 (MUC1), glycosylated cell adhesion molecule 1 (GlyCAM-1), galectin-15 and secreted phosphoprotein 1 (SPP1), also known as osteopontin. Due to this glycoprotein nature of the uterine-placental interface, integrins have become the most extensively studied adhesion molecules involved in implantation (Burghardt et al., 2009). In mice, integrin-binding RGD peptides have been shown to play a role in trophoblast attachment and outgrowth (Armant et al., 1986) and have been postulated to play a role in implantation (Sutherland et al., 1993) already quite some time ago. Treatments which block integrin attachment appeared to reduce the number of implantation sites in mice (Illera et al., 2000). In sheep and pigs, integrin subunits α_v and β_3 as well as the integrin-binding protein SPP1 have been detected on the surface of both trophectoderm and uterine luminal epithelium during the peri-implantation period of pregnancy (Burghardt et al., 2009; Erikson et al., 2009). Osteopontin or SPP1 is a RGD-containing ECM glycoprotein (for a detailed review see: Johnson et al., 2003a) which is highly upregulated during the initial stages of pregnancy in the uterus of humans and other mammalian species, including pigs (Erikson et al., 2009). In sheep, it has been demonstrated that ovine SPP1 is a component of histotroph (a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins and other substances) secreted by the uterine glandular epithelium binding to the uterine-placental interface (Johnson et al., 2003b). However, no effect on (mid- or late-gestation)

embryo resorption could be observed when using $SPP1^{-/-}$ or $SPP1^{+/-}$ mice, but $SPP1^{-/-}$ embryos were found to be significantly smaller at all gestational ages than wild-type counterparts (Weintraub et al., 2004). Although it has been commonly accepted that SPP1 principally binds to integrin $\alpha_v\beta_3$ via its RGD sequence, other receptors (i.e. integrin $\alpha_v\beta_1$, $\alpha_v\beta_5$ and $\alpha_8\beta_1$) and alternative binding sequences have been identified (Johnson et al., 2003a). *In vitro*, ovine and porcine trophectoderm and uterine luminal epithelium cells show evidence of integrin receptor activation and cytoskeletal reorganization in response to SPP1 binding (Johnson et al., 2001; Garlow et al., 2002). Results of a recent study in pigs support the hypothesis that SPP1 promotes integrin mediated implantation by mediating trophectoderm cell migration during elongation and trophectoderm cell attachment for implantation. In this model, integrin $\alpha_v\beta_3$ is assumed to be a permanent receptor for SPP1 providing (upon connection with the glycomolecule) a supportive framework for conceptus attachment and elongation via integrin $\alpha_v\beta_6$ on trophectoderm. Since SPP1 has been identified at the conceptus-maternal interface in a number of species with different types of placentation (such as pigs, ruminants, rodents and primates) and seems to be differentially regulated in a spatio-temporal manner dependent upon placentation type, it will be essential to explore different mechanisms involving SPP1 and integrins in each of these species during implantation (Erikson et al., 2009). Accordingly, differences in SPP1 expression between sheep and pigs have been detected relating to differences in placentation (Joyce et al., 2005). As the nature of ovine placentation is more invasive compared to pigs, the temporal/spatial pattern of uterine and placental SPP1 expression in sheep is remarkably similar to that observed for humans. In sheep, cooperative binding of various integrins to a rigid ECM – including large amounts of SPP1 – is believed to provide an adhesive mosaic to maintain a tight connection between uterine and placental surfaces along regions of true epitheliochorial placentation in sheep (Burghardt et al., 2009). Since SPP1-null mice are found to be fertile (while inactivation of a_v integrin caused perinatal lethality), it seems that no single ECM glycoprotein is indispensable for the implantation process (at least in mice). Loss of one protein may be compensated for by another ligand (binding a common receptor). Such redundancy in ligand/receptor molecules may ensure actual attachment of the conceptus to the uterine epithelium (Johnson et al., 1999).

In bovine, pre-treatment of spermatozoa or *in vitro* matured oocytes with purified milk SPP1 appeared to improve fertilization, cleavage and embryo development (Gonçalves et al., 2008), suggesting that both sperm and oocytes may associate with SPP1. This result was confirmed by Monaco et al. (2009) claiming that fertilization medium supplementing with 10 mg/mL SPP1 improved *in vitro* embryo production and that this glycoprotein positively

influenced sperm capacitation *in vitro*. Both research groups believe that addition of SPP1 to bovine gametes might be a useful tool to optimize the bovine embryo production system.

1.2 SELECTION OF FIT SPERMATOZOA FOR FERTILIZATION IN CATTLE

1.2.1 SELECTION OF SPERMATOZOA IN THE BOVINE GENITAL TRACT

In cattle, fertilization takes place at the ampulla of the oviduct where both male and female gamete gather (Suarez, 2002). In order to reach the fertilization site, the sperm cell is required to pass several natural selection mechanisms. Upon ejaculation, bulls deposit several billion spermatozoa into the cranial part of the vagina close to the cervical opening (López-Gatius, 2000; Hunter, 2003; Rodriguez-Martinez, 2007). As the cervix represents the primary barrier for sperm transport, the number of sperm cells eventually reaching the uterine body generally does not exceed 1%. When using artificial insemination (AI), where 5 to 20 million frozen–thawed spermatozoa are deposited directly into the uterine body, the cervix is by-passed permitting the use of relatively low sperm numbers (López-Gatius, 2000). However, cervical mucus is thought to provide some means of sperm selection, since it seems to generate a more distinct barrier to abnormal spermatozoa unable to swim efficiently or displaying a poor hydrodynamic profile than it does to morphologically normal and motile spermatozoa (Barros et al. 1984; Katz et al. 1990; Suarez, 2007). Other functions allocated to the cervix include preventing entry of seminal plasma into the uterus, excluding potentially infectious microorganisms and storage of sperm for later transportation (Abou-Haila and Tulsiani, 2009). While representing a sperm survival supporting environment, cervical mucus does not seem to induce sperm capacitation nor acrosome reaction (Barros et al. 1984). It has been established that, during the follicular phase, the mucus in the longitudinal grooves (leading from the vagina directly into the uterus) is less dense than the mucus in the central part of the cervical canal (Mullins and Saacke, 1989). Sperm cells are assumed to traverse the cervix deep inside these grooves, hence avoiding the viscous mucus in the central lumen acting to flush out uterine contents and microorganisms. After flushing cervices 19 to 24 h after mating, about 90% of the mucus and more than 90% of the luminal leukocytes, but only half the sperm cells were recovered. The residual half of the spermatozoa were detected deep in the mucosal grooves. Consequently, the cervix probably serves as a filter eliminating seminal plasma, microorganisms and abnormal spermatozoa from the semen (Suarez, 2007).
Hunter (2003) – on the other hand – believes that the cervix can be regarded as the primary but not a *functional* sperm reservoir. After mating, an impressive amount of sperm cells is trapped (oriented with their heads against the epithelium) in the prominent glands or cervical crypts, suggesting some kind of specialized storage arrangements (Hunter, 2003). However, according to him, the sperm cells specifically trapped in the cervical crypts are probably not the ones reaching the site of fertilization, considering the lack of reversing movement ability.

Therefore, another sub-population of spermatozoa, which is not directed along flow lines of mucus into cervical crypts but rather passing directly towards the uterine lumen through the central portion of the cervical canal (due to reflux of uterine fluids which are also at maximum volume during and shortly after oestrus), should be reconsidered (Hunter, 2003). The suggestion of spermatozoa escaping from cervical glands by means of longitudinal grooves and channels (Mullins and Saacke, 1989) remains hypothetical and cannot overcome the need for a reversing movement (Hunter, 2003). There has been so far no evidence that spermatozoa occupying the cervical folds and crypts could preferentially take part in fertilization (López-Gatius, 2000).

The next (anatomical, physiological and/or mucus) barrier to sperm passage in the female reproductive tract is the uterotubal junction (UTJ). Its lumen is tortuous and narrow (Suarez, 2007) and the passage through the junction is complicated by mucosal folds forming *cul-de-sacs* with their opening pointing in a uterine direction (Yániz et al., 2000). This specific orientation may trap sperm cells in order to prevent further transport. Furthermore, Wrobel et al. (1993) described the presence of a vascular plexus in the lamina propria/submucosal layer of the wall of the UTJ which might function as a sort of physiological valve: upon distension, the plexus will reduce the lumen. The substantial muscular layer in combination with the sigmoid shape of the UTJ and muscular ligaments (capable of increasing the flexure of the curve) may be able to induce extra compression (Suarez, 2007). In the mouse, the junction appears manifest shortly after coitus, but tightly closed about an hour later (Suarez, 2008). The anatomy of the UTJ suggests it is designed not only to restrict access to infectious microorganisms and leukocytes from the uterus, but also to regulate entry of sperm (Suarez, 2008). The mucus (detected in the UTJ) may exert an additional hampering effect on sperm progression (Suarez et al., 1997). Even though the UTJ expands during estrus or when stimulated by coitus, spermatozoa may not be able to pass it without exposing certain proteins on their cell surface (possibly allowing sperm to gain footholds on the wall lining the UTJ and so to progress forward by attaching reversibly to the epithelium; Suarez, 2008). Only a small subpopulation of spermatozoa is rapidly (in minutes) transported by uterine contractions towards the UTJ (Rodriguez-Martinez, 2007). Afterwards, the majority of the remaining spermatozoa are eliminated from the female genital tract by retrograde flow and intrauterine phagocytosis by infiltrating polymorphonuclear neutrophils.

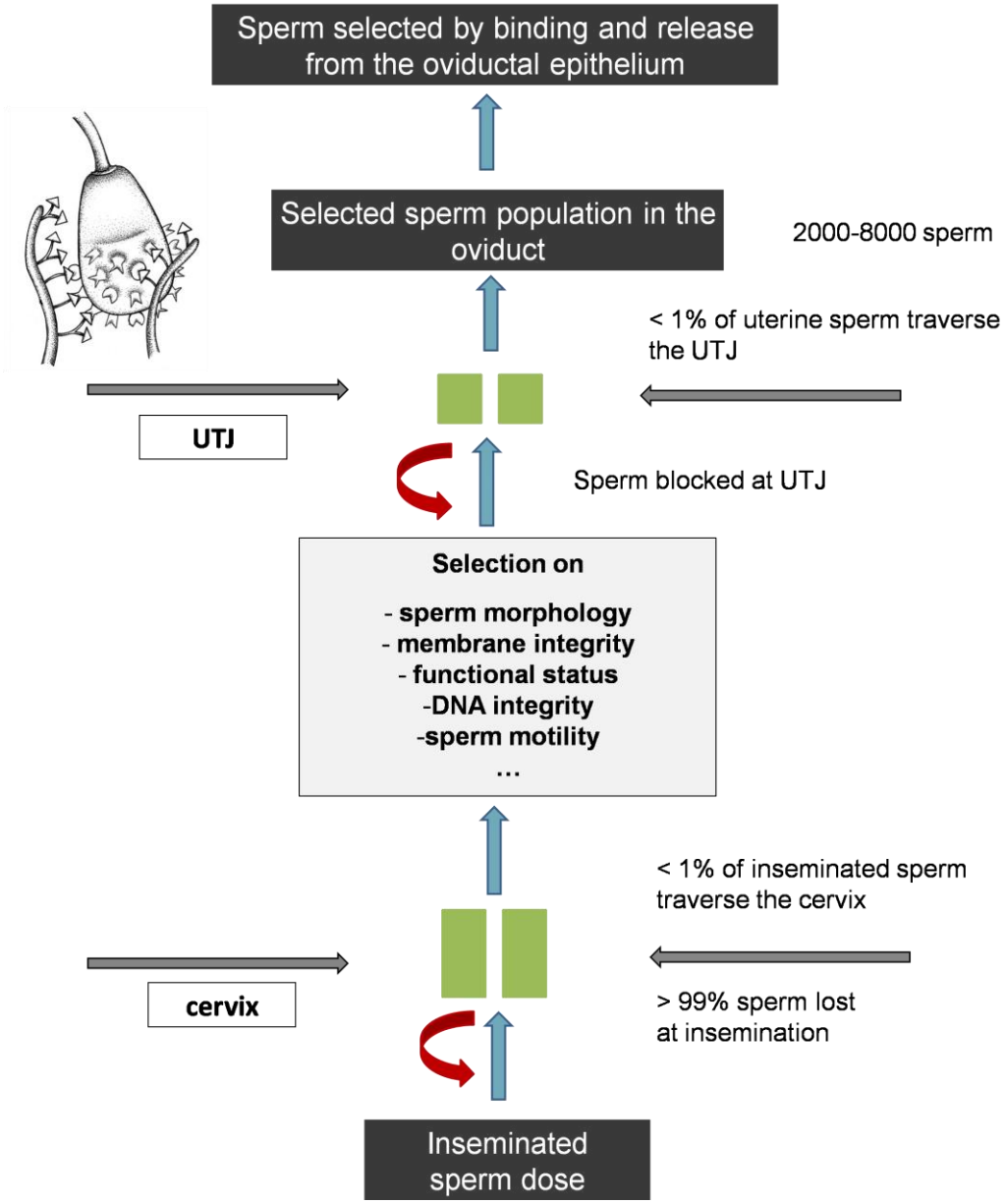


Figure 3: Schematic model for sperm selection within the female reproductive tract (adapted from Holt, 2009).

After passage through the UTJ, a number of sperm cells will reversibly bind to the epithelium of the caudal segment of the oviducal isthmus, resulting in formation of a storage reservoir (Hunter and Wilmut, 1984; Hunter, 2003). This pre-ovulatory *functional sperm reservoir* serves to guarantee successful fertilization by providing suitable numbers of sperm cells in the appropriate physiological state at the ampulla soon after ovulation (Suarez, 2002; Rodriguez-Martinez, 2007). In order to do so, the sperm reservoir may exert three different functions (Suarez, 2002). First, the reservoir is assumed to preserve the fertility of spermatozoa until ovulation is impending. Sperm incubated with oviductal epithelium *in vitro*

remain viable longer than when incubated in medium alone (Pollard et al., 1991). Second, this storage reservoir may play a role in preventing polyspermic fertilization by releasing only a small number of spermatozoa at once to further ascent to the ampulla reaching the ovulated oocyte (Suarez, 2007). Third, the physiological state of spermatozoa (namely capacitation and hyperactivation), may be regulated inside the reservoir to ensure that sperm cells are in the appropriate state when ovulation takes place (Suarez, 2002). *In vivo*, most viable spermatozoa present in the sperm reservoir – prior to ovulation – appear to be uncapacitated (Rodriguez-Martinez, 2007). The capacitation-associated increase of intracellular calcium concentration was delayed in stallion sperm attached to oviductal epithelial cells *in vitro* (Dobrinski et al., 1996). Consequently, viability may be maintained by preventing capacitation and its concurrent rise in cytoplasmic calcium (Suarez, 2008). Catalase, present in the bovine oviduct, has been proposed to serve as protector against peroxidative sperm membrane damage and thereby possibly preventing increased inward leakage of calcium (Lapointe et al., 1998).

Binding of motile bovine spermatozoa to oviductal epithelium has been observed *in vitro* already two decades ago (Suarez, 2002). After entering the oviduct, the progression of sperm cells is initially slowed down due to the narrow, convoluted lumen filled with mucus increasing their contact with the mucosal epithelium (including potential binding sites). At the level of the isthmus, the lumen is barely about the size of a sperm head and is filled with a mucopolysaccharide-rich substance (Suarez et al., 1997). This viscous secretion is assumed to strip residual male antigens from the sperm cell surface, enabling binding with the oviductal epithelium (Hunter, 2003). When contacting the mucosa (arranged in branching folds, creating channels that in some instances end blindly), sperm cells get frequently stuck (Yániz et al., 2000; Suarez, 2002). Sperm binding (via the plasma membrane overlying the acrosome) usually occurs to cilia, although association of spermatozoa with microvilli of non-ciliated cells has been observed as well (Hunter et al., 1991). Detachment of sperm cells from the reservoir may well be initiated by endocrine signals associated with imminent ovulation of a follicle through induction of capacitation and hyperactivation (Suarez, 2001; Hunter, 2003). Capacitation results in shedding of adsorbed proteins from the sperm cell surface (required for binding to the oviductal epithelium), while hyperactivation provides additional pulling force necessary for overcoming the attraction between sperm and epithelium (Suarez, 2008). Capacitated bovine spermatozoa show reduced binding to oviductal epithelium *in vitro* (Ignotz et al., 2001). After ovulation, capacitation rates considerably increase parallel with the individual and continuous sperm detachment from the reservoir. The capacity and speed of

the response to *in vitro* induction of sperm activation (capacitation) differs substantially between individual sperm cells. This diverse responsiveness among spermatozoa assures full sperm viability before ovulation and the presence of sperm cells at different stages of capacitation at the ampulla isthmic junction, hence optimizing the chance of successful fertilization (Rodriguez-Martinez, 2007). Consequently, the time for a fertilizing sperm population to reach the ipsilateral ampulla (the one on the side of the ovulatory follicle) can be expected to be significantly reduced when mating occurs closer to the time of ovulation (Hunter, 2003).

In conclusion, the female tract - particularly the oviduct - provides several filters serving to select for normal vigorously motile spermatozoa *in vivo* (Fig.3; Suarez, 2008).

1.2.2 SELECTION OF BULL SPERMATOZOA *IN VITRO*

In order to obtain adequate results *in vitro*, it is required to select for viable sperm cells prior to *in vitro* fertilization (IVF), since thorough sperm selection normally occurs in the female reproductive tract. During copulation, cervical mucus represents a barrier allowing only passage of progressively motile spermatozoa with normal morphology and high nuclear stability (Rodriguez-Martinez et al., 1997). Upon ejaculation, sperm cells are in a protective state and incapable of fertilization (Mortimer, 2000). Seminal plasma not only contains at least one decapacitation factor (preventing spontaneous capacitation upon ejaculation), but also includes one or more factors to which prolonged exposure has unfavourable effects on sperm function (such as the ability to penetrate cervical mucus and undergo the acrosome reaction *in vitro*). Accordingly, spermatozoa need to be separated from the seminal plasma as soon and as efficiently as possible after ejaculation (Mortimer, 2000). *In vivo* most sperm cells quickly enter the cervical mucus, leaving behind the seminal plasma (Suarez, 2007). *In vitro*, seminal plasma is – conversely – removed gradually by dilution and centrifugation (Suarez, 2007). Gradual dilution of seminal plasma *in vitro* could diminish the fertility of spermatozoa due to extended exposure of sperm to immune and epithelial cells, cell debris and bacteria (Morrell 2006). Furthermore, frozen-thawed bull sperm displays lower progressive motility, but the percentage of morphologically normal sperm cells seems comparable to fresh semen (Parrish et al., 1995). Cryopreservation detrimentally affects the metabolism and motility of spermatozoa as well as the status of several sperm membrane domains (Rodriguez-Martinez et al., 1997). Deleterious effects on axonemal, mitochondrial

and even chromatin structure have been described. Therefore, appropriate procedures to enrich or select viable sperm fractions after thawing are indispensable.

Although the efficiency of a sperm preparation method is regularly evaluated by its yield of progressively motile spermatozoa, it is also essential that sperm preparations for clinical use should be free of any microbiological contaminants present in semen (Mortimer, 2000). Other relevant considerations in selecting a technique include its technical complexity as well as its costs in equipment and time (Mortimer, 2000). The ideal *in vitro* sperm selection procedure should therefore be able to considerably improve the sperm quality with higher rates of progressive motility and morphologically normal spermatozoa (Rodriguez-Martinez et al., 1997; Mortimer, 2000). In order to do so, the technique is required to adequately separate motile from immotile sperm, remove seminal plasma, cryo-protective and infectious agents, and debris, and – additionally – initiate capacitation (Rodriguez-Martinez et al., 1997; Samardzija et al., 2006b). To obtain this functional state in ejaculated spermatozoa, adsorbed proteins (originating from the cauda epididymidis and the seminal plasma) have to be removed from the sperm cell surface, especially at the acrosomal region (Rodriguez-Martinez, 2007). This is usually carried out *in vitro* by washing and centrifugation of the sperm suspension. After washing, the spermatozoa are generally incubated in albumin- and bicarbonate-enriched media stimulating a sequence of capacitation-associated events (Rodriguez-Martinez, 2007). Incubation with heparin has been established to remove PDC109 (member of the bovine seminal plasma proteins) from the sperm cell surface (Suarez, 2008). Consequently, heparin is commonly supplemented to the fertilization medium during sperm-oocyte co-incubation to fully induce capacitation.

Overall, four basic approaches for separating spermatozoa from the seminal plasma and/or extender have been described (Rodriguez-Martinez et al., 1997): 1) dilution and washing (centrifugation and resuspension); 2) sperm migration (swim-up procedures); 3) adherence methods (differential filtration through glass wool, glass beads or polysaccharide beads); and 4) density gradient centrifugation (selective fractionating subpopulations).

Washing is usually carried out by dilution in relatively large volumes of culture medium followed by centrifugation and resuspension in fresh medium (Avery and Greve, 1995). Centrifugal pelleting of unselected sperm populations – however – often seems to result in generation of reactive oxygen species (ROS) within the pellet probably compromising sperm function *in vitro* (Mortimer, 2000). Irreversible damage to spermatozoa resulting in decreased fertilization rates or even fertilization failure have been reported. ROS not only appear to affect the sperm plasma membrane through induction of phospholipid

peroxidation (and consequently reduced membrane fluidity and defective sperm function), but also the sperm DNA by causing strand breaks (Mortimer, 2000). Simply diluting and centrifuging unselected sperm populations should therefore be recognized as potentially harmful and substituted by more safe procedures, as swim-up and density gradient centrifugation techniques where the ROS-generating cells are excluded from the prepared population (Mortimer, 2000). ROS are produced both by leukocytes (present in semen) and sperm cells (Aitken, 1995; Whittington and Ford, 1999). Though, only spermatozoa with excess retained spermatid cytoplasm appear to generate ROS (Aitken, 1995; Mortimer 2000). The latter – less dense – cells are exactly excluded from the ultimate sperm population by density gradient centrifugation (Mortimer, 2000). Furthermore, only density gradient methods (and not swim-up procedures) eliminate spermatozoa with nicked DNA and poorly condensed chromatin (Sakkas et al., 2000). Simple washing of spermatozoa includes a greater risk of contributing a defective genome to the embryo and might bring about the developmental failure of ICSI-derived embryos regularly observed when the embryonic genome is activated (Shoukir et al., 1998). Moreover, with this technique all types of spermatozoa (including the immotile, dead and abnormal ones) remain present in the final preparation. Inflammatory cells and bacteria can also be grouped in the sperm pellet (Rodriguez-Martinez et al., 1997).

For the swim-up procedure, thawed semen is covered with a layer of a suitable culture medium, often Tyrode's albumin lactate pyruvate (TALP, Rodriguez-Martinez et al., 1997) medium. During the subsequent incubation, the progressively motile spermatozoa migrate from the semen layer into the culture medium (Rodriguez-Martinez et al., 1997; Mortimer, 2000). This self-migration method is regarded to be functionally equivalent to the process by which spermatozoa escape from the ejaculate to colonize the cervical mucus (Mortimer, 2000). Under physiological circumstances, only highly motile spermatozoa complete the migration through the cervical and oviductal fluids (Rodriguez-Martinez et al., 1997). The ability to actively swim out of a denser medium into culture medium has been reported to be positively correlated with sperm vitality (Parrish and Foote, 1987). The preparation technique is a reliable procedure adequately selecting for proper motility and morphology, but might be suboptimal for clinical applications due to differences in chromatin quality (Rodriguez-Martinez et al., 1997; Mortimer, 2000). The rather low sperm recovery rate (10 to 20%) after applying swim-up (Shamsuddin and Rodriguez-Martinez, 1994; Correa and Zavos, 1996) is compensated by significantly higher cleavage rates after IVF than generally obtained after Percoll treatment (Parrish et al., 1995). Several beneficial effects on sperm cells have been attributed to addition of hyaluronic acid (HYA) to the swim-up medium (Mortimer, 2000).

This treatment results in a significantly increased proportion of self-migrated spermatozoa with intact and cleansed plasma membranes (Shamsuddin and Rodriguez-Martinez, 1994). Differences between bulls are no longer significant, indicating that this procedure harvests viable and motile spermatozoa, irrespective of the quality of the original semen sample (Rodriguez-Martinez et al., 1997). In addition, applying the HYA method, viable bovine spermatozoa can be selected and cleansed simultaneously since frozen-thawed sperm are allowed to migrate actively through HYA directly into the fertilization medium making it possible to add the selected spermatozoa directly to the oocytes (Fig.3; Rodriguez-Martinez et al., 1997).

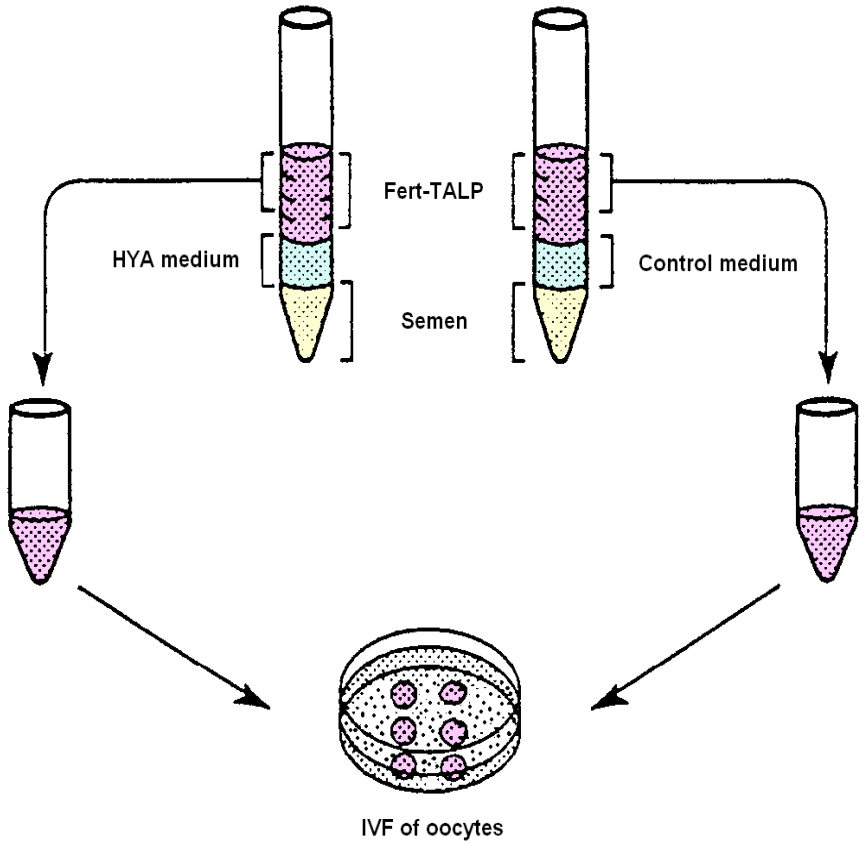


Figure 4: Swim-up with hyaluronic acid (HYA) versus control swim-up procedure (adapted from Shamsuddin and Rodriguez-Martinez, 1994).

Adherence sperm preparation techniques are based on the tendency of dead or damaged sperm cells to attach to glass surfaces or to be retarded in the filter matrix of hydrated polysaccharide beads (Rodriguez-Martinez et al., 1997). Normal spermatozoa pass through the glass filter more easily than abnormal spermatozoa (such as acrosome-damaged or immotile cells). Although this technique allows a simple and fast selection of sperm

populations, there is some evidence that glass wool can damage the plasma membrane and acrosome of human spermatozoa (Sherman et al., 1981). If considering an adherence sperm preparation technique, it is recommended to choose one which not requires a prewash step (Mortimer, 2000).

A popular sperm selection technique is density gradient centrifugation. This procedure is among others recommended by the World Health Organization for preparing human spermatozoa for assisted reproduction (WHO, 1999). Until recently, most studies concerning density gradient centrifugation are based on formulations not specific for the species, often products designed for human spermatozoa (Tanghe et al, 2002a; Somfai et al., 2002; Cesari et al., 2006; Maxwell et al., 2007). By far the most frequently used gradient substance has been Percoll (Pharmacia, Uppsala, Sweden), a medium for purification of cells, viruses and subcellular particles/organelles (Avery and Greve, 1995; Rodriguez-Martinez et al., 1997). It is based on colloidal silica particles (with a diameter of 15 to 30 nm) coated with non-dialysable polyvinyl pyrrolidone (PVP) allowing isopycnic separation of spermatozoa (Avery and Greve, 1995; Mortimer, 2000). Percoll density gradient centrifugation has been used regularly in the past to prepare human and animal spermatozoa for IVF until it was withdrawn in 1996 from the market for clinical use in human assisted reproduction (since its use was 'restricted to research purposes only') because of the potentially harmful effect of the unbound polyvinylpropylene (PVP) it contains (Mortimer, 2000). It has been suggested that PVP might cause genetic abnormalities in embryos and that some PVP solutions were toxic resulting in failed embryonic development (Mortimer, 2000). Percoll treatment of frozen-thawed semen has also been identified as the source of low cleavage and blastocyst rates in the bovine *in vitro* production system (Avery and Greve, 1995). These authors suspected that some Percoll batches could have contained an excess of free PVP (exceeding the 1 to 2% demonstrated to be commonly present), coating the spermatozoa and potentially resulting in a low oocyte penetration rate. Previously, PVP has also been found to stop motility of ejaculated bull spermatozoa and significantly reduce sperm survival and membrane integrity (Avery and Greve, 1995). However, no evidence could be found that PVP is able to cause DNA lesions (Mortimer, 2000). Additionally, it is widely acknowledged that some batches of Percoll contain high levels of endotoxin contamination, which can negatively affect sperm survival or development of fertilized oocytes (Mortimer, 2000). Additional research is – though – required to clarify the reason(s) of the suspected toxicity of Percoll (endotoxins, removal of the PVP coating, etc; Rodriguez-Martinez et al., 1997). Several intrinsic characteristics of colloidal silica made Percoll and its current replacements (PureSperm and

ISolate) optimal for preparing density gradients to select human spermatozoa (Mortimer, 2000). It has no osmotic effect (being a mineral substance) and a low viscosity (being a colloid rather than a solution) whereby it does not retard sperm cell sedimentation. The use of both continuous and discontinuous Percoll gradients have been described (Rodriguez-Martinez et al., 1997). The discontinuous gradient consists of several layers with different density (through which cells or particles penetrate and are being arrested where their density is equal to that of the gradient substance) and is the technique most commonly used in bovine IVF (Rodriguez-Martinez et al., 1997). After centrifugation, the motile sperm cells are grouped in the bottom fraction, whereas dead sperm, seminal plasma, egg yolk diluter and debris are located in the upper fraction (Avery and Greve, 1995). Percoll density gradient centrifugation – hence – efficiently separates sperm cells from foreign material (as extender remnants, cells and bacteria) and generally eliminates most tail and midpiece defects (Rodriguez-Martinez et al., 1997). The sperm recovery rate fluctuates around 50%, being 5 to 10 fold higher than for a swim-up procedure (Avery and Greve, 1995). Unlike Percoll (consisting of colloidal silica coated with PVP), its alternatives (PureSperm and ISolate) are based on colloidal silica with covalently bound silane molecules (silanized silica; Mortimer, 2000). Their clinical effectiveness appears to be equivalent to (or even better than) Percoll and they are manufactured in an isotonic ready-to-use culture medium (different from Percoll which needs to be diluted to obtain 90% v/v Percoll prior to use). Colloidal silica density gradients are very effective in identifying various subpopulations of spermatozoa (Rodriguez-Martinez et al., 1997) and overall considered as the most appropriate and commonly applicable clinical sperm preparation technique (Mortimer, 2000). Density gradients with species-specific formulation have been used for sperm preparation for diagnostic purposes (Rodriguez-Martinez et al., 1997), or for IVF (Sieren and Youngs, 2001; Samardzija et al., 2006a, 2006b), and to prepare stallion spermatozoa for research purposes (Macpherson et al., 2002; Morrell and Geraghty, 2006). Nevertheless, widespread application of the technique in processing mammalian sperm cells has been hindered by the time-consuming protocol, the scarcity of animal-specific colloid formulations (Sieren and Youngs 2001; Samardzija et al. 2006a, 2006b) and – primarily – by the difficulty of processing adequate numbers of spermatozoa for the large insemination doses required in some domestic animal species.

1.3 CONCLUSIVE REMARKS

Despite a considerable amount of knowledge gathered especially in human and mouse, the essential mechanisms of and the molecules involved in fertilization are not unequivocally identified at present. Further research into the underlying carbohydrate-mediated ligand-receptor mechanisms in bovine would not only provide information probably creating new opportunities for optimization of the bovine embryo production system, but could also contribute to the development of a non-hormonal topical contraceptive – based on glycomolecules – in the future. The bovine species has recently gained interest as an appropriate model for human reproduction.

Since Percoll (which has been used regularly in the past to prepare human and animal spermatozoa for IVF) was withdrawn in 1996 from the market for clinical use in human assisted reproduction (because of the potentially harmful effect of the unbound polyvinylpropylene it contains and the variable levels of endotoxin contamination), new sperm preparation techniques are required. Until recently, however, most studies in domestic animals concerning density gradient centrifugation are based on products designed for human spermatozoa. Thorough assessment of species specific colloid suspensions are - hence - essential for potential scaling-up of bovine artificial reproduction techniques.

REFERENCES

Abou-Haila A, Tulsiani DR. 2009. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Arch Biochem Biophys* **485** 72–81.

Aitken RJ. 1995. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* **7** 659-668.

Alfieri JA, Martin AD, Takeda J, Kondoh G, Myles DG, Primakoff P. 2003. Infertility in female mice with an oocyte-specific knockout of GPI-anchored proteins. *J Cell Sci* **116** 2149-2155.

Almeida EA, Huovila AP, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, White JM. 1995. Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. *Cell* **81** 1095-1104.

Amari S, Yonezawa N, Mitsui S, Katsumata T, Hamano S, Kuwayama M, Hashimoto Y, Suzuki A, Takeda Y, Nakano M. 2001. Essential role of the nonreducing terminal alpha-mannosyl residues of the N-linked carbohydrate chain of bovine zona pellucida glycoproteins in sperm-egg binding. *Mol Reprod Dev* **59** 221-226.

Armant DR, Kaplan HA, Mover H, Lennarz WJ. 1986. The effect of hexapeptides on attachment and outgrowth of mouse blastocysts cultured in vitro: evidence for the involvement of the cell recognition tripeptide Arg-Gly-Asp. *Proc Natl Acad Sci U S A* **83** 6751–6755.

Avery B, Greve T. 1995. Impact of Percoll on bovine spermatozoa used for *in vitro* insemination. *Theriogenology* **44** 871-878.

Barboni B, Lucidi P, Mattioli M, Berardinelli P. 2001. VLA-6 integrin distribution and calcium signalling in capacitated boar sperm. *Mol Reprod Dev* **59** 322-329.

Barros C, Vigil P, Herrera E, Arguello B, Walker R. 1984. Selection of morphologically abnormal sperm by human cervical mucus. *Arch Androl* **12** Suppl: 95-107.

Benoff S. 1997. Carbohydrates and fertilization: an overview. *Mol Hum Reprod* **3** 599–637.

Bleil JD, Wassarman PM. 1980. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20** 873-882.

Bleil JD, Wassarman PM. 1986. Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J Cell Biol* **102** 1363–1371.

Bosch P, De Avila JM, Ellington JE, Wright RW Jr. 2001. Heparin and Ca2+-free medium can enhance release of bull sperm attached to oviductal epithelial cell monolayers. *Theriogenology* **56** 247-60.

Bronson RA, Fusi FM. 1990. Evidence that an Arg-Gly-Asp (RGD) sequence play a role in mammalian fertilization. *Biol Reprod* **43** 1019-1025.

Bronson RA, Fusi FM. 1996. Integrins and human reproduction. *Mol Hum Reprod* **2** 153–168.

Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW. 2002. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells Tissues Organs* **172** 202-217.

Burghardt RC, Burghardt JR, Taylor JD 2nd, Reeder AT, Nguen BT, Spencer TE, Bayless KJ, Johnson GA. 2009. Enhanced focal adhesion assembly reflects increased mechanosensation and mechanotransduction at maternal-conceptus interface and uterine wall during ovine pregnancy. *Reproduction* **137** 567-582.

Cesari A, Kaiser GG, Mucci N, Mutto A, Vincenti A, Fornés MW, Alberio RH. 2006. Integrated morphophysiological assessment of two methods for sperm selection in bovine embryo production in vitro. *Theriogenology* **66** 1185-1193.

Camaioni A, Salustri A, Yanagishita M, Hascall VC. 1996. Proteoglycans and proteins in the extracellular matrix of mouse cumulus cell-oocyte complexes. *Arch Biochem Biophys* **325** 190-198.

Chen H, Sampson NS. 1999. Mediation of sperm-egg fusion: evidence that mouse egg $\alpha_6\beta_1$ integrin is the receptor for sperm fertilin β. *Chem Biol* **6** 1-10.

Correa JR, Zavos PM. 1996. Preparation and recovery of frozen–thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. *Theriogenology* **46** 1225–1232.

Danguy A, Decaestecker C, Genten F, Salmon I, Kiss R. 1998. Application of lectins and neoglycoconjugates in histology and pathology. *Acta Anat* **182** 206–218.

Diaz ES, Kong M, Morales P. 2007. Effect of fibronectin on proteasome activity, acrosome reaction, tyrosine phosphorylation and intracellular calcium concentrations of human sperm. *Hum Reprod* **22** 1420–1430.

Dobrinski I, Suarez SS, Ball BA. 1996. Intracellular calcium concentration in equine spermatozoa attached to oviductal epithelial cells *in vitro*. *Biol Reprod* **54** 783-788.

Ellerman DA, Brantua VS, Martinez SP, Cohen DJ, Conesa D, Cuasnicu PS. 1998. Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability. Biol Reprod 59 1029-1036.

Erikson DW, Burghardt RC, Bayless KJ, Johnson GA. 2009. Secreted Phosphoprotein 1 (SPP1, Osteopontin) binds to integrin alphavbeta6 on porcine trophectoderm cells and integrin alphavbeta3 on uterine luminal epithelial cells, and promotes trophectoderm cell adhesion and migration. *Biol Reprod* DOI:10.1095/biolreprod.109.078600.

Florman HM, Wassarman PM. 1985. *O*-Linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* **41** 313–324.

Fusi FM, Bronson RA. 1992. Sperm surface fibronectin. Expression following capacitation. *J Androl* **13** 28-35.

Fusi FM, Tamburini C, Mangili F, Montesano M, Ferrari A, Bronson RA. 1996a. The expression of alpha v, alpha 5, beta 1, and beta 3 integrin chains on ejaculated human spermatozoa varies with their functional state. *Mol Hum Reprod* **2** 169–175.

Fusi FM, Bernocchi N, Ferrari A, Bronson RA. 1996b. Is vitronectin the velcro that binds the gametes together? *Mol Hum Reprod* **2** 859–866.

Gabius H-J. 1997. Animal lectins. *Eur J Biochem* **243** 543-576.

Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW. 2002. Analysis of osteopontin at the maternaleplacental interface in pigs. *Biol Reprod* **66** 718-725.

Glander H-J, Schaller A, Rohwedder A, Henkel R. 1998. Adhesion molecules and matrix proteins on human spermatozoa. *Andrologia* **30** 289–296.

Gonçalves RF, Chapman DA, Bertolla RP, Eder I, Killian GJ. 2008. Pre-treatment of cattle semen or oocytes with purified milk osteopontin affects in vitro fertilization and embryo development. *Anim Reprod Sci* **108** 375-383.

Gougoulidis T, Trounson A, Dowsing A. 1999. Inhibition of bovine sperm-oocyte fusion by the carbohydrate GalNAc. *Mol Reprod Dev* **54** 179-185.

Gualtieri R, Mollo V, Duma G, Talevi R. 2009. Redox control of surface protein sulphhydryls in bovine spermatozoa reversibly modulates sperm adhesion to the oviductal epithelium and capacitation. *Reproduction* **138** 33-43.

Gwathmey TM, Ignotz GG, Suarez SS. 2003. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir. *Biol Reprod* **69** 809-815.

Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* **75** 501-507.

Han C, Choi E, Park I, Lee B, Jin S, Kim do H, Nishimura H, Cho C. 2009. Comprehensive analysis of reproductive ADAMs: relationship of ADAM4 and ADAM6 with an ADAM complex required for fertilization in mice. *Biol Reprod* **80** 1001-1008.

Holt WV. 2009. Is semen analysis useful to predict the odds that the sperm will meet the egg? *Reprod Domest Anim* **44** (Suppl 3) 31-38.

Hunter RHF, Wilmut I. 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. *Reprod Nutr Dev* **24** 597–608.

Hunter RH, Fléchon B, Fléchon JE. 1991. Distribution, morphology and epithelial interactions of bovine spermatozoa in the oviduct before and after ovulation: a scanning electron microscope study. *Tissue Cell* **23** 641-656.

Hunter RH. 2003. Advances in deep uterine insemination: a fruitful way forward to exploit new sperm technologies in cattle. *Anim Reprod Sci* **79** 157-170.

Ignotz GG, Lo MC, Perez CL, Gwathmey TM, Suarez SS. 2001. Characterization of a fucose-binding protein from bull sperm and seminal plasma that may be responsible for formation of the oviductal sperm reservoir. *Biol Reprod* **64** 1806-1811.

Illera MJ, Cullinan E, Gui Y, Yuan L, Beyler SA, Lessey BA. 2000. Blockade of the $\alpha_v\beta_3$ integrin adversely affects implantation in the mouse. *Biol Reprod* **62** 1285-1290.

Inoue N, Ikawa M, Isotani A, Okabe M. 2005. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* **434** 234-238.

Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. 1999. Ovine osteopontin: II. Osteopontin and $\alpha_v\beta_3$ integrin expression in the uterus and conceptus during the periimplantation period. *Biol Reprod* **61** 892-899.

Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC. 2001. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. *Biol Reprod* **65** 820-828.

Johnson GA, Burghardt RC, Bazer FW, Spencer TE. 2003a. Osteopontin: roles in implantation and placentation. *Biol Reprod* **69** 1458-1471.

Johnson GA, Burghardt RC, Joyce MM, Spencer TE, Bazer FW, Gray CA, Pfarrer C. 2003b. Osteopontin is synthesized by uterine glands and a 45-kDa cleavage fragment is localized at the uterine-placental interface throughout ovine pregnancy. *Biol Reprod* **69** 92-98.

Joyce MM, González JF, Lewis S, Woldesenbet S, Burghardt RC, Newton GR, Johnson GA. 2005. Caprine uterine and placental osteopontin expression is distinct among epitheliochorial implanting species. *Placenta* **26** 160-170.

Kanai S, Kitayama T, Yonezawa N, Sawano Y, Tanokura M, Nakano M. 2008. Disulfide linkage patterns of pig zona pellucida glycoproteins ZP3 and ZP4. *Mol Reprod Dev* **75** 847-856.

Katz DF, Morales P, Samuels SJ, Overstreet JW. 1990. Mechanisms of filtration of morphologically abnormal human sperm by cervical mucus. *Fertil Steril* **54** 513-516.

Lapointe S, Sullivan R, Sirard MA. 1998. Binding of a bovine oviductal fluid catalase to mammalian spermatozoa. *Biol Reprod* **58** 747-753.

Lefebvre R, Lo MC, Suarez SS. 1997. Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biol Reprod* **56** 1198–1204.

López-Gatius F. 2000. Site of semen deposition in cattle: a review. *Theriogenology* **53** 1407-1414.

Lorton SP, First NL. 1979. Hyaluronidase does not disperse the cumulus oophorus surrounding bovine ova. *Biol Reprod* **21** 301-308.

Macpherson M, Blanchard TL, Love CC, Brinsko SP, Thompson JA, Varner DD. 2002. Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. *Theriogenology* **58** 317–320.

Malette B, Paquette Y, Merlen Y, Bleau G. 1995. Oviductins possess chitinase-and mucin-like domains: a lead in the search for the biological function of these oviduct-specific ZP-associating glycoproteins. *Mol Reprod Dev* **41** 384–397.

Manjunath P, Lefebvre J, Jois PS, Fan J, Wright MW. 2009. New nomenclature for mammalian BSP genes. *Biol Reprod* **80** 394-397.

Mattioli M, Lucidi P, Barboni B. 1998. Expanded cumuli induce acrosome reaction in boar sperm. *Mol Reprod Dev* **51** 445-453.

Maxwell WMC, Parrilla I, Caballero I, Garcia E, Roca J, Martinez EA, Vazquez JM, Rath D. 2007. Retained functional integrity of bull spermatozoa after double freezing and thawing using PureSperm® density gradient centrifugation. *Reprod Domest Anim* **42** 489–494.

Miller BJ, Georges-Labouesse E, Primakoff P, Myles DG. 2000. Normal fertilization occurs with eggs lacking the integrin $\alpha_6\beta_1$ and is CD9-dependent. *J Cell Biol* **149** 1289-1296.

Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A, Okabe M, Mekada E. 2000. Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287** 321-324.

Monaco E, Gasparrini B, Boccia L, De Rosa A, Attanasio L, Zicarelli L, Killian G. 2009. Effect of osteopontin (OPN) on *in vitro* embryo development in cattle. *Theriogenology* **71** 450-457.

Morrell JM. 2006. Update on semen technologies for animal breeding. *Reprod Domest Anim* **41** 63–67.

Morrell JM, Geraghty RJ. 2006. Effective removal of equine arteritis virus from stallion semen. *Equine Vet J* **38** 224–229.

Mortillo S, Wassarman PM. 1991. Differential binding of gold-labeled zona pellucida glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. *Development* **113** 141–149.

Mortimer D. 2000. Sperm preparation methods. *J Androl* **21** 357-366.

Mullins KJ, Saacke RG. 1989. Study of the functional anatomy of bovine cervical mucosa with special reference to mucus secretion and sperm transport. *Ana. Rec* **225**, 106–117.

Mykles DL. 1998. Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. *Int Rev Cytol* **184** 157–289.

Nauc V, Manjunath P. 2000. Radioimmunoassays for bull seminal plasma proteins (BSP-A1/-A2, BSP-A3, and BSP-30-kilodaltons), and their quantification in seminal plasma and sperm. *Biol Reprod* **63** 1058–1066.

Nishimura H, Cho C, Branciforte DR, Myles DG, Primakoff P. 2001. Analysis of loss of adhesive function in sperm lacking cyritestin or fertilin beta. *Dev Biol* **233** 204-213.

Nixon B, Aitken RJ, McLaughlin EA. 2007. New insights into the molecular mechanisms of sperm–egg interaction. *Cell Mol Life Sci* **64** 1805–1823.

Oehninger S, Mahony MC, Swanson JR, Hodgen GD. 1993. The specificity of human spermatozoa/zona pellucida interaction under hemizona assay conditions. *Mol Reprod Dev* **35** 57–61.

Okabe M, Yagasaki M, Oda H, Matzno S, Kohama Y, Mimura T. 1988. Effect of a monoclonal anti-mouse sperm antibody (OBF13) on the interaction of mouse sperm with zona-free mouse and hamster eggs. *J Reprod Immunol* **13** 211-219.

Parrish JJ, Foote RH. 1987. Quantification of bovine sperm separation by a swim-up method. Relationship to sperm motility, integrity of acrosomes, sperm migration in polyacrylamide gel and fertility. *J Androl* **8** 259–266.

Parrish JJ, Susko-Parrish J, Winer MA, First NL. 1988. Capacitation of bovine sperm by heparin. *Biol Reprod* **38** 1171-1180.

Parrish JJ, Krogenaes A, Susko-Parrish JL. 1995. Effect of bovine sperm separation by either swim up or Percoll method on success of *in vitro* fertilization and early embryonic development. *Theriogenology* **44** 859-869.

Pollard JW, Plante C, King WA, Hansen PJ, Betteridge KJ, Suarez SS. 1991. Fertilizing capacity of bovine sperm may be maintained by binding to oviductal epithelial cells. *Biol Reprod* **44** 102–107.

Primakoff P, Myles DG. 2002. Penetration, adhesion, and fusion in mammalian sperm-egg interaction. *Science* **296** 2183-2185.

Revah I, Suarez SS, Flesch FM, Colenbrander B, Gadella BM. 2000. Changes in capacity of bull sperm to bind fucose. *Biol Reprod* **62** 1010–1015.

Rodriguez-Martinez H, Larsson B, Pertoft H. 1997. Evaluation of sperm damage and techniques for sperm clean-up. *Reprod Fertil Dev* **9** 297-308.

Rodriguez-Martinez H. 2007. Role of the oviduct in sperm capacitation. *Theriogenology* **68** Suppl 1 S138-S146.

Rubinstein E, Ziyyat A, Prenant M, Wrobel E, Wolf JP, Levy S, Le Naour F, Boucheix C. 2006. Reduced fertility of female mice lacking CD81. *Dev Biol* **290** 351-358.

Sabeur K, Cherr GN, Yudin AI, Overstreet JW. 1998. Hyaluronic acid enhances induction of the acrosome reaction of human sperm through interaction with the PH-20 protein. *Zygote* **6** 103-111.

Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG, Bianchi U. 2000. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Hum Reprod* **15** 1112-1116.

Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J, Karadjole T. 2006a. A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. *Anim Reprod Sci* **91** 237–247.

Samardzija M, Karadjole M, Getz I, Makek Z, Cergolj M, Dobranic T. 2006b. Effects of bovine spermatozoa preparation on embryonic development *in vitro*. *Reprod Biol Endocrinol* **4** 58.

Seidah NG, Manjunath P, Rochemont J, Sairam MR, Chrétien M. 1987. Complete amino acid sequence of BSP-A3 from bovine seminal plasma. Homology to PDC-109 and to the collagen-binding domain of fibronectin. *Biochem J* **243** 195-203.

Shamsuddin M., Rodriguez-Martinez H. 1994. A simple, non-traumatic swim-up method for the selection of spermatozoa for *in vitro* fertilization in the bovine. *Anim Reprod Sci* **36** 61–75.

Sherman JK, Paulson JD, Liu KC. 1981. Effect of glass wool filtration on ultrastructure of human spermatozoa. *Fertil Steril* **36** 643–650.

Shoukir Y, Chardonnens D, Campana A, Sakkas D. 1998. Blastocyst development from supernumerary embryos after intracytoplasmic sperm injection: a paternal influence? *Hum Reprod* **13** 1632-1637.

Shur BD. 1998. Is sperm galactosyltransferase a signaling subunit of a multimeric gamete receptor? *Biochem Biophys Res Commun* **250** 537-543.

Shur BD, Rodeheffer C, Ensslin MA, Lyng R, Raymond A. 2006. Identification of novel gamete receptors that mediate sperm adhesion to the egg coat. *Mol Cell Endocrinol* **250** 137 – 148.

Sieren KR, Youngs CR. 2001. Evaluation of Bovipure™ for *in vitro* production of bovine embryos. Theriogenology **55** 438.

Sinowatz F, Wessa E, Neumüller C, Palma G. 2003. On the species specificity of sperm binding and sperm penetration of the zona pellucida. *Reprod Domest Anim* **38** 141-146.

Somfai T, Bodó S, Nagy S, Papp AB, Iváncsics J, Baranyai B, Gócza E, Kovács A. 2002. Effect of swim up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. *Reprod Domest Anim* **37** 285-290.

Sostaric E, van de Lest CH, Colenbrander B, Gadella BM. 2005. Dynamics of carbohydrate affinities at the cell surface of capacitating bovine sperm cells. *Biol Reprod* **72** 346-357.

Spencer TE, Johnson GA, Bazer FW, Burghardt RC. 2004. Implantation mechanisms: insights from the sheep. *Reproduction* **128** 657-668.

Suarez SS, Brockman K, Lefebvre R. 1997. Distribution of mucus and sperm in bovine oviducts after artificial insemination. *Biol Reprod* **56** 447–453.

Suarez SS. 2001. Carbohydrate-mediated formation of the oviductal sperm reservoir in mammals. *Cells Tissues Organs* **168** 105-112.

Suarez SS. 2002. Formation of a reservoir of sperm in the oviduct. *Reprod Domest Anim* **37** 140-143.

Suarez SS, Pacey AA. 2006. Sperm transport in the female reproductive tract. *Hum Reprod Update* **12** 23-37.

Suarez SS. 2007. Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. *Reprod Fertil Dev*. **19** 103-110.

Suarez SS. 2008. Regulation of sperm storage and movement in the mammalian oviduct. *Int J Dev Biol* **52** 455-462.

Sutherland AE, Calarco PG, Damsky CH. 1993. Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* **119** 1175–1186.

Talbot P, Shur BD, Myles DG. 2003. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. *Biol Reprod* **68** 1-9.

Talevi R, Gualtieri R. 2001. Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium in vitro. *Biol Reprod* **64** 491-498.

Tanghe S, Van Soom A, Sterckx V, Maes D, de Kruif A. 2002a. Assessment of different sperm quality parameters to predict in vitro fertility of bulls. *Reprod Domest Anim* **37** 127-132.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. 2002b. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* **61** 414–424.

Tanghe S, Van Soom A, Mehrzad J, Maes D, Duchateau L, de Kruif A. 2003. Cumulus contributions during bovine fertilization *in vitro*. *Theriogenology* **60** 135-149.

Tanghe S, Van Soom A, Duchateau L, De Kruif A. 2004a. Inhibition of bovine sperm-oocyte fusion by the p-aminophenyl derivative of D-mannose. *Mol Reprod Dev* **67** 224-232.

Tanghe S, Van Soom A, Duchateau L, Nauwynck H, de Kruif A. 2004b. Carbohydrates and glycoproteins involved in bovine fertilization *in vitro*. *Mol Reprod Dev* **68** 492-499.

Töpfer-Petersen E. 1999a. Molecules on the sperm's route to fertilization. *J Exp Zool* **285** 259-266.

Töpfer-Petersen E. 1999b. Carbohydrate-based interactions on the route of a spermatozoon to fertilization. *Hum Reprod Update* **5** 314-329.

Tulsiani DR, Yoshida-Komiya H, Araki Y. 1997. Mammalian fertilization: a carbohydrate-mediated event. *Biol Reprod* **57** 487–494.

Vjugina U, Evans JP. 2008. New insights into the molecular basis of mammalian sperm–egg membrane interactions. *Front Biosci* **13** 462–476.

Voorhees PM, Orlowski RZ. 2006. The proteasome and proteasome inhibitors in cancer therapy. *Annu Rev Pharmacol Toxicol* **46** 189-213.

Wassarman PM. 1988. Zona pellucida glycoproteins. *Ann Rev Biochem* **57** 415–442.

Weintraub AS, Lin X, Itskovich VV, Aguinaldo JG, Chaplin WF, Denhardt DT, Fayad ZA. 2004. Prenatal detection of embryo resorption in osteopontin-deficient mice using serial noninvasive magnetic resonance microscopy. *Pediatr Res* **55** 419-424.

Whittington K, Ford WC. 1999. Relative contribution of leukocytes and of spermatozoa to reactive oxygen species production in human sperm suspensions. *Int J Androl* **22** 229-235.

World Health Organisation. 1999. WHO laboratory manual for the analysis of human semen and sperm–cervical mucus interaction. 4th edn, Cambridge University Press, Cambridge, UK.

Wrobel K-H, Kujat R, Fehle G. 1993. The bovine tubouterine junction: general organization and surface morphology. *Cell Tissue Res* **271** 227–239.

Yániz JL, Lopez-Gatius F, Santolaria P, Mullins KJ. 2000. Study of the functional anatomy of bovine oviductal mucosa. *Anat Rec* **260**, 268–278.

Yin L, Chung CM, Huo R, Liu H, Zhou C, Xu W, Zhu H, Zhang J, Shi Q, Wong HY, Chen J, Lu Y, Bi Y, Zhao C, Du Y, Ma M, Cai Y, Chen WY, Fok KL, Tsang LL, Li K, Ni Y, Chung YW, Zhou Z, Sha J, Chan HC. 2009. A sperm GPI-anchored protein elicits sperm-cumulus cross-talk leading to the acrosome reaction. *Cell Mol Life Sci* **66** 900-908.

Yonezawa N, Fukui N, Kuno M, Shinoda M, Goko S, Mitsui S, Nakano M. 2001. Molecular cloning of bovine zona pellucida glycoproteins ZPA and ZPB and analysis for sperm-binding component of the zona. *Eur J Biochem* **268** 3587 –3594.

Yuan R, Primakoff P, Myles DG. 1997. A role for the disintegrin domain of cyritestin, a sperm surface protein belonging to the ADAM family, in mouse sperm-egg plasma membrane adhesion and fusion. *J Cell Biol* **137** 105-112.

Ziyyat A, Rubinstein E, Monier-Gavelle F, Barraud V, Kulski O, Prenant M, Boucheix C, Bomsel M, Wolf JP. 2006. CD9 controls the formation of clusters that contain tetraspanins and the integrin alpha 6 beta 1, which are involved in human and mouse gamete fusion. *J Cell Sci* **119** 416 – 424.

CHAPTER 2

AIMS OF THE STUDY

It has commonly been recognized that carbohydrates and glycoproteins are involved in several reproductive processes such as sperm-oviduct adhesion, sperm-oocyte interaction and embryo implantation. Previous research identifying which carbohydrate complexes can actually interfere with bovine *in vitro* fertilization (IVF) demonstrated that supplementation of fibronectin (Fn) and vitronectin (Vn) – both extracellular matrix glycoproteins – to a certain extent inhibited sperm penetration of *in vitro* matured cumulus-oocyte-complexes. Both glycomolecules are ubiquitously present in body fluids and extracellular matrices of a variety of animal species and are involved in diverse cell-to-cell and cell-to-matrix adhesion processes (such as blood clotting, wound healing, cell migration and embryonic development). Accordingly, these glycoconjugates represent interesting candidates for further research to explore which fertilization step(s) are exactly affected by their presence and to locate the carbohydrate receptor sites and corresponding ligands on male and female gametes (**Chapter 3 and 4**). Elucidation of the molecular basis underlying their inhibitory effect during bovine IVF might contribute to a better understanding of the fertilization process and eventually to further optimization of the bovine *in vitro* embryo production system.

In order to obtain adequate results *in vitro*, it is required to select for viable sperm cells prior to IVF, since thorough sperm selection normally occurs in the female reproductive tract. Furthermore, cryopreservation detrimentally affects the metabolism, membranes and motility of spermatozoa. Since Percoll was withdrawn in 1996 from the market for clinical use in human assisted reproduction (because of the potentially harmful effect of the unbound polyvinylpropylene it contains and the variable levels of endotoxin contamination), new sperm preparation techniques are required. Therefore, a new *in vitro* sperm selection technique was compared with the conventional Percoll gradient centrifugation (**Chapter 5**). This new sperm selection technique involves one single layer of a species-specific colloid suspension. Compared to the double layer (of different density) required for Percoll centrifugation, this technique can provide a technically simplified way to scale-up the standard IVF-procedure. The single-layer technique would allow faster selection of large numbers of viable sperm to be used for artificial insemination (AI) or IVF.

The specific scientific aims of the present thesis were:

1. to assess the expression of endogenous Fn and its receptor (integrin $\alpha_5\beta_1$) in bovine male and female gametes (**Chapter 3**),
2. to elucidate the role of endogenous Fn during bovine fertilization (**Chapter 3**),
3. to map the expression of endogenous Vn and its receptor (integrin $\alpha_v\beta_3$) in bovine male and female gametes (**Chapter 4**),
4. to explore the function of endogenous Vn during bovine fertilization (**Chapter 4**),
5. to evaluate the *in vitro* fertilizing capacity of frozen-thawed bull spermatozoa selected by single-layer (glycerolpropylsilane, GS) silane-coated silica colloidal centrifugation (**Chapter 5**).

CHAPTER 3

EXPRESSION AND PUTATIVE FUNCTION OF FIBRONECTIN AND ITS RECEPTOR (INTEGRIN $\alpha_5\beta_1$) IN MALE AND FEMALE GAMETES DURING BOVINE FERTILIZATION *IN VITRO*

Modified from

EXPRESSION AND PUTATIVE FUNCTION OF FIBRONECTIN AND ITS RECEPTOR (INTEGRIN $\alpha_5\beta_1$) IN MALE AND FEMALE GAMETES DURING BOVINE FERTILIZATION *IN VITRO*.

Thys M., Nauwynck H., Maes D., Hoogewijs M., Vercauteren D., Rijsselaere T., Favoreel H., Van Soom A.

Reproduction 2009; **138** 471-482.

ABSTRACT

Fibronectin (Fn) is a 440 kDa glycoprotein assumed to participate in sperm-egg interaction in human. Recently, it has been demonstrated that Fn - when present during bovine *in vitro* fertilization (IVF) - strongly inhibits sperm penetration. The present study was conducted 1) to evaluate the expression of Fn and its integrin receptor ($\alpha_5\beta_1$) on male and female bovine gametes using indirect immunofluorescence and 2) to determine the function of Fn during bovine IVF. Endogenous Fn was detected underneath the zona pellucida (ZP) and integrin subunit α_5 on the oolemma of cumulus-denuded oocytes. Bovine spermatozoa displayed integrin subunit α_5 at their equatorial segment after acrosome reaction. We established that the main inhibitory effect of exogenously supplemented Fn was located at the sperm-oolemma binding, with a (concurrent) effect on fusion, and this can probably be attributed to the binding of Fn to spermatozoa at the equatorial segment, as shown by means of AlexaFluor®488-conjugated Fn. Combining these results, the inhibitory effect of exogenously supplemented Fn seemed to be exerted on the male gamete by binding to the exposed integrin $\alpha_5\beta_1$ receptor after acrosome reaction. The presence of endogenous Fn underneath the ZP together with integrin subunit α_5 expression on oolemma and acrosome reacted sperm cell surface, suggests a reversible adhesive interaction between the endogenous Fn ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating sperm-egg binding.

INTRODUCTION

Fertilization is the union of a single sperm cell and an oocyte, and involves a complex series of molecular interactions in order to be successful (Benoff, 1997). Although a considerable amount of knowledge has been gained especially in human and mouse (Almeida et al., 1995; Bronson and Fusi, 1996; Fusi et al., 1996*a*; Fusi et al., 1996*b*; Nixon et al., 2007; Vjugina and Evans, 2008), the exact mechanisms of and the molecules involved in bovine fertilization are not unequivocally identified at present. Carbohydrates and glycoproteins are assumed to modulate adhesion and binding events during consecutive reproductive processes, like sperm-oviduct adhesion (Lefebvre et al., 1997; Suarez et al., 1998; Revah et al., 2000; Talevi and Gualtieri, 2001; Sostaric et al., 2005; Ignotz et al., 2007), sperm-oocyte interactions (Tulsiani et al., 1997; Gougoulidis et al., 1999; Amari et al., 2001; Tanghe et al., 2004*a*; 2004*b*) and embryo implantation (Whyte and Allen, 1985; Biermann et al., 1997).

In order to successfully fertilize an oocyte, sperm cells must overcome several barriers in the female genital tract. Besides escaping mechanical entrapment in the cervical folds, spermatozoa have to reversibly bind the oviductal epithelium (resulting in the formation of a functional sperm reservoir) ensuring that fit sperm cells reach the oocyte at the right time. One of the final hurdles is the extracellular cumulus matrix surrounding the mammalian oocyte. This matrix is - like other extracellular matrices - composed of a variety of molecules among which adhesive glycoproteins such as laminin, fibronectin and collagen type IV (Diaz et al., 2007).

Fibronectin (Fn) is a dimeric filament-forming 440 kDa glycoprotein consisting of two similar 200-250-kDa subunits connected by disulfide bridges (McKeown-Longo and Mosher, 1984; Fusi and Bronson, 1992; Wennemuth et al., 2001). It is present in a soluble form in plasma and other body fluids, and in an insoluble (cellular/fibrillar) form in the fibrin clot, the loose connective tissue and basement membranes (McKeown-Longo and Mosher, 1984; Fusi and Bronson, 1992; Olorundare et al., 2001). The Fn molecule displays several binding sites for binding to heparin/heparan sulfate, fibrin, collagen and receptors expressed on the cell surface, rendering the glycoprotein the possibility to mediate a broad variety of biological functions (Lyon et al., 2000; Wennemuth et al., 2001). Fibronectin is consequently involved in diverse processes, such as blood clotting (through binding fibrin to form a plug preventing further blood loss), wound healing (through interaction with collagen), cell migration, phagocytosis, embryonic development, malignant transformation, metastasis, cell-to-cell and cell-to-matrix adhesion (Mosher, 1984; Ruoslahti and Pierschbacher, 1986; Fusi and Bronson, 1992; Akiyama et al., 1995; Sandeman et al., 2000; Midwood et al., 2004). Binding of Fn to the cell surface is mediated by integrins, which are transmembrane protein receptors recognizing the Arg-Gly-Asp (RGD) amino acid sequence in Fn (Ruoslahti and Pierschbacher, 1987; Fusi and Bronson, 1992; Fusi et al., 1992).

Involvement of Fn and its $\alpha_5\beta_1$ integrin receptor in *in vitro* fertilization (IVF) has previously been demonstrated in several species. Oligopeptides containing the RGD integrin-binding sequence inhibit binding of human and hamster spermatozoa to zona-free hamster eggs (Bronson and Fusi, 1996). Furthermore, human spermatozoa express Fn on their surface following capacitation (Bronson and Fusi, 1996), and Fn is also secreted during cumulus expansion (Sutovsky et al., 1995; Relucenti et al., 2005). The Fn receptor integrin $\alpha_5\beta_1$ is detected on both male and female human gametes (Fusi et al., 1993; Bronson and Fusi, 1996), suggesting a possible role of the glycoprotein in sperm-egg interaction in human. Although Tanghe et al. (2004*b*) already established that Fn - when present during bovine IVF - causes a

high inhibition of sperm penetration, the exact functions of Fn and its receptor in the bovine fertilization process remained unclear. Therefore, the present study was conducted 1) to evaluate the expression of Fn and its integrin receptor ($\alpha_5\beta_1$) on male and female gametes in bovine, and 2) to determine the function of Fn during bovine IVF.

MATERIALS AND METHODS

Oocyte and semen preparation

Oocytes were derived from bovine ovaries randomly collected at a local abattoir and prepared following the protocol of Tanghe et al. (2004*b*). Frozen-thawed bull semen from the same ejaculate was used for all inhibition experiments. Straws were thawed in a water bath (37°C) for 60 s. Subsequently, the semen was centrifuged on a discontinuous Percoll gradient (90% and 45%; Pharmacia, Uppsala, Sweden) as described by Thys et al. (2009).

Chemicals and media were analogous to those used by Tanghe et al. (2004*a*; for details see Addendum). Fibronectin from bovine plasma (F4759) used in all experiments was purchased from Sigma-Aldrich (Bornem, Belgium).

Localization of Fn on female and male bovine gametes

With respect to the female bovine gamete, immature COCs, *in vitro* matured COCs, cumulus-denuded oocytes as well as zona-free oocytes were sampled following the protocol of Tanghe et al. (2004*a*). Cumulus cells were removed mechanically by vortexing (8 min), and the zona pellucida (ZP) was dissolved by incubation of the cumulus-denuded oocytes in 0.1% (w/v) protease (P5147, Sigma-Aldrich, Bornem, Belgium) in phosphate-buffered saline (PBS) for 5 to 15 min. As a positive control, a cumulus monolayer was grown *in vitro* to confirm the presence of Fn in bovine cumulus cells (following the protocol described by Vandaele et al. (2007)). All female gamete samples were fixed with 4% (w/v) paraformaldehyde (P6118, Sigma-Aldrich, Bornem, Belgium) in PBS for 1 h (4°C) and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min at room temperature (RT), except half of the cumulus-denuded oocytes (which were processed without permeabilization). Subsequently, they were incubated with 10% (v/v) goat serum (16210-064, Invitrogen, Merelbeke, Belgium) in polyvinyl pyrrolidone (PVP, 0.1% (w/v) in PBS) solution for 30 min (37°C), with mouse monoclonal antibody [A17] to Fibronectin (Abcam, Cambridge, UK) (1/100) for 1 to 2 h (37°C) and with goat-anti-mouse FITC antibody

(Molecular Probes, Leiden, The Netherlands) (1/100) for 1 h (37°C). To stain the nuclei, all oocyte types were treated with 2% (v/v) Propidium Iodide (Molecular Probes, Leiden, The Netherlands) in PBS for 30 min. Between each treatment the samples were washed in PVP. They were mounted in a droplet of glycerol with (25 mg/ml) 1,4-diazabicyclo (2.2.2) octane (DABCO; Acros, Ghent, Belgium) and evaluated for the presence of Fn using a Leica DM/RBE fluorescence microscope (Leica Microsystems, Groot-Bijgaarden, Belgium) and a Nikon C1 confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels, Belgium) equipped with a Plan Apo 40x 0.95 NA air objective and suitable optical elements to acquire differential interference contrast (DIC) transmission images.

To assess the presence of Fn on the male gamete, frozen-thawed semen was centrifuged on a discontinuous Percoll gradient. Next, the sample was split into 3 fractions. Fraction 1 was diluted to a concentration of $10x10^6$ sp/ml (with medium consisting of a HEPES-buffered Tyrode balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg/ml gentamycin sulphate) prior to indirect immunofluorescence, and represented non-treated (NT) sperm. Fraction 2 and 3 were diluted to a concentration of $5x10^6$ sp/ml (with medium containing Tyrode balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg/ml gentamycin sulphate, 6 mg/ml fatty acid-free BSA, and 20 µg/ml heparin) and subsequently incubated for 30 min (39°C; 5% CO_2) to induce capacitation. Then, fraction 2 (representing capacitated – CAP – sperm) was processed in the same way as fraction 1. Fraction 3 was supplemented with 100 µg/ml lysophosphatidyl choline (LPC; L5004, Sigma-Aldrich, Bornem, Belgium) and incubated for 15 min (39°C; 5% CO_2) in order to induce the acrosome reaction (acrosome reacted – AR – sperm). All three sperm fractions were fixed with 1% (w/v) paraformaldehyde (in PBS) for 30 min (at 4°C) and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min (at RT). Subsequently, they were incubated with 10% (v/v) goat serum in PVP for 30 min (37°C), with mouse monoclonal antibody [A17] to Fibronectin (Abcam, Cambridge, UK) (1/100) for 1 to 2 h (37°C) and with goat-anti-mouse FITC antibody (Molecular Probes, Leiden, The Netherlands) (1/100) for 1 h (37°C). To stain the nuclei, all sperm fractions were treated with 10 µg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) for 10 min (RT). Between each treatment the sperm fractions were centrifuged (10 min, 200g) and re-suspended in PVP. They were mounted in glycerol with 25 mg/ml DABCO and evaluated for the presence of Fn using fluorescence microscopy

(Olympus IX81 inverted fluorescence microscope and a Hamamatsu Orca B/W camera using Olympus Cell*R software, Aartselaar, Belgium). A second set of samples was processed without fixation and permeabilization to check if potential membrane expression altered due to these treatments. The latter samples were processed on ice. To assess the efficacy of sperm capacitation, the chlortetracycline staining was used (as described by Fraser *et al.* 1993).

To evaluate the specificity of the mouse monoclonal antibody [A17] to Fibronectin (Abcam, Cambridge, UK), a negative control was included: a sample incubated with an isotype-matched mouse IgG_1 antibody prior to the FITC-labeled secondary antibody treatment. To check for unspecific binding of the FITC-labeled secondary antibody, an additional negative control was prepared: a sample processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody.

Localization of the α_5 subunit of the Fn-receptor ($\alpha_5\beta_1$ integrin) on female and male bovine gametes

With respect to the female bovine gamete, *in vitro* matured cumulus-denuded oocytes were sampled (as described above) and fixed with 2% (w/v) paraformaldehyde in PBS for 30 min (4°C) prior to indirect immunofluorescence.

To assess the presence of α_5 on the male gamete, frozen-thawed semen originating from the same ejaculate was centrifuged on a discontinuous Percoll gradient, and the sperm pellet was diluted to a concentration of $10x10^6$ sp/ml. Subsequently, the sample was split into 3 fractions. Each fraction was processed as described before, resulting in a non-treated (NT), capacitated (CAP) and acrosome reacted (AR) sperm fraction. All sperm samples were fixed with ice-cold methanol during 15 min.

This time, the primary antibody used was rabbit anti-human polyclonal antibody to CD49e/integrin subunit α_5 (USBioLogical, Swampscott, MA, USA) (1/100), which was fluorescently labeled with goat anti-rabbit FITC antibody (Molecular Probes, Leiden, The Netherlands) (1/100). To evaluate the specificity of the rabbit anti-human polyclonal antibody to CD49e/integrin subunit α_5 (USBioLogical), a negative control was included: a sample incubated with heat-inactivated rabbit serum prior to the FITC-labeled secondary antibody treatment. To check for unspecific binding of the FITC-labeled secondary antibody, an

additional negative control was prepared: a sample processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody.

Dose-response effect of Fn on sperm penetration after bovine IVF

To reconfirm whether the Fn concentration (500 nM) applied in the study of Tanghe *et al.* (2004b) was the most appropriate, a preliminary experiment was conducted to assess the dose-respons effect of Fn on sperm penetration after bovine IVF. *In vitro* matured COCs were randomly assigned to 6 different fertilization media (Tanghe *et al.* 2004*a*): fertilization medium supplemented with 0 nM, 10 nM, 100 nM, 250 nM, 500 nM and 1 µM Fn. The oocytes were co-incubated with sperm at a final concentration of 10^6 spermatozoa/ml for 20 h (39°C; 5% CO_2). Prior to overnight fixation (2% (w/v) paraformaldehyde – 2% (v/v) glutaraldehyde in PBS) and staining with 10 µg/ml Hoechst 33342 (Molecular Probes) for 10 min, the presumed zygotes were vortexed for 3 min to remove excess spermatozoa and/or cumulus cells. Zygotes were mounted in glycerol with 25 mg/ml DABCO and evaluated using a Leica DMR fluorescence microscope (Leica Microsystems Belgium). Penetration percentage was defined as the sum of fertilization (presence of two pronuclei) and polyspermy (more than two pronuclei) percentage.

Effect of Fn on sperm penetration of the cumulus oophorus

In vitro matured COCs were randomly assigned to 4 groups (3 replicates). Half of the oocytes were denuded by vortexing for 8 min (cumulus denuded or CD) and the other half was kept cumulus-enclosed (CE). Both CD and CE oocytes were fertilized under control conditions (in standard fertilization medium) or in the presence of 500 nM Fn. The oocytes were coincubated with sperm at a final concentration of 10^6 sp/ml for 20 h (39°C; CO_2). Prior to overnight fixation (2% (w/v) paraformaldehyde – (v/v) 2% glutaraldehyde in PBS) and staining with 10 µg/ml Hoechst 33342 for 10 min, the presumed zygotes were vortexed for 3 min to remove excess spermatozoa. Zygotes were mounted in glycerol with 25mg/ml DABCO and sperm penetration was evaluated using a Leica DMR fluorescence microscope (Leica Microsystems Belgium).

Effect of Fn on sperm-zona binding

In vitro matured COCs were denuded by vortexing and randomly assigned to two groups (4 replicates). The first group was fertilized under control conditions and the second

group in the presence of 500 nM Fn. The oocytes were coincubated with sperm at a final concentration of 10^5 sp/ml. After 4 h of coincubation (Fazeli et al., 1993), the oocytes were washed 3 times to remove loosely attached spermatozoa, and subsequently fixed and stained (as described in the previous experiment). Per presumed zygote, the number of spermatozoa bound to the ZP was determined.

Effect of Fn on sperm-oolemma binding and fusion

The sperm pellet – obtained after Percoll centrifugation – was diluted with fertilization medium to a concentration of 5 x 10^5 sp/ml and incubated for 30 min (39°C; 5% CO_2) to allow capacitation of the sperm cells. Subsequently, the acrosome reaction was induced by incubation of the sperm suspension for 15 min in 100 µg/ml LPC (39°C; 5% CO_2) (Tanghe *et al.* 2004*a*).

After *in vitro* maturation of COCs, the cumulus oophorus was removed mechanically from the COCs by vortexing (8 min). Subsequently, the cumulus-denuded oocytes were incubated in 0.1% (w/v) protease in PBS for 5 to 15 min in order to dissolve their ZP. The ZP-free oocytes (3 replicates) were randomly assigned to two different media: standard fertilization medium or fertilization medium supplemented with 500 nM Fn. The female gametes were coincubated with sperm at a final concentration of 2.5 x 10^5 sp/ml in 50 µl droplets of medium (10 oocytes/droplet) covered with paraffin oil. One hour after insemination half of the oocytes from each group were washed 3 times to remove loosely attached spermatozoa, fixed and stained with Hoechst 33342 (as described earlier). Per presumed zygote the number of spermatozoa bound to the oolemma was evaluated. The other half of the oocytes were fixed and stained after 20 h of coincubation. These presumed zygotes were evaluated for sperm-egg fusion (defined as the presence of two or more pronuclei).

Pre-incubation of male gametes with Fn prior to fertilization

Two groups of about 100 *in vitro* matured COCs were fertilized in standard fertilization medium (3 replicates). The first group was inseminated with spermatozoa ($1x10^6$ sp/ml) previously incubated with 500 nM Fn for 30 min. The second group was fertilized with spermatozoa (from the same ejaculate) which were incubated with standard fertilization medium. At 20 hours post insemination (hpi), all presumed zygotes were fixed, stained (with Hoechst 33342) and evaluated for fertilization and polyspermy. Two additional experiments –

where the sperm cells were incubated for 2 h respectively 4 h – were performed to evaluate the effect of time of sperm pre-incubation on inhibition of sperm penetration.

Pre-incubation of female gametes with Fn prior to fertilization

After *in vitro* maturation, about 200 COCs were divided into two groups (3 replicates). The first group was fertilized under standard conditions. The COCs of the second group were incubated with 500 nM Fn for 30 min prior to fertilization. At 20 hpi all oocytes were fixed, stained (with Hoechst 33342) and evaluated for fertilization and polyspermy. Subsequently, a similar setup was applied on zona-free oocytes. After removal of the ZP (as described earlier), two groups of about 85 oocytes were incubated (39°C; 5% CO_2) for 3 h in standard fertilization medium to allow recovery of the oolemma. Again, the first group was fertilized under standard conditions, whereas the second group was incubated with 500 nM Fn for 30 min prior to fertilization.

Incubation of bovine spermatozoa with Alexa Fluor® 488-conjugated Fn

Fibronectin was labeled with Alexa Fluor® 488 using the Alexa Fluor® 488 Protein Labeling Kit (A10235; Molecular Probes, Leiden, The Netherlands) according to the manufacturer's suggestions. After Percoll centrifugation, frozen-thawed semen (originating from the same ejaculate as the one used in the inhibition experiments) was incubated for 20 h in fertilization medium supplemented with 500 nM of Alexa Fluor® 488-conjugated Fn (10^6 sp/ml). At 4 h and at 20 h of incubation respectively, sperm cells were fixed for 30 min with 1 % (w/v) paraformaldehyde in PBS and stained for 10 min with 10 µg/ml Hoechst 33342. Putative presence of Alexa Fluor®488-labeled Fn on bovine spermatozoa was assessed by means of fluorescence microscopy (using an Olympus IX81 inverted fluorescence microscope connected to a Hamamatsu Orca B/W camera with Olympus Cell*R software).

Effect of sperm incubation with Fn on membrane integrity, acrosomal reaction and sperm motility

Frozen-thawed bull semen originating from the same ejaculate (3 replicates) was centrifuged on a discontinuous Percoll gradient and diluted to a concentration of $60x10^6$ spermatozoa/ml (with medium containing Tyrode balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg/ml gentamycin sulphate, 6 mg/ml fatty acid-free BSA, and 20 µg/ml heparin), prior to a 30 min incubation

(39°C; 5% CO_2) to allow capacitation. Subsequently, the sperm suspension was split into 2 fractions: the first fraction was diluted (1:1) with the modified Tyrode balanced salt solution, while the second fraction was diluted (1:1) with the modified Tyrode balanced salt solution supplemented with 1 μM Fn. Four aliquots from each sperm fraction were incubated (39°C; 5% CO_2), and at four different time points (0 h, 1 h, 4 h and 6 h, respectively) one aliquot per fraction was evaluated for membrane integrity, motility and acrosomal status.

Membrane integrity was evaluated using a fluorescent SYBR14-Propidium Iodide (PI) staining technique (L7011; Molecular Probes, Leiden, The Netherlands). A stock solution of 1 mmol/l SYBR14 reagent was diluted (1:50) in HEPES-TALP, stored frozen at –20°C and thawed just before use. From each sperm aliquot, 100 μl was used and 1 μL SYBR14 was added. After 5 min of incubation (at 37°C), 1 μL PI was added prior to another 5 min incubation (at 37°C). Per aliquot 200 spermatozoa were examined using a Leica DMR fluorescence microscope. Three populations of sperm cells were identified: living (membrane intact; stained green), dead (membrane damaged; stained red), and moribund (double stained; green-orange) spermatozoa. The moribund sperm cells were considered to be part of the dead sperm population.

Acrosome integrity was evaluated using fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (PSA-FITC; L0770; Sigma-Aldrich, Bornem, Belgium), whereas total and progressive motility were determined by means of computer-assisted sperm analysis (Hamilton-Thorne CEROS 12.3) (Tanghe et al., 2004a).

Effect of an Arg-Gly-Asp sequence (RGD)-containing oligopeptide on sperm penetration of bovine COCs

In vitro matured COCs were randomly assigned to 7 different fertilization media: standard fertilization medium, fertilization medium supplemented with 2 μM, 10 μM and 50 μM of a custom made GRGDdSP-oligopeptide (Thermo Fisher Scientific, Ulm, Germany) and fertilization medium supplemented with 2 μM, 10 μM and 50 μM of a non-RGD containing (GRGES) oligopeptide (Thermo Fisher Scientific, Ulm, Germany) according to Fusi et al. (1996b). The oocytes were co-incubated with sperm at a final concentration of 10^6 spermatozoa/ml for 20 h (39°C; 5% CO_2). Prior to overnight fixation (2% (w/v) paraformaldehyde – 2% (v/v) glutaraldehyde in PBS) and staining with 10 μg/ml Hoechst 33342 for 10 min, the presumed zygotes were vortexed for 3 min to remove excess

spermatozoa and/or cumulus cells. Zygotes were mounted in glycerol with 25 mg/ml DABCO and evaluated using a Leica DMR fluorescence microscope. Penetration percentage was defined as the sum of fertilization (presence of two pronuclei) and polyspermy (more than two pronuclei) percentage.

Statistical analyses

Differences in fertilization, polyspermy and penetration percentage were analyzed using binary logistic regression (including the effect of replicate). Differences in mean number of spermatozoa bound to the ZP and the oolemma were analyzed using the non parametric Kruskal Wallis test, since the data were not normally distributed. Differences in membrane integrity, acrosomal status, total and progressive motility were evaluated using repeated measures analysis. Hypothesis testing was performed using a significance level of 5% (SPSS 15.0). Data were reported as mean ± SEM.

RESULTS

Localization of Fn on female and male bovine gametes

Immature and matured cumulus-oocyte-complexes (COCs). Immature and matured bovine COCs expressed Fn at the level of the cytoplasm in 42.3% and 58.2% of the cumulus cells respectively (Fig. 5). When staining the COCs without fixation and permeabilization, Fn expression was observed in the extracellular matrix as well. Concerning the cumulus monolayer culture, the cytoplasm of 64.5% of the cells and the extracellular matrix stained positive.

Cumulus-denuded and ZP-free oocytes. The cumulus denuded oocytes which were not treated with protease showed a fluorescent band underneath the ZP at the perivitelline space (Fig. 6) which was not present in the negative controls. The oolemma of the ZP-free oocytes did not stain fluorescently. The weak immunoreactivity within the oocyte may be due to intracellular production of Fn. Since it has been demonstrated that the Fn present in the cumulus oophorus is a different splice variant than the Fn underneath the ZP (Goossens et al., 2009), the Fn at the perivitelline space cannot originate from the cumulus cells. The rare fluorescent spots at the exterior side of the ZP should likely be considered as remnants of Fn molecules present at the extracellular corona radiata matrix.

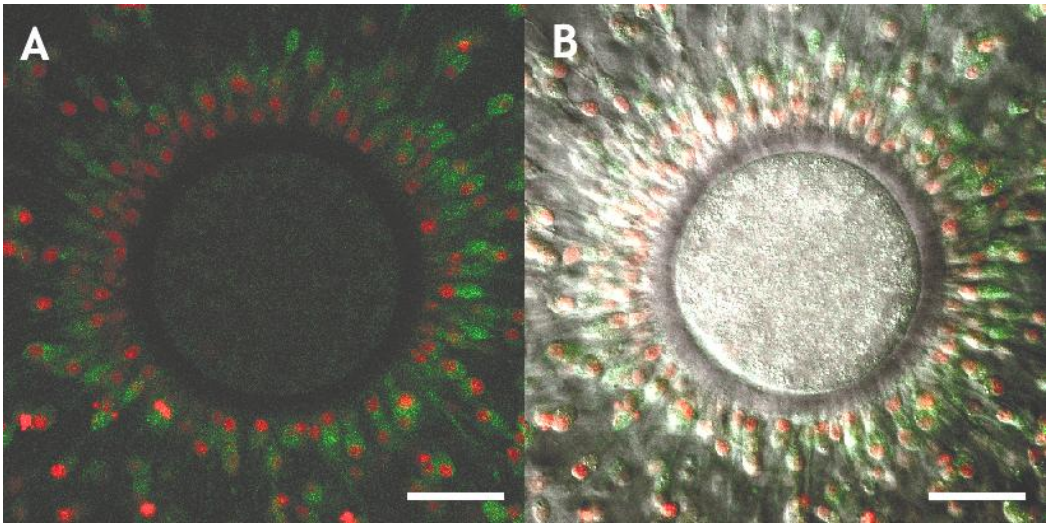


Figure 5**:** *In vitro* matured bovine COC labeled with mouse monoclonal to Fn and goat-anti-mouse FITC combined with Propidium Iodide staining after fixation and permeabilization (A): confocal fluorescent image, (B): overlay with DIC image (Original Magnification x400; Bar = 50 µm).

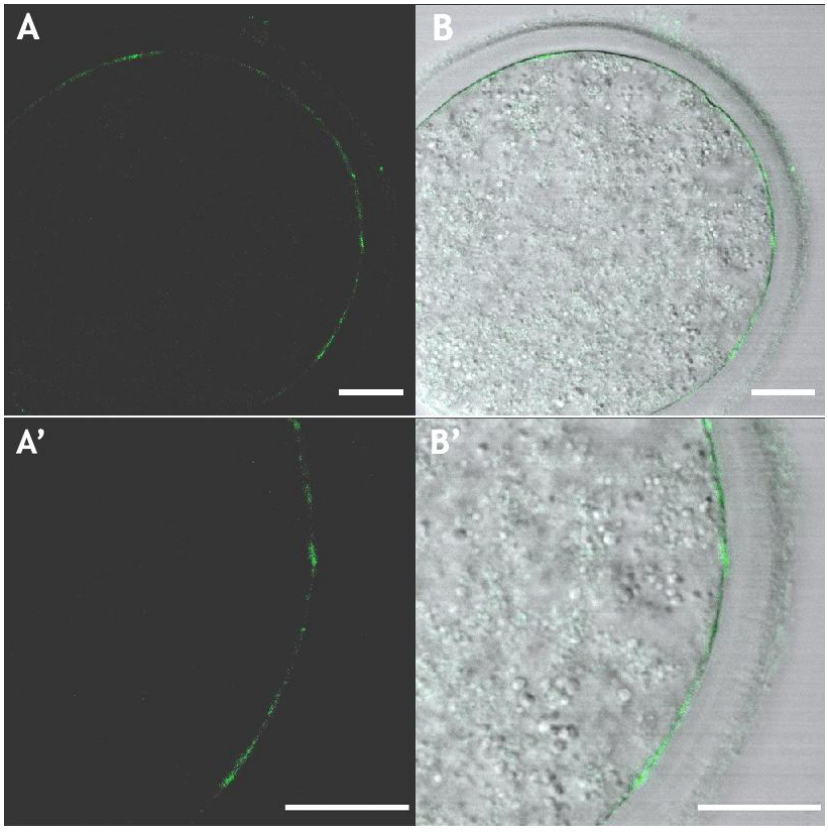


Figure 6: Cumulus-denuded ZP intact bovine oocyte labeled with mouse monoclonal to Fn and goat-anti-mouse FITC combined with Propidium Iodide staining after fixation, (A) and (A'): confocal fluorescent image, (B) and (B'): overlay with DIC image (Original Magnification x400; Bar = 20 µm).

Sperm cells. Using indirect immunofluorescence, we could not detect Fn expression on the surface of non-treated (NT), capacitated (CAP) nor acrosome reacted (AR) frozen-thawed bovine sperm cells after fixation and permeabilization. Assuming detrimental effects of fixation and permeabilization procedures on the cell surface structure, frozen-thawed semen was additionally stained without prior fixation and permeabilization, resulting in negligible percentages of sperm cells displaying faintly fluorescent spots at the acrosomal region (data not shown).

Localization of the α_5 subunit of the Fn-receptor ($\alpha_5\beta_1$ integrin) on female and male bovine gametes

Cumulus-denuded oocytes. Expression of α_5 could clearly be detected on the oolemma of *in vitro* matured cumulus denuded oocytes (Fig. 7). The localization of Fn (at the level of the perivitelline space) and α_5 (on the oolemma) suggests the existence of an Fn-$\alpha_5\beta_1$ integrin ligand-receptor complex underneath the ZP. The few fluorescent dots at the exterior side of the ZP are assumed to be remnants of molecules present at the cell surface of corona radiata cells which have been shown to have cellular projections traversing the ZP and terminating upon the oolemma (Tanghe et al., 2002). Integrin expression has been observed in bovine cumulus cells (Sutovsky et al., 1995).

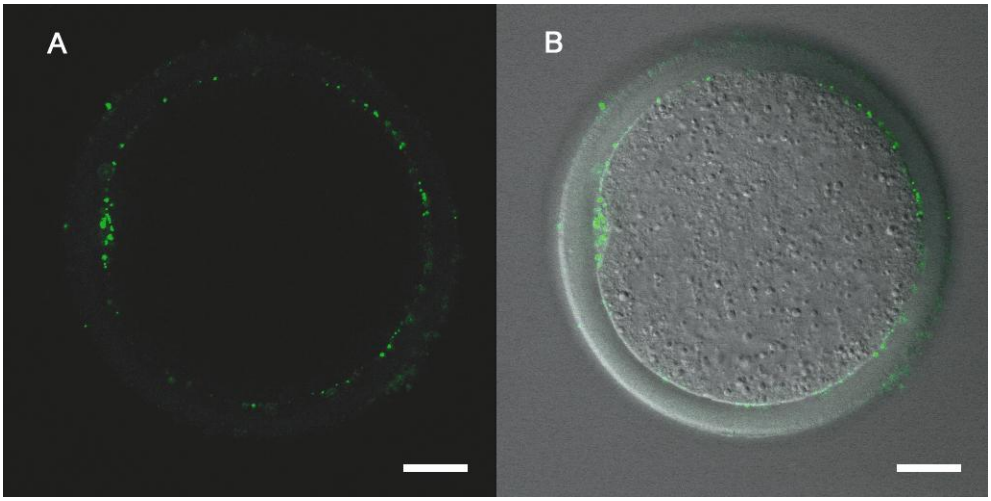


Figure 7: Cumulus-denuded ZP intact bovine oocyte labeled with rabbit-anti-human polyclonal to CD49e/integrin subunit α_5 and goat-anti-rabbit FITC combined with Propidium Iodide staining after fixation, (A): confocal fluorescent image, (B): overlay with DIC image (Original Magnification x400; bar = 25 μm).

Sperm cells. All sperm cells (in each of the three fractions) displayed green fluorescence. In the NT and CAP fraction fluorescence at the rostral sperm head was observed (Fig. 8, A-D), while a fluorescent band at the equatorial segment was noted in the AR spermatozoa (Fig. 8, E-F). Since the same pattern was noted when pre-incubating the NT and CAP sperm fraction with heat-inactivated rabbit serum (instead of the rabbit anti-human polyclonal antibody to integrin subunit α_5), this fluorescent signal was due to non-specific binding of rabbit serum to the sperm. Combining these results, integrin subunit α_5 appears to be only expressed at the equatorial segment of sperm cells after acrosome reaction.

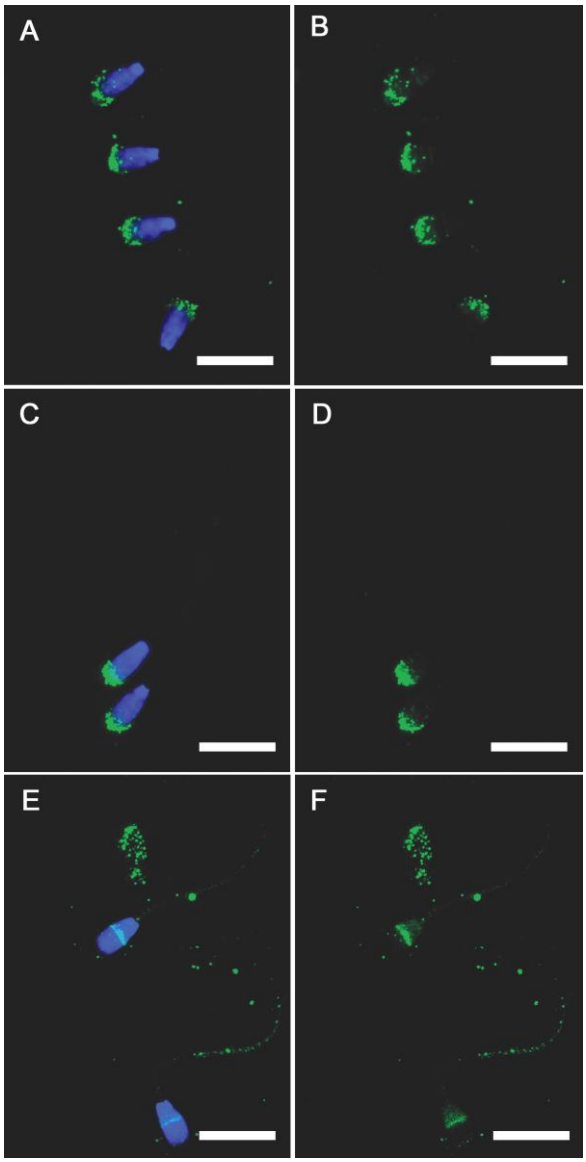


Figure 8: Fluorescent images of frozen-thawed bovine semen labeled with rabbit-anti-human polyclonal to CD49e/integrin subunit α_5 and goat-anti-rabbit FITC (B, D and F) combined with Hoechst 33342 staining (A, C and E) after fixation (Original Magnification x600; Bar = 10 μm) (A-B): non-treated sperm; (C-D): capacitated sperm; (E-F): acrosome reacted sperm.

Dose-response effect of Fn on sperm penetration after bovine IVF

Compared to the control (0 nM Fn), sperm penetration decreased significantly ($P < 0.01$) when Fn was supplemented at a concentration of 250 nM or higher. The inhibition of sperm penetration by exogenous Fn seemed to be dose-dependent (Fig. 9). Further increase of the Fn concentration to 1 µM did not result in a significantly different sperm penetration percentage ($P = 0.20$) compared to the 500 nM Fn group.

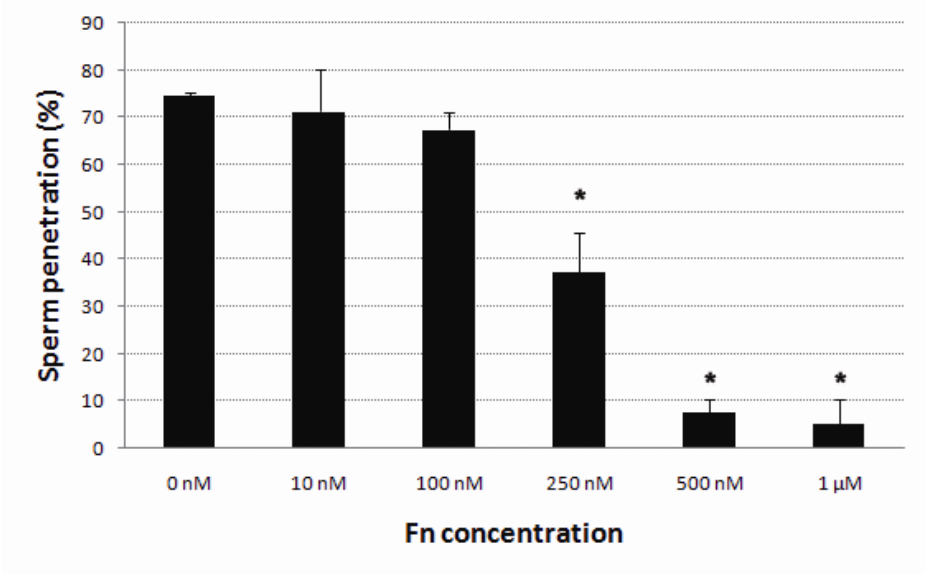


Figure 9: Dose-response effect of fibronectin (Fn) on sperm penetration after bovine IVF. Data represent mean ± SEM. *Values significantly different from control with 0 nM Fn ($P < 0.01$).

Effect of Fn on sperm penetration of the cumulus oophorus

The sperm penetration and fertilization percentages in both cumulus-enclosed and denuded oocytes decreased significantly when 500 nM Fn was supplemented to the fertilization medium compared to the respective control group (Table 1). In this experimental set-up, Fn induced inhibition of sperm penetration independent of the presence of the cumulus cells. Therefore, the observed inhibitory effect of Fn on bovine IVF was further explored by assessing whether its presence negatively affected the sperm-zona binding, the sperm-oolemma binding or the sperm-oocyte fusion.

Table 1: Fertilization, polyspermy and penetration percentages of cumulus-denuded (CD) and cumulus-enclosed (CE) bovine oocytes inseminated in standard fertilization medium and in fertilization medium supplemented with 500 nM of fibronectin (Fn).

Oocytes	Fn (nM)	No.	Fertilization (%)	Polyspermy (%)	Penetration (%)	Inhibition of penetration (%)
CD	0	249	$15.0^{a} \pm 2.07$	$0.4^{a} \pm 0.43$	$15.4^{a} \pm 1.86$	-
	500	258	$1.5^{b} \pm 0.30$	$2.3^{a} \pm 1.22$	$3.8^{b} \pm 1.47$	75.3
CE	0	296	$62.9^{a} \pm 7.19$	$7.7^{a} \pm 1.04$	$70.6^{a} \pm 7.72$	-
	500	290	$5.8^{b} \pm 1.19$	$2.8^{b} \pm 1.26$	$8.6^{b} \pm 0.60$	87.8

Data represent mean ± SEM.

[a,b] Values with a different superscript in the same column within the CD and the CE groups differ significantly ($P < 0.05$).

Effect of Fn on sperm-zona binding

The number of spermatozoa bound to the ZP decreased significantly (from 20.9 ± 2.86 to 13.3 ± 2.18 sp/oocyte) when Fn was added to the fertilization medium ($P<0.05$). This decrease in sperm-zona binding might only partially account for the substantial inhibition of sperm penetration in the presence of 500 nM Fn.

Effect of Fn on sperm-oolemma binding and -fusion

A 4-fold reduction in the number of spermatozoa bound to the oolemma was observed (from 28.1 ± 1.89 to 6.7 sp/oocyte ± 0.89) when 500 nM Fn was present during fertilization compared to the control group ($P<0.05$). Furthermore, fertilization and sperm penetration were negatively influenced ($P<0.05$) when Fn was supplemented (Table 2). Compared to the control group, the sperm penetration was inhibited with 60.4%.

Combining the previously described results, we can assume that the main inhibitory effect of Fn was located at the level of sperm-oolemma binding, with a (concurrent) effect on fusion. To identify whether Fn interacts with either male or female gametes, two additional experiments were conducted incubating either sperm cells or cumulus oocyte complexes with Fn prior to IVF.

Table 2: Fertilization, polyspermy and penetration percentages of ZP-free bovine oocytes inseminated in standard fertilization medium and in fertilization medium supplemented with 500 nM of fibronectin.

Oocytes	Fibronectin (nM)	No.	Fertilization (%)	Polyspermy (%)	Penetration (%)	Inhibition of penetration (%)
ZP-free	0	113	$25.4^{a} \pm 6.53$	$2.6^{a} \pm 1.47$	$28.0^{a} \pm 7.82$	-
	500	116	$6.1^{b} \pm 1.01$	$5.0^{a} \pm 2.27$	$11.1^{b} \pm 1.79$	60.4

Data represent mean ± SEM.

[a,b] Values with a different superscript in the same column differ significantly ($P<0.05$).

ZP, zona pellucida.

Pre-incubation of male gametes with Fn prior to fertilization

Pre-incubation of sperm cells with 500 nM Fn (for 30 min) prior to fertilization significantly decreased the sperm penetration compared to that of the control (75.2% vs 87.0%) resulting in an inhibition of sperm penetration of 13.6% ($P<0.001$). The same tendency was observed for fertilization with or without Fn pre-incubated sperm (68.6 % vs 78.2 %; $P < 0.01$). Prolonging the duration of sperm pre-incubation caused more prominent inhibition of penetration (22.2% inhibition after 2 h versus 42.8% after 4 h).

Pre-incubation of female gametes with Fn prior to fertilization

Pre-incubation of COCs with 500 nM Fn prior to fertilization did not significantly decrease the sperm penetration and fertilization percentages (76.2% respectively 67.3%) compared to the ones of the control group (83.0% respectively 75.4%; $P=0.10$ respectively $P=0.09$). Furthermore, also the zona-free oocytes did not show a decreased sperm penetration (42.0% versus 46.9%) nor fertilization percentage (37.1% versus 37.0%) when pre-incubated with Fn.

The results of both pre-incubation experiments suggested that Fn inhibits sperm penetration in bovine COCs mainly through interaction with the sperm cell.

Incubation of bovine spermatozoa with Alexa Fluor® 488-conjugated Fn

To investigate whether Fn supplementation during IVF results in binding of the glycoprotein to the male gamete – as suggested by our previous findings – bovine spermatozoa were incubated in the presence of 500 nM Alexa Fluor® 488-conjugated Fn. After 4 h of incubation, 49% of the sperm cells displayed green fluorescence at the equatorial segment. A brighter fluorescent signal was observed in 83% of the spermatozoa evaluated after 20 h of incubation (Fig. 10). Since the α_5 subunit of the Fn integrin receptor was also observed at the level of the equatorial segment of acrosome reacted sperm cells, binding of exogenously supplemented Fn to the acrosome reacted male gamete – resulting in a defective sperm-oolemma binding – seems the main mode of action. The sperm cells are no longer able to interact with the Fn-integrin $\alpha_5\beta_1$ ligand-receptor complex on the oolemma.

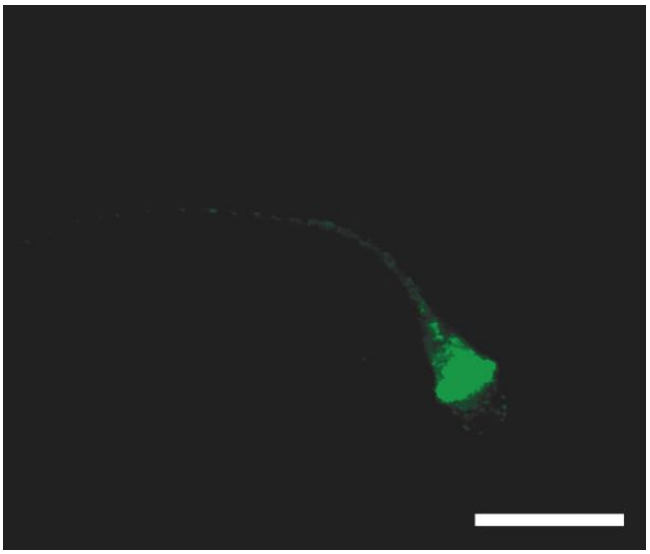


Figure 10: Fluorescent image of frozen-thawed bovine sperm cell (incubated for 20h with 500 nM of Alexa Fluor® 488-conjugated fibronectin) displaying fluorescence at the equatorial segment (Original Magnification x600; Bar = 10 μm).

Effect of sperm incubation with Fn on membrane integrity, acrosomal reaction and sperm motility

Sperm incubation with 500 nM Fn did not significantly affect membrane integrity nor acrosomal reaction at any of the evaluated time points ($P > 0.05$; Fig. 11-12). Total and progressive motility were significantly lower in the presence of 500 nM Fn (Fig. 13). However, the rather small decrease in motility cannot account for the strong inhibition of sperm penetration (as described in Table 1).

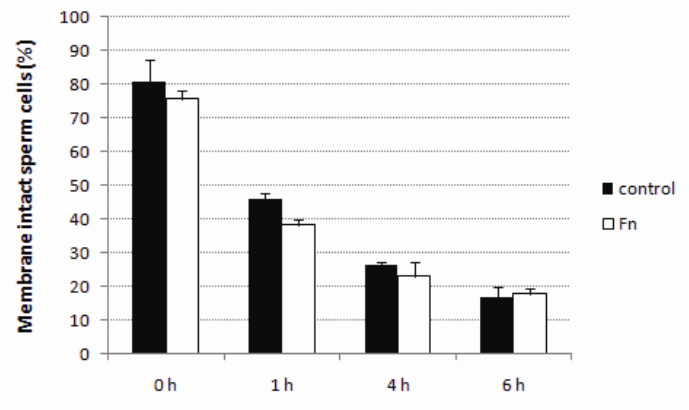


Figure 11: Effect of 500 nM fibronectin on membrane integrity of bovine frozen-thawed spermatozoa during incubation (evaluated by means of SYBR14-PI staining).
Data represent mean ± SEM.

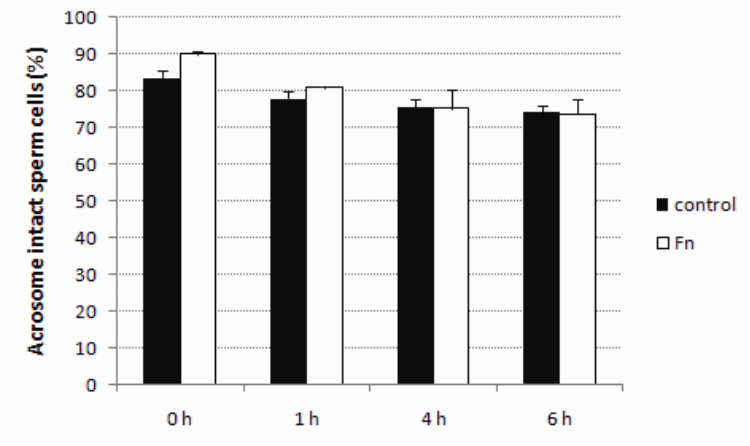


Figure 12: Effect of 500 nM fibronectin on the acrosome reaction of bovine frozen-thawed spermatozoa during incubation (evaluated by means of PSA staining).
Data represent mean ± SEM.

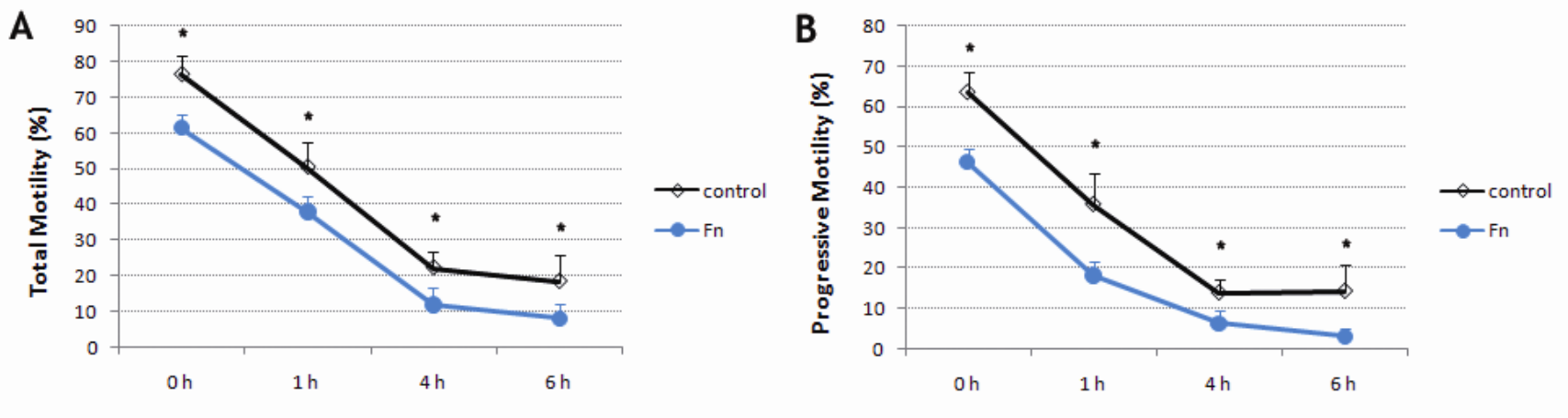


Figure 13: Effect of 500 nM fibronectin on total motility (A) and progressive motility (B) of bovine frozen-thawed spermatozoa during incubation (evaluated by means of CASA). Data represent mean ± SEM.

Effect of an Arg-Gly-Asp sequence (RGD)-containing oligopeptide on sperm penetration of bovine COCs

To investigate whether the inhibitory effect of exogenous Fn on sperm penetration during IVF was exerted through interaction of its RGD-sequences with integrin receptors, different concentrations of a custom made GRGDdSP-oligopeptide (a peptide known to block Fn receptors only) were supplemented to the fertilization medium (Fusi et al., 1996b). A non-RGD containing oligopeptide (GRGES) was used as a negative control at the same concentrations. Compared to the control, sperm penetration was significantly decreased in the presence of the RGD-peptide at all tested concentrations ($P < 0.05$), but was not affected when the non-RGD-peptide was supplemented (Fig. 14).

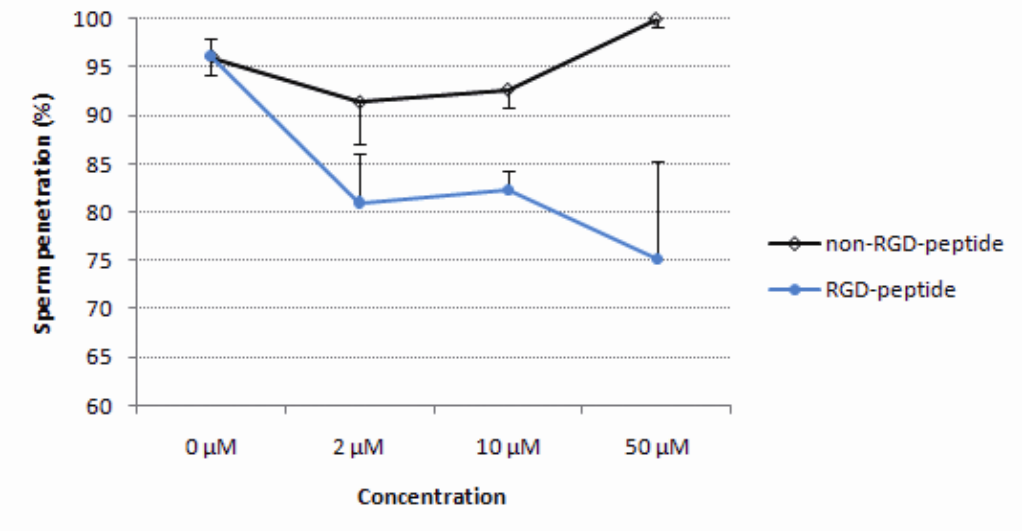


Figure 14: Effect of an Arg-Gly-Asp (RGD)-containing oligopeptide and a control (non-RGD) oligopeptide on sperm penetration of bovine cumulus-oocyte-complexes. Data represent mean ± SEM.

DISCUSSION

In the present study, we confirmed our earlier findings that exogenously supplemented fibronectin has a substantial negative influence on bovine sperm-oocyte interaction (Tanghe et al., 2004*b*). Moreover, we were able to demonstrate that the main inhibitory effect of Fn was located at the level of sperm-oolemma binding, with a (concurrent) effect on fusion. In accordance, we detected endogenous Fn underneath the zona pellucida (at the level of the peri-vitelline space) and α_5 (subunit of Fn-receptor integrin $\alpha_5\beta_1$) on the oolemma of cumulus

denuded bovine oocytes. In addition, bovine spermatozoa displayed integrin subunit α_5 at the equatorial segment after acrosome reaction. Correspondingly, incubation of sperm cells with Alexa Fluor®488-conjugated Fn resulted in green fluorescence at the equatorial segment which increased with time. Combining these results, the inhibitory effect of exogenously supplemented Fn seemed to be exerted on the male gamete by binding to the exposed integrin $\alpha_5\beta_1$ receptor after acrosome reaction. In order to put forward a hypothesis on the putative function of Fn in bovine fertilization, it is important to understand that the soluble plasma Fn (exogenous Fn) and the cumulus Fn are different splice variants than the Fn which can be detected underneath the zona pellucida. Plasma Fn (or exogenous Fn for that matter) lacks the IIICS segment which displays a second RGD sequence, and as such cannot function as a connecting molecule between sperm cell and oocyte. To exert this type of dual reversible binding interaction, a second RGD sequence is required, and this second RGD sequence is present in the biological variant of Fn which is located underneath the ZP (Goossens et al., 2009).

Different splice variants can react with the same antibody, but may exert different biological functions. Since the Fn splice variant found underneath the ZP displays an extra RGD-sequence, it may interact with another (integrin) receptor.

Additionally, a modest negative influence of Fn supplementation on sperm-ZP binding was observed. In human, Fn (Fusi and Bronson, 1992) – and integrin receptors for Fn ($\alpha_5\beta_1$) (Fusi et al., 1996*a*) – appear on the surface of spermatozoa after capacitation, suggesting that supplemented – exogenous – Fn possibly competes with those Fn molecules expressed on the sperm surface in binding complementary receptors on the ZP. Nevertheless, in the present study, Fn could not be detected on the surface of frozen-thawed bovine spermatozoa irrespective of their functional state. Pre-incubation of bovine oocytes with Fn prior to IVF did not result in an inhibition of sperm penetration and we did not perceive integrin subunit α_5 expression in the ZP. Taking into account the finding that exogenous Fn seems to bind to the equatorial segment (of AR sperm cells), the moderate decrease in sperm-ZP binding (observed in presence of Fn) might be due to the inability of already partially acrosome reacted sperm cells to bind the ZP (in this case in a biologically irrelevant manner). In human, experimental models have demonstrated that acrosome reacting sperm are still capable of binding to the ZP (Morales et al., 1989). Molecules on the sperm cell surface required for sperm-ZP binding might be sterically hindered by the glycoprotein sticking to the corresponding integrin receptor. Unlike in human (Diaz et al., 2007) and pig (Mattioli et al.,

1998), incubation of bovine sperm with Fn did not seem to induce important levels of premature acrosome reaction, which could also have been a plausible explanation for a decrease in sperm-ZP binding. Combining all these results, it is very likely that Fn is not essential for successful sperm-ZP interaction in bovine.

The main inhibitory effect of exogenous Fn was observed at sperm oolemma interaction. Fertilization of zona-free oocytes in the presence of Fn resulted in a significantly (4-fold) decreased sperm oolemma binding. The presence of endogenous Fn at the perivitelline space of cumulus-denuded oocytes combined with integrin subunit α_5 expression on the oolemma and on the surface of acrosome reacted spermatozoa implies a potential reversible dual binding interaction between the endogenous Fn ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating sperm-egg binding (Fig. 15). Binding of Alexa Fluor® 488-conjugated Fn to the equatorial segment of spermatozoa provides additional support for this hypothesis. It has been established quite some time that sperm cells first bind to the oolemma by the tip of the sperm head and that this initial binding is subsequently converted to a lateral binding when the spermatozoon turns parallel to the oocyte surface. The fusion of the sperm plasma membrane with the oolemma takes place at the equatorial region (Myles, 1993). A possible involvement of Fn in sperm-oocyte fusion was put forward by the fact that supplementation of Fn appeared to significantly inhibit sperm penetration of zona-free oocytes. However, this fusion effect could simply be due to the observed inhibition of sperm binding to the oolemma.

The results of our pre-incubation experiments provide additional data in favour of our hypothesis concerning the reversible dual binding action of fibronectin during bovine IVF. Since there was no effect observed on sperm penetration/fertilization when cumulus-enclosed oocytes were pre-treated with Fn prior to fertilization, and a significant – time-dependent – effect was found after pre-incubating spermatozoa with Fn, it will be likely that Fn binds to the corresponding integrin $\alpha_5\beta_1$ receptor on the sperm cell surface. The fact that the effect increased over time, suggests that binding of exogenous Fn to the male gamete is more prominent in acrosome reacted sperm cells. Further support for this assumption is provided by the finding that binding of Alexa Fluor® 488-conjugated Fn to sperm also increases over time (from 49% fluorescent spermatozoa after 4 h to 83% after 20 h). The observation that pre-incubation of ZP-free oocytes with exogenous Fn did not enhance sperm penetration and that Fn treatment of acrosome reacted sperm cells prior to IVF of ZP-free oocytes resulted in a

significantly decreased sperm-oolemma binding (data not shown), seems – however – not to be in accordance with our hypothesis. The latter experimental outcome may be explained by the absence of a second RGD-binding site in the exogenous Fn variant, by which the Fn molecule can only bind to one single integrin (in this case the one on the sperm cell). The lack of effect of pre-treatment of ZP-free oocytes clearly indicates that Fn is not capable of binding the oolemmal integrin receptor. Possibly a conformational modification of the Fn-ligand (induced by binding to the sperm integrin receptor) is required for successful connection to the receptor on the oocyte. Matured cumulus-denuded ZP-intact oocytes did not display an exact co-localization of Fn and integrin subunit α_5 after performing a double staining procedure (data not shown), demonstrating that the endogenous Fn is not bound to its receptor. Additionally, ZP-free oocytes (treated with protease) did show integrin subunit α_5 on their oolemma (but no Fn), indicating that the receptor was present on the membrane (data not shown).

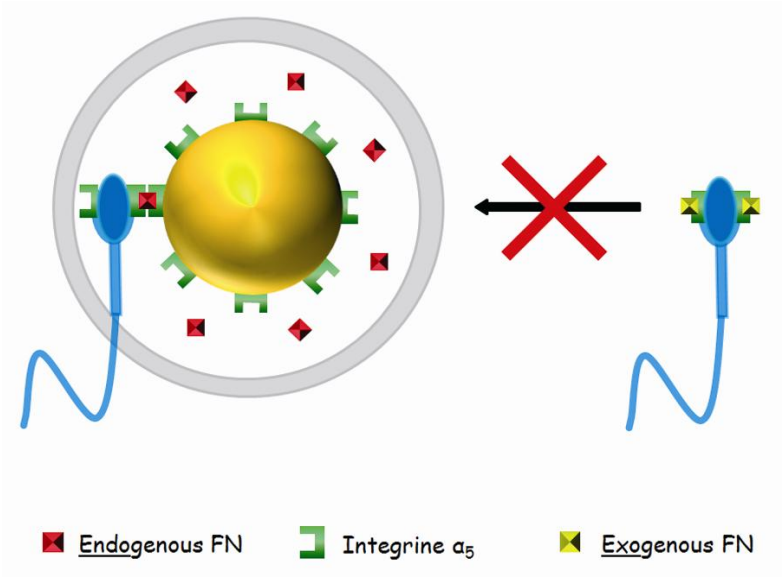


Figure 15: Hypothetical model of function of endogenous fibronectin during bovine sperm-oocyte interaction and interference of exogenously supplemented fibronectin during bovine IVF.

Nonetheless, the diffuse pattern of Alexa Fluor® 488-conjugated Fn at the equatorial and posterior sperm head (Fig. 10) differs from that of the discrete equatorial immunolabeling of integrin subunit α_5 (Fig. 8E-F). Possibly the Fn occupies simultaneously different receptors. At least ten different integrins are able to serve as Fn receptors. Some are specifically binding Fn, but others have multiple ligands (Goossens et al., 2009). Similarly,

the observation that after 4 h of incubation, 49% of the sperm cells displayed Fn fluorescence at the equatorial segment seems inconsistent with the fact that approximately 75% of the sperm cells were still acrosome intact at this stage of incubation. This discrepancy is in favour of the previous statement that several transmembrane proteins can act as Fn receptors. Several of these receptors may be present on the sperm cell surface prior to acrosome reaction.

Since sperm incubation with exogenous Fn did not affect membrane integrity at any of the evaluated time points, the possibility of simply exerting a toxic effect on spermatozoa could be excluded. Total and progressive sperm motility were significantly lower in the presence of Fn. However, the rather small decrease in motility cannot account for the strong inhibition of sperm penetration during IVF.

Actual involvement of the RGD-sequence in sperm-egg interaction was assessed to verify whether the inhibitory effect of exogenously supplemented Fn was indeed due to binding to the α_5 integrin. A Fn-specific RGD-containing peptide (Fusi et al., 1996b) negatively affected sperm penetration at all tested concentrations, whereas a non-RGD control peptide did not seem to have any effect.

In conclusion, the present study demonstrates that exogenously supplemented fibronectin negatively influences bovine IVF by inhibiting sperm-oolemma binding and (concurrently) sperm-egg fusion. The presence of endogenous Fn underneath the ZP together with integrin subunit α_5 expression on the oolemma and the acrosome reacted sperm cell surface, suggests a reversible dual binding interaction between the endogenous Fn ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating sperm-egg binding. Further research (ideally making use of the specific Fn splice variant present underneath the ZP), identifying the effect of Fn binding to its integrin $\alpha_5\beta_1$ receptor on the intracellular signal transduction in male and female gamete, is indispensable to elucidate the exact underlying mechanism of interaction in order to validate our model and to create a non-hormonal topical contraceptive – based on the glycoprotein – in the future.

REFERENCES

Akiyama SK, Olden K, Yamada KM. 1995. Fibronectin and integrins in invasion and metastasis. *Cancer Metastasis Rev* **14** 173-189.

Almeida EA, Huovila AP, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, White JM. 1995. Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. *Cell* **81** 1095-1104.

Amari S, Yonezawa N, Mitsui S, Katsumata T, Hamano S, Kuwayama M, Hashimoto Y, Suzuki A, Takeda Y, Nakano M. 2001. Essential role of the nonreducing terminal alpha-mannosyl residues of the N-linked carbohydrate chain of bovine zona pellucida glycoproteins in sperm-egg binding. *Mol Reprod Dev* **59** 221-226.

Benoff S. 1997. Carbohydrates and fertilization: an overview. *Mol Hum Reprod* **3** 599-637.

Biermann L, Gabius HJ, Denker HW. 1997. Neoglycoprotein-binding sites (endogenous lectins) in the Fallopian tube, uterus and blastocyst of the rabbit during the preimplantation phase and implantation. *Acta Anat (Basel)* **160** 159-171.

Bronson RA, Fusi FM. 1996. Integrins and human reproduction. *Mol Hum Reprod* **2** 153-168.

Diaz ES, Kong M, Morales P. 2007. Effect of fibronectin on proteasome activity, acrosome reaction, tyrosine phosphorylation and intracellular calcium concentrations of human sperm. *Hum Reprod* **22** 1420-1430.

Fazeli AR, Steenweg W, Bevers MM, de Loos FA, van den Broek J, Colenbrander B. 1993. Development of a sperm zona pellucida binding assay for bull semen. *Vet Rec* **32** 14-16.

Fraser LR, Umar G, Sayed S. 1993. Na^+-requiring mechanisms modulate capacitation and acrosomal exocytosis in mouse spermatozoa. *J Reprod Fert* **97** 539-549.

Fusi FM, Bronson RA. 1992. Sperm surface fibronectin. Expression following capacitation. *J Androl* **13** 28-35.

Fusi FM, Lorenzetti I, Vignali M, Bronson RA. 1992. Sperm surface proteins after capacitation. Expression of vitronectin on the spermatozoan head and laminin on the sperm tail. *J Androl* **13** 488-497.

Fusi FM, Vignali M, Gailit J, Bronson RA. 1993. Mammalian oocytes exhibit specific recognition of the RGD (Arg-Gly-Asp) tripeptide and express oolemmal integrins. *Mol Reprod Dev* **36** 212-219.

Fusi FM, Tamburini C, Mangili F, Montesano M, Ferrari A, Bronson RA. 1996*a.* The expression of alpha v, alpha 5, beta 1, and beta 3 integrin chains on ejaculated human spermatozoa varies with their functional state. *Mol Hum Reprod* **2** 169-175.

Fusi FM, Bernocchi N, Ferrari A, Bronson RA. 1996*b.* Is vitronectin the velcro that binds the gametes together? *Mol Hum Reprod* **2** 859-866.

Goossens K, Van Soom A, Van Zeveren A, Favoreel H, Peelman LJ. 2009. Quantification of Fibronectin 1 (FN1) splice variants, including two novel ones, and analysis of integrins as candidate FN1 receptors in bovine preimplantation embryos. *BMC Dev Biol* **9** 1 (doi:10.1186/1471-213X-9-1).

Gougoulidis T, Trounson A, Dowsing A. 1999. Inhibition of bovine sperm-oocyte fusion by the carbohydrate GalNAc. *Mol Reprod Dev* **54** 179-185.

Ignotz GG, Cho MY, Suarez SS. 2007. Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir. *Biol Reprod* **77** 906-913.

Lefebvre R, Lo MC, Suarez SS. 1997. Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biol Reprod* **56** 1198-1204.

Lyon M, Rushton G, Askari JA, Humphries MJ, Gallagher JT. 2000. Elucidation of the structural features of heparan sulfate important for interaction with the Hep-2 domain of fibronectin. *J Biol Chem* **275** 4599-4606.

Mattioli M, Lucidi P, Barboni B. 1998. Expanded cumuli induce acrosome reaction in boar sperm. *Mol Reprod Devel* **51** 445-453.

McKeown-Longo PJ, Mosher DF. 1984. Mechanism of formation of disulfide-bonded multimers of plasma fibronectin in cell layers of cultured human fibroblasts. *J Biol Chem* **259** 12210-12215.

Midwood KS, Williams LV, Schwarzbauer JE. 2004. Tissue repair and the dynamics of the extracellular matrix. *Int J Biochem Cell Biol* **36** 1031-1037.

Morales P, Cross NL, Overstreet JW, Hanson FW. 1989. Acrosome-intact and acrosome-reacted human sperm can initiate binding to the zona pellucida. *Dev Biol* **133** 385-392.

Mosher DF. 1984. Physiology of fibronectin. *Annu Rev Med* **35** 561-575.

Myles DG. 1993. Molecular mechanisms of sperm-egg membrane binding and fusion in mammals. *Dev Biol* **158** 35-45.

Nixon B, Aitken RJ, McLaughlin EA. 2007. New insights into the molecular mechanisms of sperm-egg interaction. *Cell Mol Life Sci* **64** 1805-1823.

Olorundare OE, Peyruchaud O, Albrecht RM, Mosher DF. 2001. Assembly of a fibronectin matrix by adherent platelets stimulated by lysophosphatidic acid and other agonists. *Blood* **98** 117-124.

Relucenti M, Heyn R, Correr S, Familiari G. 2005. Cumulus oophorus extracellular matrix in the human oocyte: a role for adhesive proteins. *It J Anat Embryol* **110** (2 Suppl 1) 219-224.

Revah I, Gadella BM, Flesch FM, Colenbrander B, Suarez SS. 2000. Physiological state of bull sperm affects fucose- and mannose-binding properties. *Biol Reprod* **62** 1010-1015.

Ruoslahti E, Pierschbacher MD. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell* **44** 517-518.

Ruoslahti E, Pierschbacher MD. 1987. New perspectives in cell adhesion: RGD and integrins. *Science* **238** 491-497.

Sandeman SR, Allen MC, Liu C, Faragher RG, Lloyd AW. 2000. Human keratocyte migration into collagen gels declines with *in vitro* ageing. *Mech Ageing Dev* **119** 149-157.

Sostaric E, van de Lest CH, Colenbrander B, Gadella BM. 2005. Dynamics of carbohydrate affinities at the cell surface of capacitating bovine sperm cells. *Biol Reprod* **72** 346-357.

Suarez SS, Revah I, Lo M, Kolle S. 1998. Bull sperm binding to oviductal epithelium is mediated by a Ca2+-dependent lectin on sperm that recognizes Lewis-a trisaccharide. *Biol Reprod* **59** 39-44.

Sutovsky P, Flechon JE, Pavlok A. 1995. F-actin is involved in control of bovine cumulus expansion. *Mol Reprod Dev* **41** 521-529.

Talevi R, Gualtieri R. 2001. Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium *in vitro*. *Biol Reprod* **64** 491-498.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* **61** 414-424.

Tanghe S, Van Soom A, Duchateau L, de Kruif A. 2004*a.* Inhibition of bovine sperm-oocyte fusion by the p-aminophenyl derivative of D-mannose. *Mol Reprod Dev* **67** 224-232.

Tanghe S, Van Soom A, Duchateau L, Nauwynck H, de Kruif A. 2004*b.* Carbohydrates and glycoproteins involved in bovine fertilization *in vitro*. *Mol Reprod Dev* **68** 492-499.

Thys M, Vandaele L, Morrell JM, Mestach J, Van Soom A, Hoogewijs M, Rodriguez-Martinez H. 2009. *In vitro* fertilising capacity of frozen-thawed bull spermatozoa selected by single-layer (glycerolpropylsilane, GS) silane-coated silica colloidal centrifugation. *Reprod Dom Anim* **44** 390-394.

Tulsiani DR, Yoshida-Komiya H, Araki Y. 1997. Mammalian fertilization: a carbohydrate-mediated event. *Biol Reprod* **57** 487-494.

Vandaele L, Mateusen B, Maes DG, de Kruif A, Van Soom A. 2007. Temporal detection of caspase-3 and -7 in bovine *in vitro* produced embryos of different developmental capacity. *Reproduction* **133** 709-718.

Vjugina U, Evans JP. 2008. New insights into the molecular basis of mammalian sperm-egg membrane interactions. *Front Biosci* **13** 462-476.

Wennemuth G, Meinhardt A, Mallidis C, Albrecht M, Krause W, Renneberg H, Aumuller G. 2001. Assessment of fibronectin as a potential new clinical tool in andrology. *Andrologia* **33** 43-46.

Whyte A, Allen WR. 1985. Equine endometrium at pre-implantation stages of pregnancy has specific glycosylated regions. *Placenta* **6** 537-542.

CHAPTER 4

VITRONECTIN AND ITS RECEPTOR (INTEGRIN $\alpha_v\beta_3$) DURING BOVINE FERTILIZATION *IN VITRO*

Modified from

VITRONECTIN AND ITS RECEPTOR (INTEGRIN $\alpha_v\beta_3$) DURING BOVINE FERTILIZATION *IN VITRO*

Thys M., Nauwynck H., Maes D., Favoreel H., Van Soom A.

Reproduction, Fertility and Development, *submitted*.

ABSTRACT

When present during bovine *in vitro* fertilization (IVF), vitronectin (Vn) has been observed to inhibit sperm penetration. This study was conducted to evaluate the expression of Vn and its integrin receptor ($\alpha_v\beta_3$) on male and female bovine gametes and to determine their function during IVF. Vitronectin was identified as an intrinsic sperm protein, exposed during acrosome reaction, and was observed at the cumulus oophorus, especially near the exterior side of the zona pellucida (ZP). Integrin subunit α_v was expressed on the anterior sperm cell surface vaguely after capacitation but distinctly after acrosome reaction. Integrin subunit α_v was also present at the oolemma of all sampled oocytes, and at the exterior side of the ZP in the majority of these cells. A low dose of exogenous Vn (100 nM) enhanced sperm penetration of bovine cumulus-oocyte complexes, whereas 500 nM had an inhibitory effect: sperm-oocyte fusion was significantly hampered. Low doses of Vn may well improve sperm penetration through boosting initial sperm-ZP binding. The presence of Vn at the sperm cell surface after acrosome reaction together with integrin subunit α_v expression on the oolemma suggests a reversible dual binding interaction between the Vn ligand and corresponding receptors on both sperm cell and oolemma, mediating sperm-oocyte interaction.

INTRODUCTION

The complex series of molecular interactions between male and female gametes required for successful fertilization have interested several research groups for many years (Benoff, 1997). Even though substantial insights about these interactions have been established in particular in human and mouse (Fusi et al., 1992; 1996*a*; 1996*b*; Almeida et al., 1995; Evans et al., 1995; Bronson and Fusi, 1996), a clear identification of the underlying mechanisms and molecules implicated in bovine fertilization is still required. The involvement of numerous carbohydrates and glycoproteins in adhesion and binding events during several reproductive processes has been described in ruminants, ranging from roles in sperm-oviduct adhesion (Revah et al., 2000; Talevi and Gualtieri, 2001; Sostaric et al., 2005; Gwathmey et al., 2006; Ignotz et al., 2007), sperm-oocyte interactions (Gougoulidis et al., 1999; Amari et al., 2001; Tanghe et al., 2004*a*; 2004*b*) to embryo implantation (Spencer et al., 2004).

A convenient way to study receptor-ligand interactions is to incubate sperm and/or oocytes with possible ligands in order to inhibit fertilization. Using this approach, Tanghe et

al. (2004*b*) demonstrated that vitronectin – among other glycoproteins and carbohydrates – when present during bovine *in vitro* fertilization (IVF) causes an inhibition of sperm penetration.

Vitronectin (Vn) is a multifunctional 75 kDa glycoprotein - rather exclusively secreted by the liver into the plasma in a monomeric form - and abundantly stored in an essentially multimeric form in diverse extracellular matrices (Stockmann et al., 1993; Gechtman et al., 1997; Francois et al., 1999). Like other adhesive proteins (e.g. fibronectin), Vn possesses a heparin binding site and interacts via its Arg-Gly-Asp (RGD) amino acid sequence with integrin receptors (mainly the $\alpha_v\beta_3$ integrin) at the cell surface (Bronson et al., 2000). Interaction of this glycoprotein with a wide range of macromolecules has been described allowing it to participate in several physiological processes, among which complement-mediated cell lysis, cell surface proteolysis, cell adhesion, coagulation and fibrinolysis (Gibson et al., 1999; Bronson et al., 2000).

RGD sequences, present in Vn as well as in other extracellular matrix proteins (Fusi et al., 1992), are believed to take part in various integrin-mediated recognition systems involved in cell-to-cell and cell-to-matrix adhesion (Ruoslahti and Pierschbacher, 1986). Since - in human - integrins have been detected on both male and female gametes and spermatozoa express Vn on their surface following capacitation (Bronson and Fusi, 1996), vitronectin may be involved in sperm-egg interaction. The present study was conducted to determine whether the inhibitory effect of exogenously supplemented Vn on bovine IVF appeared during a) the sperm penetration of the cumulus oophorus, b) the sperm-zona binding, c) the sperm-oolemma binding or d) the sperm-oocyte fusion. Subsequently, the expression of endogenous Vn and integrin subunit α_v (subunit of the Vn receptor) on bovine oocytes and sperm cells was evaluated using indirect immunofluorescence, and the effect of exogenous Vn on sperm membrane integrity and sperm motility was assessed.

MATERIALS AND METHODS

Oocyte and semen preparation

Oocytes were derived from bovine ovaries randomly collected at a local abattoir and prepared following the protocol of Tanghe et al. (2004*a*). Immature cumulus-oocyte complexes (COC) were aspirated from follicles with a diameter ranging from 2 to 8 mm. Only COCs displaying a multilayered compact cumulus and a homogeneous ooplasm were selected. Frozen-thawed bull semen from the same ejaculate was used for all inhibition

experiments. Straws were thawed in a water bath (37°C) for 60 s. Subsequently, the semen was centrifuged on a discontinuous Percoll gradient (90% and 45%; Pharmacia, Uppsala, Sweden) as described by Thys et al. (2009*a*).

Chemicals and media were analogous to those used by Tanghe et al. (2004*a*; for details see Addendum). Vitronectin from bovine plasma (V9881) used in all experiments was purchased from Sigma-Aldrich (Bornem, Belgium).

Removal of the zona pellucida

The cumulus oophorus of the matured COCs was removed mechanically by vortexing (8 min). Subsequently, the cumulus-denuded oocytes were incubated in 0.1% protease (P5147, Sigma-Aldrich, Bornem, Belgium) in phosphate-buffered saline (PBS) for 5 to 15 min at 37°C to dissolve their zona pellucida (ZP). Afterwards the oocytes were washed and transferred to the incubator to allow recovery of the oolemma for at least 30 min (Tanghe et al., 2004*a*).

Fixation and staining of oocytes

After fertilization, the presumed zygotes were fixed overnight (2% paraformaldehyde – 2% glutaraldehyde in PBS) and stained with 10 µg mL^{-1} Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) for 10 min. Zygotes were mounted in a droplet of glycerol with (25 mg mL^{-1}) 1,4-diazabicyclo (2.2.2) octane (DABCO; Acros, Ghent, Belgium) and evaluated using a Leica DMR fluorescence microscope (Leica Microsystems, Groot-Bijgaarden, Belgium). The presence of two pronuclei was indicative for a successful fertilization of the oocyte. Penetration percentage was defined as the sum of the fertilization and polyspermy (more than two pronuclei) percentage per experimental group.

Dose-response effect of Vn on sperm penetration after bovine IVF

To reconfirm whether the Vn concentration (500 nM) applied in the study of Tanghe et al. (2004*b*) was the most appropriate, a preliminary experiment was conducted to assess the dose-respons effect of Vn on sperm penetration after bovine IVF. *In vitro* matured COCs were randomly assigned to 6 different fertilization media (Tanghe et al., 2004*a*): fertilization medium supplemented with 0 nM, 10 nM, 100 nM, 250 nM, 500 nM and 1 µM Vn. The oocytes were co-incubated with sperm at a final concentration of 10^6 spermatozoa mL^{-1} for 20

h (39°C; 5% CO_2). Prior to overnight fixation (2% paraformaldehyde – 2% glutaraldehyde in PBS) and staining with 10 µg mL^{-1} Hoechst 33342 for 10 min, the presumed zygotes were vortexed for 3 min to remove excess spermatozoa and/or cumulus cells. Zygotes were mounted in glycerol with 25 mg mL^{-1} DABCO and evaluated for sperm penetration using a Leica DMR fluorescence microscope.

Effect of Vn on sperm penetration of the cumulus oophorus

In vitro matured COCs were randomly assigned to 4 groups (3 replicates). Half of the oocytes (2 groups) were denuded by vortexing (cumulus denuded or CD) and the other half (2 groups) were kept cumulus-enclosed (CE). Both CD and CE oocytes were fertilized under control conditions (in standard fertilization medium) and in the presence of 500 nM Vn (Tanghe et al., 2004b). The oocytes were coincubated with sperm at a concentration of 10^6 sp mL^{-1} for 20 h (39°C; 5% CO_2). Each group contained 76 to 100 oocytes. Prior to fixation the presumed zygotes were vortexed to remove excess spermatozoa. After staining, the oocytes were evaluated for fertilization and polyspermy.

Effect of Vn on sperm-zona binding

In vitro matured COCs were denuded by vortexing and randomly assigned to two groups (4 replicates). The first group was fertilized under control conditions (in standard fertilization medium), the second group in the presence of 500 nM Vn. The oocytes were coincubated with sperm at a concentration of 10^5 sp mL^{-1}. This sperm concentration was previously determined in order to allow unambiguous counting of the number of spermatozoa bound to the ZP. Each group consisted of 77 to 100 oocytes. After 20 h of coincubation, the oocytes were washed 3 times to remove loosely attached spermatozoa, and subsequently fixed and stained with Hoechst 33342. Per presumed zygote the number of spermatozoa bound to the ZP was determined.

Effect of Vn on sperm-oolemma binding

The sperm pellet – obtained after Percoll® centrifugation – was diluted with fertilization medium to a concentration of 5 x 10^5 sp mL^{-1} and incubated for 30 min (39°C; 5% CO_2) to allow capacitation of the sperm cells. Subsequently, the acrosome reaction was

induced by incubation of the sperm suspension for 15 min (39°C; 5% CO_2) in 100 µg mL^{-1} lysophosphatidyl choline (LPC; L5004, Sigma-Aldrich, Bornem, Belgium).

In vitro matured ZP-free oocytes (3 replicates) were randomly assigned to two different media: standard fertilization medium or fertilization medium supplemented with 500 nM Vn. The female gametes were coincubated with sperm at a final concentration of 2.5 x 10^5 sp mL^{-1} in 50 µl droplets of medium (10 oocytes per droplet) covered with paraffin oil (Tanghe et al., 2004*a*). The number of oocytes per experimental group ranged from 28 to 44. One hour after insemination the oocytes were washed 3 times to remove loosely attached spermatozoa, fixed and stained. Of each presumed zygote the number of spermatozoa bound to the oolemma was evaluated.

Effect of Vn on sperm-oocyte fusion

The experimental setup was identical to the one described above, except that the ZP-free oocytes were fixed 20 h after insemination. All presumed zygotes were evaluated for sperm-oolemma fusion (defined as the presence of two or more pronuclei).

Localization of endogenous Vn on female and male bovine gametes

With respect to the female bovine gamete, immature COCs, *in vitro* matured COCs, CD oocytes as well as ZP-free oocytes were sampled following the protocol of Tanghe et al. (2004*a*). Cumulus cells were removed mechanically by vortexing (8 min), and the ZP was dissolved by incubation of the CD oocytes in 0.1% protease in PBS for 5 to 15 min (at 37°C). Since Vn is an extracellular matrix protein, expression of Vn was also analyzed in cumulus monolayers that were grown *in vitro* for a week as described before (Vandaele et al., 2007). All female gamete samples were fixed with 4% paraformaldehyde (P6118, Sigma-Aldrich, Bornem, Belgium) in PBS for 1 h (4°C) and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, Bornem, Belgium) in PBS for 30 min at room temperature (RT). Subsequently, they were incubated with 10% goat serum (16210-064, Invitrogen, Merelbeke, Belgium) in polyvinyl pyrrolidone (PVP, 0.1% in PBS) solution for 30 min (37°C), with mouse monoclonal antibody A18 to Vitronectin (Abcam, Cambridge, UK) (1/300) for 1 to 2 h (37°C) and with goat-anti-mouse FITC antibody (Molecular Probes, Leiden, The Netherlands) (1/100) for 1 h (37°C). To stain the nuclei, all oocyte types were treated with 2% Propidium Iodide (Molecular Probes, Leiden, The Netherlands) in PBS for 30 min. Between each treatment the samples were washed in PVP. They were mounted in a droplet of glycerol with

25 mg mL^{-1} DABCO and evaluated for the presence of Vn using a Leica DM/RBE laser scanning confocal fluorescence microscope (Leica Microsystems, Groot-Bijgaarden, Belgium).

To evaluate the expression of Vn on the male gamete, frozen-thawed semen was thawed in water of 37°C for 60 s and centrifuged on a discontinuous Percoll® gradient. Next, the sample was split into 3 fractions. Fraction 1 was diluted to a concentration of $10x10^6$ sp mL^{-1} (with medium consisting of a HEPES-buffered Tyrode balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg mL^{-1} gentamycin sulphate) prior to indirect immunofluorescence, and represented non-treated (NT) sperm. Fraction 2 and 3 were diluted to a concentration of $5x10^6$ sp mL^{-1} (with medium containing Tyrode balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg mL^{-1} gentamycin sulphate, 6 mg mL^{-1} fatty acid-free BSA, and 20 µg mL^{-1} heparin) and subsequently incubated for 30 min (39°C; 5% CO_2) to induce capacitation. Then, fraction 2 (representing capacitated – CAP – sperm) was processed in the same way as fraction 1. Fraction 3 was supplemented with 100 µg mL^{-1} LPC and incubated for 15 min (39°C; 5% CO_2) in order to induce the acrosome reaction (acrosome reacted – AR – sperm). All three sperm fractions were fixed with 1% paraformaldehyde (in PBS) for 30 min (at 4°C) and permeabilized with 0.5% Triton X-100 in PBS for 30 min (at RT). Subsequently, they were incubated with 10% goat serum in PVP for 30 min (37°C), with mouse monoclonal antibody A18 to Vitronectin (1/300) for 1 to 2 h (37°C) and with goat-anti-mouse FITC antibody (1/100) for 1 h (37°C). To stain the nuclei, all sperm fractions were treated with 10 µg mL^{-1} Hoechst 33342 for 10 min (RT). Between each treatment the sperm fractions were centrifuged (10 min, 200g) and re-suspended in PVP. They were mounted in glycerol with 25 mg mL^{-1} DABCO and evaluated for the presence of Vn using fluorescence microscopy (Olympus IX81 inverted fluorescence microscope and a Hamamatsu Orca B/W camera using Olympus Cell*R software, Aartselaar, Belgium) and flow cytometry (FacsCanto II, BD, Belgium). Additionally, frozen-thawed semen originating from the same ejaculate was stained to evaluate Vn-expression (as described above) without previous fixation and permeabilization. The latter samples were processed on ice.

The mouse monoclonal antibody A18 is claimed to be highly specific for vitronectin, since there is no evidence for cross reactivity with other connective tissue proteins (fibronectin, elastin, collagen, laminin). Nevertheless, a negative control was included: a sample incubated with an isotype-matched mouse IgG1 antibody prior to the FITC-labeled secondary goat-anti-mouse antibody treatment. To check for unspecific binding of the FITC-

labeled secondary antibody, an additional negative control was prepared: a sample processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody.

Localization of α_v (subunit of the Vn integrin receptor) on female and male bovine gametes

With respect to the female bovine gamete, *in vitro* matured CD oocytes were sampled (as described above) and fixed with 2% paraformaldehyde in PBS for 30 min (4°C) prior to indirect immunofluorescence.

To assess the presence of α_v on the male gamete, frozen-thawed semen originating from the same ejaculate was centrifuged on a discontinuous Percoll gradient, and the sperm pellet was diluted to a concentration of $10x10^6$ sp mL^{-1}. Subsequently, the sample was split into 3 fractions. Each fraction was processed as described before, resulting in a non-treated (NT), capacitated (CAP) and acrosome reacted (AR) sperm fraction. All sperm samples were fixed with ice-cold methanol during 15 min.

This time, the primary antibody used was rabbit polyclonal antibody to integrin subunit α_v (AB1930; Chemicon – Millipore, Belgium) (1/100), which was fluorescently labeled with goat anti-rabbit FITC antibody (Molecular Probes, Leiden, The Netherlands) (1/100). The primary antibody is guaranteed to have no-cross reactivity with α_1, α_2, α_3, α_4 or α_6 integrin subunits. To evaluate the specificity of the rabbit polyclonal antibody to integrin subunit α_v, a sample incubated with heat-inactivated rabbit serum prior to the FITC-labeled secondary antibody treatment was included as negative control. To check for unspecific binding of the FITC-labeled secondary antibody, an additional negative control was prepared: a sample processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody.

Effect of sperm incubation with Vn on membrane integrity and sperm motility

Frozen-thawed bull semen originating from the same ejaculate (3 replicates) was centrifuged on a discontinuous Percoll gradient and diluted to a concentration of $60x10^6$ spermatozoa mL^{-1} (with medium containing Tyrode balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 10 µg mL^{-1} gentamycin sulphate, 6 mg mL^{-1} fatty acid-free BSA, and 20 µg mL^{-1} heparin). Subsequently, the sperm suspension was split into three fractions, which were diluted (1:1) respectively with the modified Tyrode balanced salt solution (control), modified Tyrode balanced salt solution supplemented with 200 nM Vn (100 nM Vn) and modified Tyrode balanced salt solution

supplemented with 1 μM Vn (500 nM Vn). Three aliquots from each sperm fraction were incubated (39°C; 5% CO_2), and at three different time points of incubation (1 h, 3 h and 6 h, respectively) one aliquot per fraction was evaluated for membrane integrity and total versus progressive sperm motility.

Membrane integrity was evaluated using a fluorescent SYBR14-Propidium Iodide (PI) staining technique (L7011; Molecular Probes, Leiden, The Netherlands). A stock solution of 1 mmol L^{-1} SYBR14 reagent was diluted (1:50) in HEPES-TALP, stored frozen at –20°C and thawed just before use. From each sperm aliquot, 100 μl was used and 1 μL SYBR14 was added. After 5 min of incubation (at 37°C), 1 μL PI was added prior to another 5 min incubation (at 37°C). Per aliquot 200 spermatozoa were examined using a Leica DMR fluorescence microscope. Three populations of sperm cells were identified: living (membrane intact; stained green), dead (membrane damaged; stained red), and moribund (double stained; green-orange) spermatozoa. The moribund sperm cells were considered to be part of the dead sperm population.

Total and progressive motility were determined by means of computer-assisted sperm analysis (Hamilton-Thorne CEROS 12.3) (Tanghe et al., 2004*a*).

Statistical analyses

Differences in fertilization and penetration percentages, and differences in number of Vn-positive cells were analyzed by means of binary logistic regression (including the effect of replicate). To evaluate the differences in mean number of spermatozoa bound to the ZP, the non parametric Kruskal Wallis test was applied, since the concerning variable was not normally distributed. Differences in mean number of sperm cells binding the oolemma were analyzed using ANOVA. Differences in membrane integrity and (total and progressive) sperm motility were evaluated using repeated measures analysis of variance. Hypothesis testing was performed using a significance level of 5% (2-sided test) and results were cited as mean ± S.E.M. (SPSS 15.0).

RESULTS

Dose-response effect of Vn on sperm penetration after bovine IVF

Compared to the control (0 nM Vn), sperm penetration significantly improved when supplementing 100 nM Vn during IVF ($P<0.05$; Fig.16). Sperm penetration was significantly

inhibited in a concentration-dependent manner starting from 500 nM Vn. This suggests that at higher concentrations, the inhibiting effect of Vn dominates the beneficial effect observed at lower concentrations. In order to determine the mechanism underlying the inhibitory effect of Vn on sperm penetration, subsequent experiments were performed using 500 nM Vn.

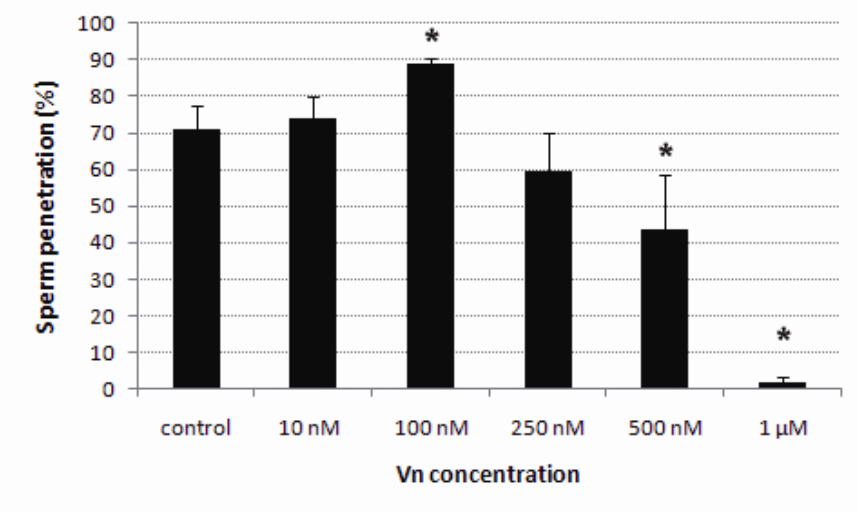


Figure 16: Dose-response effect of vitronectin (Vn) on sperm penetration after bovine IVF. Data represent mean ± SEM. *Values significantly different from control with 0 nM Vn ($P < 0.05$).

Effect of Vn on sperm penetration of the cumulus oophorus

When 500 nM Vn was supplemented to the fertilization medium, sperm penetration percentages and fertilization percentages in both CE and CD oocytes decreased significantly compared to the respective control group (Table 3). The difference in reduction of sperm penetration was not statistically significant when comparing the CE and the CD groups ($P=0.106$). Nevertheless, considering the small sample size ($n=6$), the mean difference of 30.2% in inhibition of penetration between cumulus-enclosed and cumulus-denuded groups suggests a relevant effect of cumulus denudation.

Effect of Vn on sperm-zona binding

The number of spermatozoa bound to the ZP in the Vn supplemented group was slightly but significantly higher than that in the control group (50.1 ± 2.1 versus 42.9 ± 1.9 spermatozoa per oocyte; $P<0.05$).

Table 3: Fertilization, polyspermy and penetration percentages of cumulus-denuded (CD) and cumulus-enclosed (CE) oocytes inseminated in standard fertilization medium and in fertilization medium supplemented with 500 nM of vitronectin.

Oocytes	Vitronectin (nM)	No.	Fertilization (%)	Polyspermy (%)	Penetration (%)	Inhibition of penetration (%)
CD	0	249	$15.0^{a} \pm 2.06$	$0.4^{a} \pm 0.43$	$15.4^{a} \pm 1.86$	-
	500	258	$1.6^{b} \pm 1.60$	$0.4^{a} \pm 0.40$	$2.0^{b} \pm 1.44$	87.0
CE	0	296	$62.9^{a} \pm 7.23$	$7.7^{a} \pm 1.04$	$70.6^{a} \pm 7.71$	-
	500	273	$26.8^{b} \pm 12.16$	$3.7^{b} \pm 0.87$	$30.5^{b} \pm 12.42$	56.8

[a,b] Values with a different superscript in the same column within the CD and the CE groups differ significantly ($P<0.05$).

Effect of Vn on sperm-oolemma binding

Vitronectin supplementation did not significantly influence the sperm-oolemmabinding. However, a slight numerical decrease in sperm adherence (from 27.4 ± 1.9 to 23.0 ± 2.8 spermatozoa per oocyte) was observed in the presence of Vn ($P>0.05$).

Effect of Vn on sperm-oocyte fusion

A significant decrease in fertilization percentage (from 25.4% to 14.2%) and sperm penetration percentage (from 28.0% to 16.0%) was found when 500 nM Vn was supplemented during IVF ($P<0.05$). Compared to the ZP-free control group, the sperm penetration was inhibited with 42.9%.

Localization of endogenous Vn on female and male bovine gametes

After fixation and permeabilization, the percentage of Vn positive sperm cells was very high and consistent in all three sperm fractions (≥ 99.4% positive cells for NT, CAP and AR sperm). However, the intensity of fluorescence was 3 times higher in the AR group compared to the NT and CAP group (Table 4) and the predominant fluorescent pattern observed in the AR fraction (Fig.17E – fluorescence at the acrosomal region and midpiece) also differed from the one mainly observed in the NT and CAP groups (Fig.17A and 17C – fluorescence at the postacrosomal region and midpiece). When using fluorescence microscopy

for the evaluation of unfixed spermatozoa, fluorescence was only observed in the AR fraction, displaying a green signal at the apical sperm head region (Fig.17F). No fluorescence could be visualized in the NT and CAP sperm fractions (Fig.17B-D). Subsequently, flow cytometry (a far more sensitive technique) was applied, resulting in detection of fluorescent spermatozoa in all three sperm fractions: 5.8% in NT sperm, 14.4% in CAP sperm and 49.5% in AR sperm respectively. The intensity of fluorescence was much lower compared to the equivalent fixed and permeabilized sperm fractions (Table 4).

Table 4: Percentage of vitronectin positive cells, mean and median of relative fluorescence intensity per sperm fraction after flow cytometric evaluation of frozen-thawed bovine sperm cells labeled with mouse monoclonal to vitronectin and goat-anti-mouse FITC.

Treatment	Sperm fraction	No.	Positive cells (%)	Mean	Median
+ fixation + permeabilization	NT	10 000	99.4	1586	1452
	CAP	10 000	99.7	1458	1226
	AR	10 000	99.8	4635	4728
- fixation - permeabilization	NT	10 000	5.8	134	116
	CAP	10 000	14.4	172	139
	AR	10 000	49.5	284	214

NT: non-treated sperm; CAP: capacitated sperm; AR: acrosome-reacted sperm.

With respect to the cumulus monolayer, the cytoplasm of approximately 100% of the cumulus cells stained positively for Vn, and fluorescent mesh forming structures were observed in the extracellular matrix (Fig.18A). In immature COCs a relatively small number of cumulus cells expressed Vn in their cytoplasm. After *in vitro* maturation, the number of Vn positive cumulus cells was considerably increased in the COCs (Fig.18B). Cumulus-denuded oocytes appeared to express Vn on the surface of the ZP, including fluorescent spurs penetrating the ZP (Fig.19). Protease treated (ZP-free) oocytes did not show membrane expression.

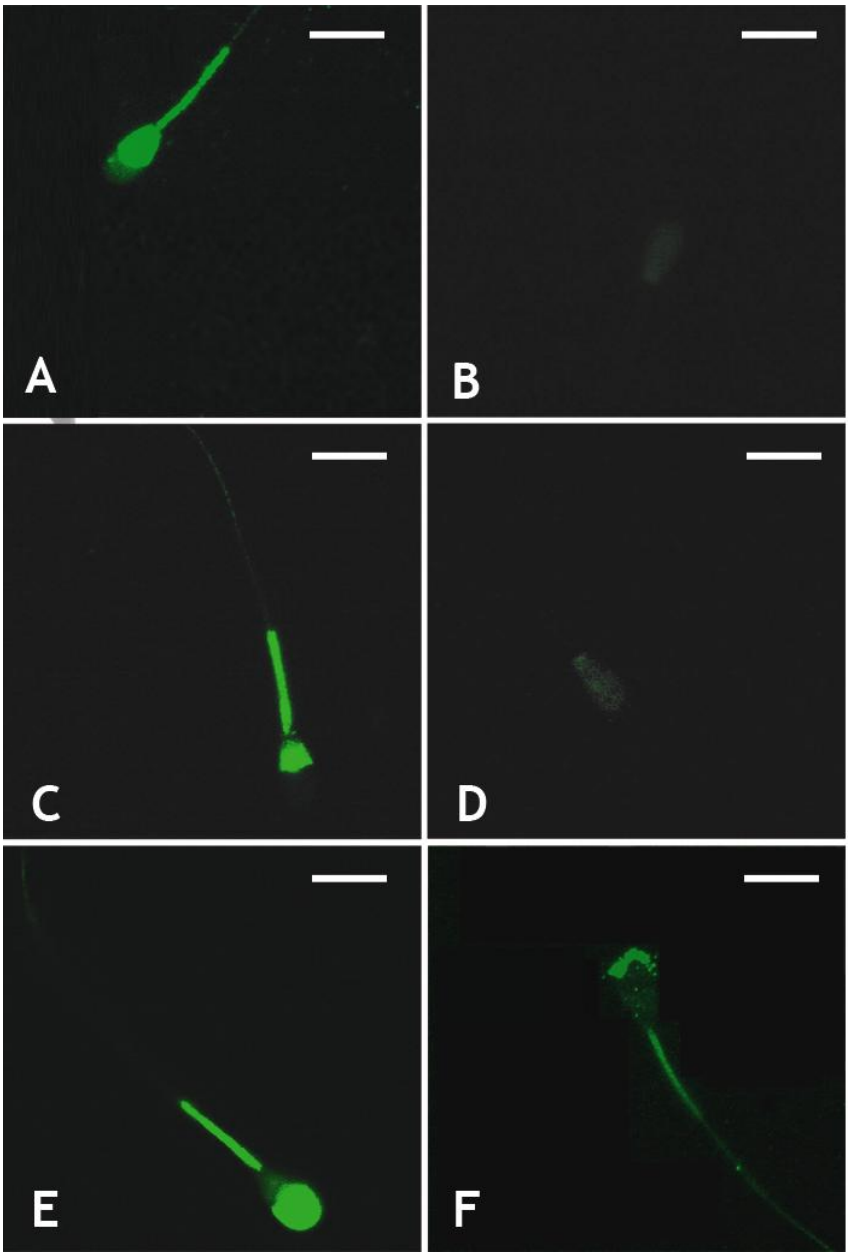


Figure 17: Fluorescent images of frozen-thawed bovine semen labeled with mouse monoclonal to vitronectin and goat-anti-mouse FITC after fixation and permeabilization (A, C and E) and without prior fixation and permeabilization (B, D and F) (Original Magnification x600; Bar = 10 μm) (A-B): non-treated sperm; (C-D): capacitated sperm; (E-F): acrosome reacted sperm.

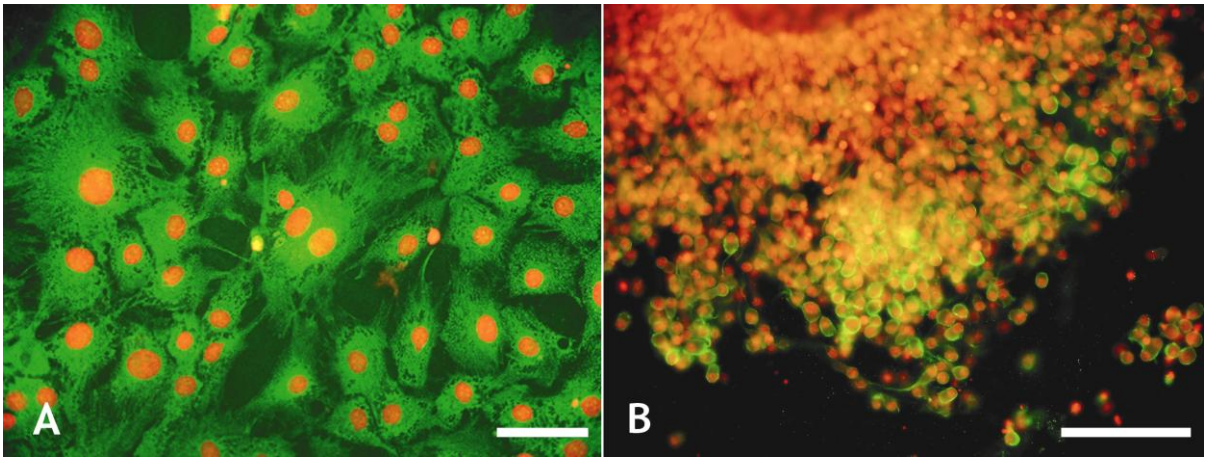


Figure 18: Indirect immunofluorescent staining with mouse monoclonal to vitronectin (Vn) and goat-anti-mouse FITC combined with Propidium Iodide staining after fixation and permeabilization, (A): cumulus cell monolayer (Original Magnification x600; Bar = 25 µm), (B): *in vitro* matured COC (Original Magnification x400; Bar = 50 µm).

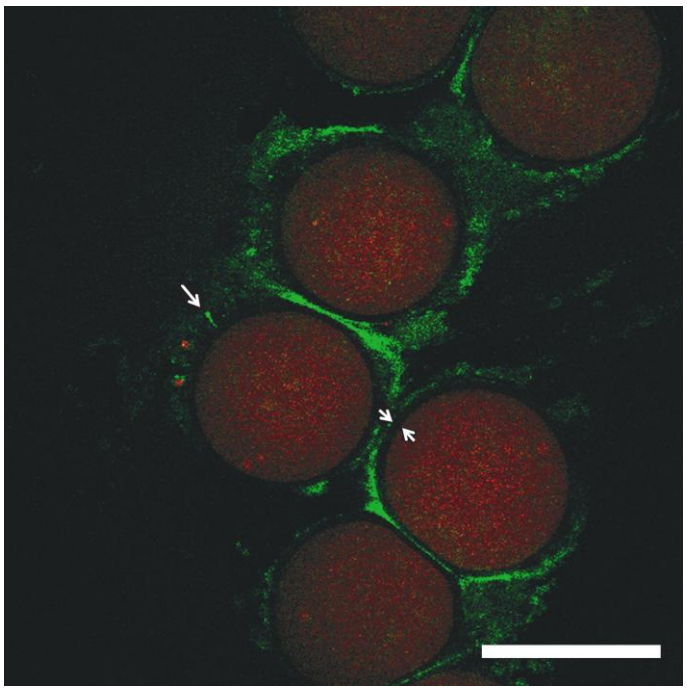


Figure 19: Confocal fluorescent image of cumulus-denuded ZP intact bovine oocytes labeled with mouse monoclonal to vitronectin (Vn) and goat-anti-mouse FITC combined with Propidium Iodide staining after fixation and permeabilization (Original Magnification x200; Bar = 100 µm).

⇨ fluorescent spur penetrating the ZP;

⇨ ⇦ ZP

Localization of α_v (subunit of the Vn integrin receptor) on female and male bovine gametes

After fixation and permeabilization, all sampled sperm cells stained positively, irrespective of their functional state (NT, CAP or AR sperm). All spermatozoa displayed the same fluorescent pattern at their apical surface (Fig.20A, 20C and 20E). When staining NT, CAP and AR sperm without prior fixation and permeabilization, no integrin expression was visually observed in the NT group (Fig.20B). The CAP sperm fraction showed faint fluorescence (Fig.20D), whereas a bright signal was detected in the AR sperm cells (Fig.20F). Flow cytometric evaluation of unfixed sperm cells confirmed these subjective observations (Table 5). The number of integrin subunit α_v positive cells increased after heparin treatment, whereas the relative fluorescence intensity was substantially increased after artificial induction of the acrosome reaction.

However, incubation of the same *fixed* sperm fractions with heat-inactivated rabbit serum - instead of the primary rabbit polyclonal antibody - appeared to induce a similar fluorescent pattern in NT and CAP sperm cells (data not shown).

Table 5: Percentage of integrin subunit α_v positive cells, mean and median of relative fluorescence intensity per sperm fraction after flow cytometric evaluation of unfixed frozen-thawed bovine sperm cells labeled with rabbit polyclonal to integrin subunit α_v and goat-anti-rabbit FITC.

Sperm fraction	No.	Positive cells (%)	Mean	Median
NT	10 000	55.7	5105	4964
CAP	10 000	69.4	6373	6273
AR	10 000	69.9	8018	7808

NT: non-treated sperm; CAP: capacitated spermatozoa; AR: acrosome-reacted sperm.

Integrin subunit α_v was detected in *in vitro* matured CD bovine oocytes (Fig.21). The fluorescent pattern varied between the sampled cells: some oocytes (12.5%) expressed integrin subunit α_v only at their oolemma (Fig.21A), whereas the greater part (87.5%) appeared to express the subunit of the Vn receptor also at (the exterior side of) the ZP, including fluorescent spurs penetrating the ZP (Fig.21B). ZP-free bovine oocytes displayed green fluorescent spots at their surface.

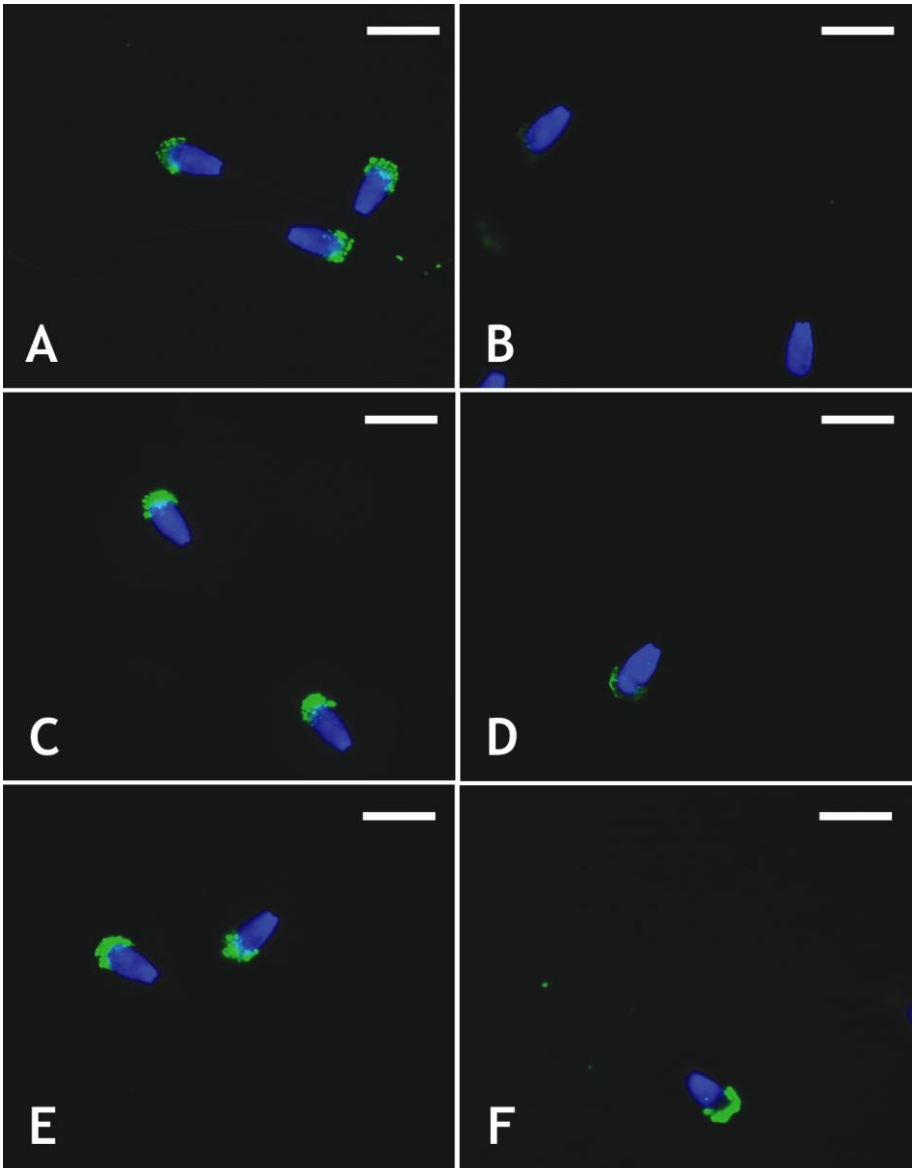


Figure 20: Fluorescent images of frozen-thawed bovine semen labeled with rabbit polyclonal antibody to integrin subunit α_v and goat-anti-rabbit FITC combined with Hoechst 33342 staining after fixation (A-C-E) and without prior fixation (B-D-F) (Original Magnification x600; Bar = 10 µm). (A-B): non-treated sperm; (C-D): capacitated sperm; (E-F): acrosome reacted sperm.

Effect of sperm incubation with Vn on membrane integrity and sperm motility

Sperm membrane integrity was negatively affected in the presence of 500 nM Vn ($P < 0.05$), but was not altered by sperm incubation with 100 nM Vn (Fig.22). Total and progressive motility differed significantly ($P < 0.05$) between all sampled groups (control, 100 nM Vn and 500 nM Vn; Fig.23). However, especially the substantial twofold decrease in

progressive motility in presence of 500 nM Vn should be considered as an important factor contributing to the inhibitory effect of Vn supplementation on sperm penetration during IVF.

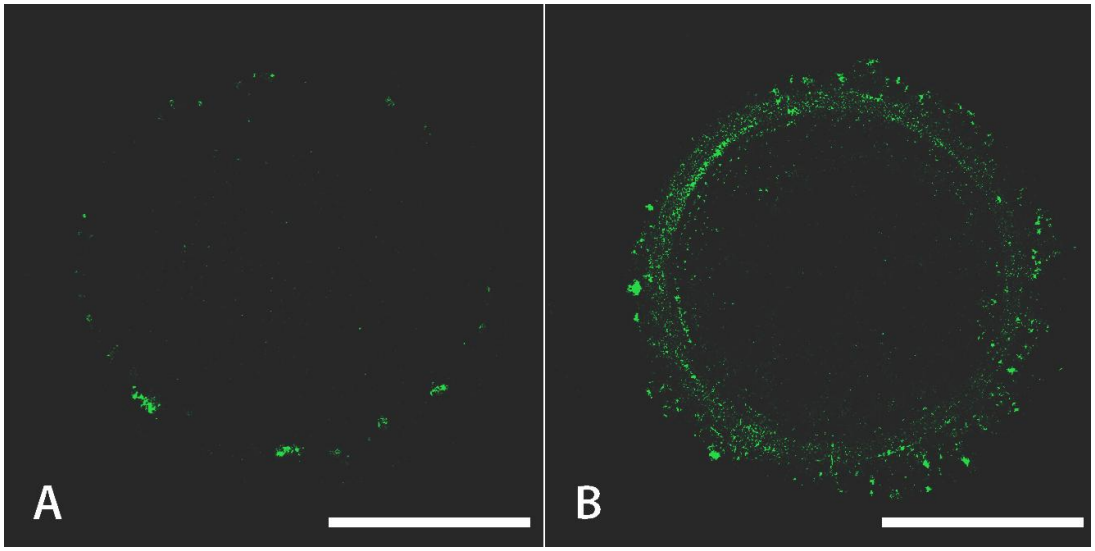


Figure 21: Confocal fluorescent images of cumulus-denuded ZP intact bovine oocytes labeled with rabbit polyclonal antibody to integrin subunit α_v and goat-anti-rabbit FITC after fixation, (A): fluorescent signal confined to the oolemma, (B): fluorescent signal at the level of the oolemma, the ZP and exterior side of the ZP (Original Magnification x400; bar = 50 µm).

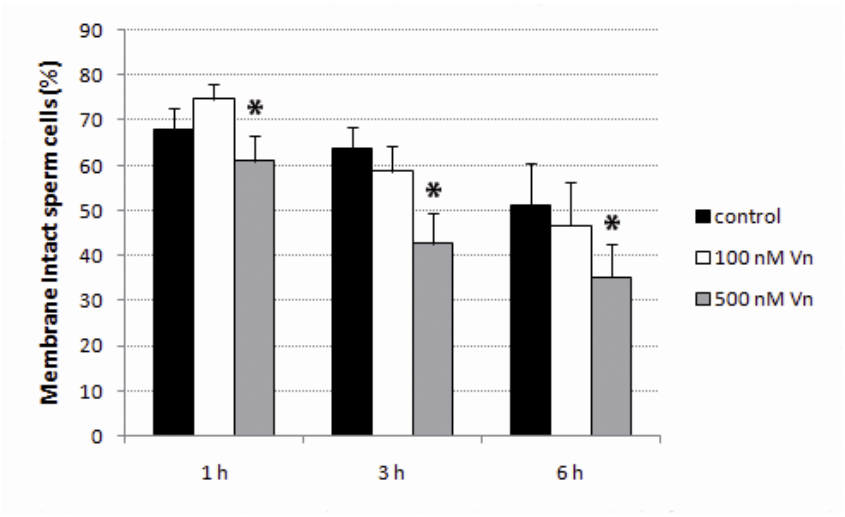


Figure 22: Effect of 100 nM and 500 nM vitronectin on membrane integrity of bovine frozen-thawed spermatozoa during incubation (evaluated by means of SYBR14-PI staining). Data represent mean ± SD. *Values significantly different from control with 0 nM Vn ($P < 0.05$).

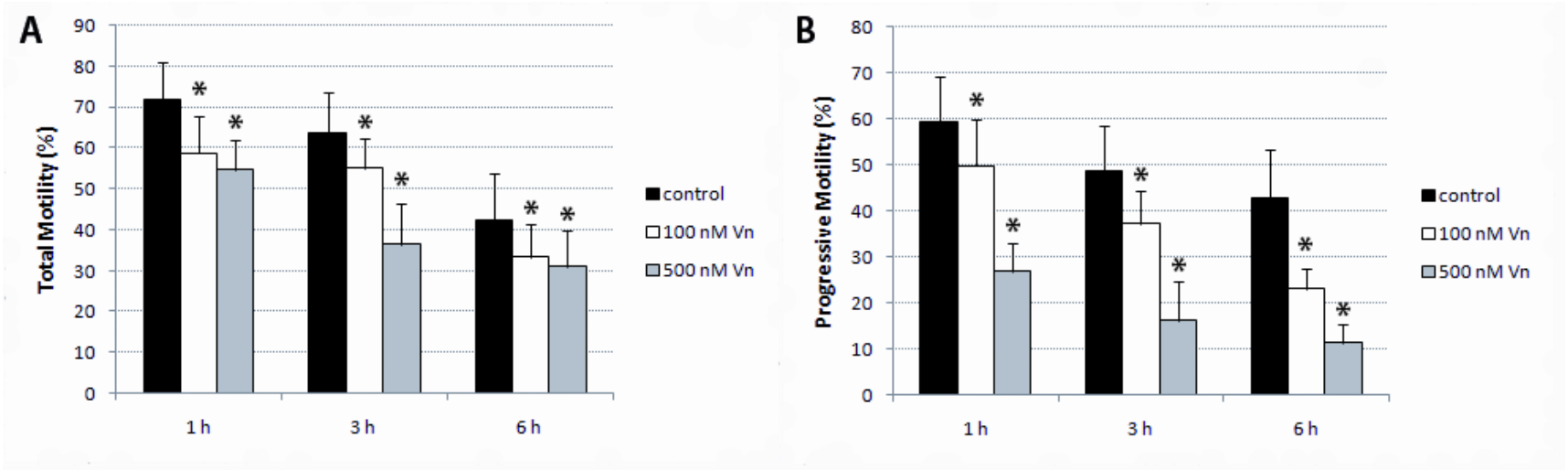


Figure 23: Effect of 100 nM and 500 nM vitronectin on total motility (A) and progressive motility (B) of bovine frozen-thawed spermatozoa during incubation (evaluated by means of CASA). Data represent mean ± SD. *Values significantly different from control with 0 nM Vn ($P < 0.05$).

DISCUSSION

In cattle, cumulus cells are shed from the oocyte in the oviduct within a few hours (Lorton and First, 1979) to 10 h after ovulation (Hyttel et al., 1988). However, it is not entirely clear whether the cumulus cells or the matrix are necessary for bovine fertilization *in vivo*: sperm cells probably need to penetrate the cumulus matrix first, before they can pass through the ZP and subsequently fuse with the oolemma (Van Soom et al., 2002). The molecular basis of each of these processes has not been resolved yet, but it is now accepted that sperm-egg binding requires a multitude of receptor-ligand interactions (Lyng and Shur, 2007). In the present study, we have confirmed that high concentrations of the extracellular matrix glycoprotein Vn had a negative effect on bovine sperm-oocyte interaction (Tanghe et al., 2004*b*). Furthermore, the main inhibitory effect of 500 nM exogenous Vn was observed at the level of sperm-oolemma fusion, implicating that Vn might be one of the ligands involved in sperm-egg recognition in cattle.

Vitronectin seemed to strongly reduce sperm penetration in CD oocytes, but only moderately so in CE oocytes. These results suggest that the cumulus oophorus is able to capture a substantial part of the supplemented Vn allowing still a fair sperm penetration rate. Vitronectin (serving as a cell-to-substrate adhesion molecule) is likewise known to interact with glycosaminoglycans and proteoglycans and is recognized by certain members of the integrin family (http://www.uniprot.org/uniprot/P04004). Integrin expression has been observed in bovine cumulus cells before (Sutovsky et al., 1995).

A slightly augmented number of spermatozoa binding the ZP was noted in the presence of 500 nM Vn. This was an intriguing finding, since despite the increased sperm binding in the presence of Vn, oocyte penetration was substantially reduced. In mice, it has been demonstrated that sperm binding to the ZP is not sufficient to induce acrosomal exocytosis (Baibakov et al., 2007). The actual sperm passage through the pores of the ZP is believed to mechanically trigger the acrosome reaction: contact between motile sperm and the small ZP-pores would generate sufficient shear force to bring forth a mechanosensory signal and acrosomal exocytosis. Binding to the ZP is suggested to slow down the forward progression of motile sperm and the forceful thrusting of the tail in order to transduce a mechanosensory signal mobilizing acrosomal Ca^{2+} stores and – consequently – to induce the acrosome reaction (Baibakov et al., 2007). The substantial twofold decrease in progressive sperm motility noted in presence of 500 nM Vn may therefore well be responsible for a defective sperm-ZP penetration. Furthermore, considerable head-to-head agglutination was observed when

incubating bovine spermatozoa in the presence of Vn, especially at the high concentration of 500 nM (data not shown). Probably, the sperm is able to bind the ZP (assisted by the exogenous Vn connecting the α_v integrin at the sperm cell surface to the α_v integrin at the exterior side of the ZP), but is not capable of successful penetration of the ZP. When supplementing low concentrations of Vn (100 nM), sperm penetration of COCs was enhanced, possibly through the increased sperm-ZP binding. Compared to 500 nM Vn, such low doses did not affect sperm membrane integrity and did not have the same impact on progressive sperm motility. Possibly, the forward progression of these ZP bound sperm cells was still sufficient for proper penetration.

A reversible dual binding function connecting both the male and female gamete – as suggested in human by Fusi et al. (1996*b*) – is even more plausible in the bovine species, since ruminant Vn apparently displays two integrin binding RGD sequences, in contrast to only one RGD site in human Vn (Suzuki et al., 1985; Mahawar and Joshi, 2008). Furthermore, the presumed reversible dual binding function could additionally be exerted through spontaneous multimerization of several Vn molecules as described by Stockmann et al. (1993). The C-terminal half of the molecule comprises two hemopexin-like domains, able to interact with the acidic residues of the connecting segment of the same molecule, consequently allowing intramolecular and intermolecular linking to form Vn polymers (Royce and Steinmann, 2002).

Vitronectin supplementation to the fertilization medium did not lead to a statistically significant inhibition of the sperm-oolemma binding in our experiment. Fusi et al. (1996*b*) previously suggested that Vn was well suited to play a significant role in human sperm-egg adhesion. These authors found a promotion of oolemmal adherence of spermatozoa, following addition of Vn to the medium over a certain concentration range. Supplementation of Vn enhanced oolemmal adherence of spermatozoa over a concentration range of 2.2 nM to 100 nM (Fusi et al., 1996*b*). Higher Vn concentrations – like in the present study – reduced the number of spermatozoa adhering to the egg (Fusi et al., 1996*b*), possibly due to vitronectin-mediated sperm aggregation within the culture dish. During the sperm incubation experiment, substantial head-to-head agglutination was observed after 4 h incubation in the presence of 100 nM and the agglutination was even more distinct when supplementing 500 nM Vn. Nevertheless, we could merely detect a slight – statistically insignificant – decrease in sperm-oolemma binding during bovine IVF when 500 nM Vn was supplemented to the fertilization medium. However, the physiological relevance of the number of sperm cells bound to a ZP-free oocyte is debatable (Talbot et al., 2003). The underlying assumption is that spermatozoa

are bound to the oolemma by a mechanism that can result in sperm-egg fusion. This *in vitro* assay may – though – include a heterogeneous population of bound sperm cells, including non-physiologically bound sperm, besides the specific population of physiologically relevant sperm that are tethered or docked before fusion. Some of the sperm cells may well be bound via interactions that will not result into fusion. Acrosome-intact sperm are – for instance – known to be able to bind to ZP-free oocytes, but not to fuse. If the non-specifically bound sperm fraction outnumbers the specifically bound fraction, variations in the number of physiologically relevant bound sperm will not be measured (Talbot et al., 2003).

In the present experiment, Vn supplementation inhibited 42.9% of sperm-oolemma fusion. Accordingly, Fusi et al. (1996*b*) observed a decrease in the number of penetrating sperm cells in human oocytes in the presence of increasing Vn concentrations starting from 100 nM.

Furthermore, a dual (concentration-dependent) effect of exogenous Vn supplementation on bovine IVF was observed. Low Vn concentrations (10 nM – 100 nM) appeared to enhance sperm penetration, whereas a negative influence was noted in the presence of high concentrations (500 nM – 1 µM). This inhibitory effect may well be – at least partially – due to compromised membrane integrity. Furthermore, the observed substantial twofold decrease in progressive motility in the presence of 500 nM Vn (compared to the control) should most likely be considered as an important factor contributing to the inhibitory effect of Vn supplementation on sperm penetration during IVF. Combining these results with the explicit head-to-head agglutination noted when incubating sperm with 500 nM of Vn, high concentrations of Vn should obviously be regarded as detrimental for successful fertilization.

As in human (Fusi et al., 1994), vitronectin also appears to be an intrinsic protein of bovine sperm cells (Fig.24A-B). After fixation and permeabilization, practically all sampled spermatozoa did show fluorescence when applying indirect immunofluorescence and flow cytometry. The observed shift in fluorescent pattern after LPC treatment suggests that the vitronectin sequestered inside the sperm head is exposed at the sperm cell surface after acrosomal reaction (Fig.24C). With respect to the biological relevance of the moderate increase of Vn expression in unfixed sperm cells, we have to note that LPC treatment does not induce acrosomal reaction in all spermatozoa of the samples. Only penetration of the sperm cells through the ZP leads to nearly 100 % acrosome reaction. LPC treatment typically induces an increase in the percentage of acrosome reacted sperm ranging from about 26% to 38% (compared to a negative control group) in frozen-thawed bovine semen (O'Flaherty et al., 2005). With respect to the expression of the α_v subunit of the Vn receptor, all fixed and

permeabilized sperm cells seemed to display fluorescence at their apical surface, irrespective of their functional state (NT, CAP or AR). However, incubation of the same *fixed* sperm fractions with heat-inactivated rabbit serum (instead of the primary rabbit polyclonal antibody to integrin subunit α_v) appeared to induce a similar fluorescent pattern in NT and CAP sperm cells (data not shown). Some degree of non-specific binding of the rabbit antibodies should therefore be acknowledged. Nevertheless, the finding that AR sperm cells did not show non-specific fluorescence (when using heat-inactivated rabbit serum) indicates that there is specific binding of the primary anti-integrin subunit α_v antibody as well. In addition, the equivalent *unfixed*, not permeabilized, sperm fractions displayed a different pattern. Only the AR fraction was visually fluorescent, which also supports the specificity of the primary antibody to integrin subunit α_v. The present findings, including a clear quantitative increase in fluorescence in unfixed AR spermatozoa measured by means of flow cytometry, are in accordance with the work of Fusi et al. (1996*a*) stating that α_v expression was maximal following ionophore-exposure, used as inducer of the acrosome reaction. They assumed that α_v may be located on the inner acrosomal membrane, which becomes accessible to anti-integrin subunit antibody both during the acromose reaction and following permeabilization of capacitated spermatozoa.

A fascinating observation was the abundant presence of endogenous Vn at the exterior side of the ZP including fluorescent spurs penetrating the ZP. Probably, the glycoprotein is bound to the $\alpha_v\beta_3$ integrin, of which the α_v subunit was also detected at the level of the ZP in a similar pattern. These fluorescent signals are assumed to be remnants of molecules present at the cell surface of corona radiata cells which have been shown to have cellular projections traversing the ZP and terminating upon the oolemma (Tanghe et al., 2002). Integrin expression has been observed in bovine cumulus cells before (Sutovsky et al., 1995), and these transmembrane receptors are assumed to be easily ripped out of the cell with bits of attached membrane, when internal anchorage with the cytoskeleton is disturbed (Alberts et al., 1994). This disconnection may have occurred through the mechanical force exerted on the cell by vortexing. Since integrin subunit α_v was already observed to some extent in CAP spermatozoa (69.4% of that sperm fraction), it could be speculated that capacitated sperm cells reversibly bind to the endogenous Vn at the level of the ZP resulting in penetration of this acellular egg vestment. The finding that low concentrations of exogenously supplemented Vn improve sperm penetration during bovine IVF, might be attributed to an additional reversible adhesion function exerted by this molecule. A similar function could be exerted by the endogenous Vn located at the level of the extracellular cumulus matrix, in this case

assisting the sperm cell in traversing the cumulus oophorus (Fig.24D). In order to elucidate the beneficial effect of low Vn concentrations on sperm penetration, further studies are required.

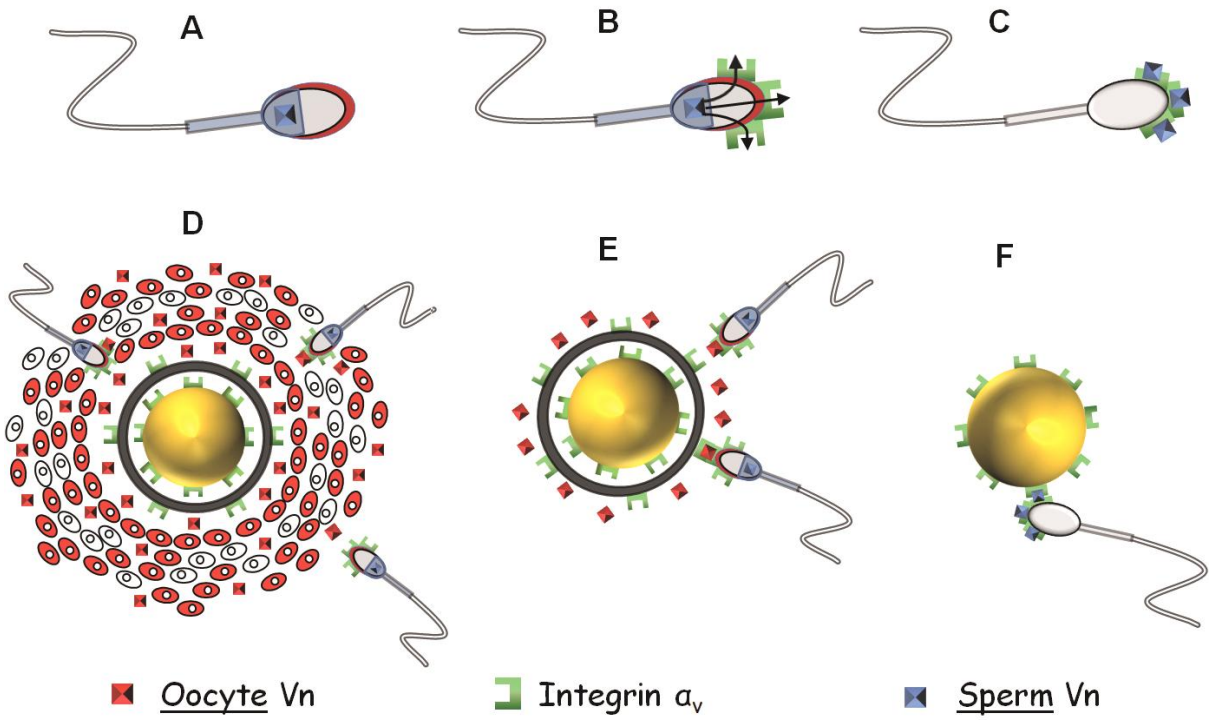


Figure 24: Hypothetical model concerning the function of endogenous vitronectin (Vn) during bovine IVF. (A): non-treated sperm cell, (B): capacitated sperm cell, (C): acrosome-reacted sperm cell, (D): capacitated spermatozoa traversing the cumulus oophorus, (E): spermatozoa binding the zona pellucida through endogenous oocyte Vn, (F): sperm-oocyte interaction through endogenous sperm Vn liberated after acrosome reaction.

Since integrin subunit α_v was present at the oolemma of ZP-free bovine oocytes and spermatozoa express Vn at their surface after acrosomal reaction, this receptor-ligand might play a role in sperm-oocyte interaction. To confirm this hypothesis additional experiments investigating whether supplementation of low Vn concentrations to an IVF system with ZP-free oocytes and acrosome reacted sperm effectively inhibit sperm penetration are necessary. Previously, detection of fibronectin (another extracellular matrix glycoprotein) underneath the ZP together with observed expression of the α_5 subunit of its corresponding receptor on oolemma and acrosome reacted sperm cell surface already indicated a reversible dual binding

interaction between the fibronectin ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating bovine sperm-egg binding (Thys et al., 2009*b*).

Bearing in mind the present findings, the following hypothesis can be put forward concerning the interaction of vitronectin during bovine fertilization. Detection of endogenous Vn and integrin subunit α_v at the exterior side of the ZP and integrin subunit α_v on the sperm cell surface, combined with an increased sperm-ZP binding in presence of exogenous Vn, suggests at least some intervention of this glycoprotein in initial sperm-ZP interaction (Fig.24E). Since the α_v subunit of the Vn receptor was identified on the oolemma, and spermatozoa appeared to express both integrin subunit α_v and Vn after acrosomal reaction, this receptor-ligand mechanism may play a role in sperm-oocyte interaction (Fig.24F). The inhibitory effect of exogenously supplemented Vn on sperm penetration of ZP-free oocytes could then be explained by the competition between the exogenous Vn and the Vn liberated from the sperm cell following the acrosome reaction. Further research is required to distinguish the dual effect of low versus high concentrations of exogenous Vn during bovine IVF.

REFERENCES

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. 1994. Cell Junctions, Cell Adhesion, and the Extracellular Matrix. In 'Molecular Biology of the Cell', Third Edition. (Eds B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson.) pp. 949-1009. (Garland Publishing: New York.)

Almeida EA, Huovila AP, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, White JM. 1995. Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. *Cell* **81** 1095-1104.

Amari S, Yonezawa N, Mitsui S, Katsumata T, Hamano S, Kuwayama M, Hashimoto Y, Suzuki A, Takeda Y, Nakano M. 2001. Essential role of the nonreducing terminal alpha-mannosyl residues of the N-linked carbohydrate chain of bovine zona pellucida glycoproteins in sperm-egg binding. *Mol Reprod Dev* **59** 221-226.

Baibakov B, Gauthier L, Talbot P, Rankin TL, Dean J. 2007. Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. *Development* **134** 933-943.

Benoff S. 1997. Carbohydrates and fertilization: an overview. *Mol Hum Reprod* **3** 599-637.

Bronson RA, Fusi FM. 1996. Integrins and human reproduction. *Mol Hum Reprod* **2** 153-168.

Bronson R, Peresleni T, Golightly M, Preissner K. 2000. Vitronectin is sequestered within human spermatozoa and liberated following the acrosome reaction. *Mol Hum Reprod* **6** 977-982.

Evans JP, Schultz RM, Kopf GS. 1995. Mouse sperm-egg plasma membrane interactions: analysis of roles of egg integrins and the mouse sperm homologue of PH-30 (fertilin) beta. *J Cell Sci* **108** 3267-3278.

Francois PP, Preissner KT, Herrmann M, Haugland RP, Vaudaux P, Lew DP, Krause KH. 1999. Vitronectin interaction with glycosaminoglycans. Kinetics, structural determinants, and role in binding to endothelial cells. *J Biol Chem* **274** 37611-37619.

Fusi FM, Lorenzetti I, Vignali M, Bronson RA. 1992. Sperm surface proteins after capacitation. Expression of vitronectin on the spermatozoan head and laminin on the sperm tail. *J Androl* **13** 488-497.

Fusi FM, Lorenzetti I, Mangili F, Herr JC, Freemerman AJ, Gailit J, Bronson RA. 1994. Vitronectin is an intrinsic protein of human spermatozoa released during the acrosome reaction. *Mol Reprod Dev* **39** 337-343.

Fusi FM, Tamburini C, Mangili F, Montesano M, Ferrari A, Bronson RA. 1996*a*. The expression of alpha v, alpha 5, beta 1, and beta 3 integrin chains on ejaculated human spermatozoa varies with their functional state. *Mol Hum Reprod* **2** 169-175.

Fusi FM, Bernocchi N, Ferrari A, Bronson RA. 1996*b*. Is vitronectin the velcro that binds the gametes together? *Mol Hum Reprod* **2** 859-866.

Gechtman Z, Belleli A, Lechpammer S, Shaltiel S. 1997. The cluster of basic amino acids in vitronectin contributes to its binding of plasminogen activator inhibitor-1: evidence from thrombin-, elastase- and plasmin-cleaved vitronectins and anti-peptide antibodies. *Biochem J* **325** 339-349.

Gibson AD, Lamerdin JA, Zhuang P, Baburaj K, Serpersu EH, Peterson CB. 1999. Orientation of heparin-binding sites in native vitronectin. Analyses of ligand binding to the primary glycosaminoglycan-binding site indicate that putative secondary sites are not functional. *J Biol Chem* **274** 6432-6442.

Gougoulidis T, Trounson A, Dowsing A. 1999. Inhibition of bovine sperm-oocyte fusion by the carbohydrate GalNAc. *Mol Reprod Dev* **54** 179-185.

Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* **75** 501-507.

Hyttel P, Greve T, Callesen H. 1988. Ultrastructure of in-vivo fertilization in cattle. *J Reprod Fertil* **82** 1–13.

Ignotz GG, Cho MY, Suarez S.S. 2007. Annexins are candidate oviductal receptors for bovine sperm surface proteins and thus may serve to hold bovine sperm in the oviductal reservoir. *Biol Reprod* **77** 906-913.

Liu YX. 2007. Involvement of plasminogen activator and plasminogen activator inhibitor type 1 in spermatogenesis, sperm capacitation, and fertilization. *Semin Thromb Hemost* **33** 29-40.

Lorton SF, First NL. 1979. Hyaluronidase does not disperse the cumulus oophorus surrounding bovine ova. *Biol Reprod* **21** 301–308.

Lyng R, Shur BD. 2007. Sperm-egg binding requires a multiplicity of receptor-ligand interactions: new insights into the nature of gamete receptors derived from reproductive tract secretions. *Soc Reprod Fertil* Suppl **65** 335-351.

Mahawar M, Joshi P. 2008. Goat vitronectin: characterization and binding to Staphylococcus aureus. *Comp Biochem Physiol B Biochem Mol Biol* **149** 410-418.

O'Flaherty C, Breininger E, Beorlegui N, Beconi MT. 2005. Acrosome reaction in bovine spermatozoa: role of reactive oxygen species and lactate dehydrogenase C4. *Biochim Biophys Acta* **1726** 96-101.

Revah I, Gadella BM, Flesch FM, Colenbrander B, Suarez SS. 2000. Physiological state of bull sperm affects fucose- and mannose-binding properties. *Biol Reprod* **62** 1010-1015.

Royce PM, Steinmann B. 2002. Connective tissue and its heritable disorders. Molecular, Genetic, and Medical Aspects. Second Edition. p. 305. (Wiley: New York.)

Ruoslahti E, Pierschbacher MD. 1986. Arg-Gly-Asp: a versatile cell recognition signal. *Cell* **44** 517-518.

Sostaric E, van de Lest CH, Colenbrander B, Gadella BM. 2005. Dynamics of carbohydrate affinities at the cell surface of capacitating bovine sperm cells. *Biol Reprod* **72** 346-357.

Spencer TE, Johnson GA, Bazer FW, Burghardt RC. 2004. Implantation mechanisms: insights from the sheep. *Reproduction* **128** 657-668.

Stockmann A, Hess S, Declerck P, Timpl R, Preissner KT. 1993. Multimeric vitronectin. Identification and characterization of conformation-dependent self-association of the adhesive protein. *J Biol Chem* **268** 22874-22882.

Sutovsky P, Flechon JE, Pavlok A. 1995. F-actin is involved in control of bovine cumulus expansion. *Mol Reprod Dev* **41** 521-529.

Suzuki S, Oldberg A, Hayman EG, Pierschbacher MD, Ruoslahti E. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO J* **4** 2519-2524.

Talbot P, Shur BD, Myles DG. 2003. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. *Biol Reprod* **68** 1-9.

Talevi R, Gualtieri R. 2001. Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium *in vitro*. *Biol Reprod* **64** 491-498.

Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* **61** 414-424.

Tanghe S, Van Soom A, Duchateau L, and de Kruif A. 2004*a*. Inhibition of bovine sperm-oocyte fusion by the p-aminophenyl derivative of D-mannose. *Mol Reprod Dev* **67** 224-232.

Tanghe S, Van Soom A, Duchateau L, Nauwynck H, de Kruif A. 2004*b*. Carbohydrates and glycoproteins involved in bovine fertilization *in vitro*. *Mol Reprod Dev* **68** 492-499.

Thys M, Vandaele L, Morrell JM, Mestach J, Van Soom A, Hoogewijs M, Rodriguez-Martinez H. 2009*a*. *In vitro* Fertilizing Capacity of Frozen-thawed Bull Spermatozoa Selected by Single-layer (Glycidoxypropyltrimethoxysilane) Silane-coated Silica Colloidal Centrifugation. *Reprod Domest Anim* **44** 390-394.

Thys M, Nauwynck H, Maes D, Hoogewijs M, Vercauteren D, Rijsselaere T, Favoreel H, Van Soom A. 2009*b*. Expression and putative function of fibronectin and its receptor (integrin $\alpha_5\beta_1$) in male and female gametes during bovine fertilization *in vitro*. *Reproduction* **138** 471-482.

Vandaele L, Mateusen B, Maes DG, de Kruif A, Van Soom A. 2007. Temporal detection of caspase-3 and -7 in bovine *in vitro* produced embryos of different developmental capacity. *Reproduction* **133** 709-718.

Van Soom A, Tanghe S, De Pauw I, Maes D, de Kruif A. 2002. Function of the cumulus oophorus before and during mammalian fertilization. *Reprod Dom Anim* **37** 141-151.

CHAPTER 5

IN VITRO FERTILIZING CAPACITY OF FROZEN-THAWED BULL SPERMATOZOA SELECTED BY SINGLE-LAYER (GLYCIDOXYPROPYLTRIMETHOXYSILANE) SILANE-COATED SILICA COLLOIDAL CENTRIFUGATION

Modified from

IN VITRO FERTILIZING CAPACITY OF FROZEN-THAWED BULL SPERMATOZOA SELECTED BY SINGLE-LAYER (GLYCIDOXYPROPYLTRIMETHOXYSILANE) SILANE-COATED SILICA COLLOIDAL CENTRIFUGATION

Thys M., Vandaele L., Morrell J.M., Mestach J., Van Soom A., Hoogewijs M., Rodriguez-Martinez H.

Reproduction in Domestic Animals 2009; **44** 390-394.

ABSTRACT

Barriers to the use of density gradient centrifugation for preparing animal spermatozoa for artificial insemination (AI) include the scarcity of animal-specific formulations and the daunting prospect of processing large volumes of ejaculate in small aliquots (1.5 ml extended semen). Recently, new colloid formulations have been tested in vitro in a modified procedure, centrifugation on a single layer of colloid. The present study investigated the fertilizing ability during in vitro fertilization (IVF) of frozen-thawed bovine spermatozoa following centrifugation through a single layer of glycerolpropylsilane (GS)-coated silica colloid with a species-specific formulation (patent applied for; treatment, T). Controls (C) included centrifugation through gradients of either the same colloid (C1) or Percoll^{TM} (C2). Sperm recovery surpassed 50% for both C1–C2 and T (n.s.). Mean values of various parameters of computerized analysis of sperm motility did not differ between T and C1 (n.s.), and only the proportions of path straightness and linearity were lower in T vs C2 ($p < 0.05$). In T, the mean (±SD) percentages of fertilization rate, blastocyst development rate and the total number of blastomeres were 58.1 ± 23.3%, 24.5 ± 14.3% and 94.6 ± 23.4%, respectively. The proportions did not differ significantly from controls (C1⁄C2). Therefore, centrifugation through a single layer of colloid offers an alternative method to density gradient centrifugation for selection of viable, potentially fertile frozen-thawed bull spermatozoa. This single-layer technique is gentle, versatile and convenient because it facilitates scaling-up the process of sperm preparation to allow larger numbers of spermatozoa (for instance, whole ejaculates) to be processed for AI.

INTRODUCTION

The technique of density gradient centrifugation through colloids is one of those recommended by the World Health Organization for preparing human spermatozoa for assisted reproduction (WHO, 1999). This method has also been suggested as a potential means of improving the quality of animal sperm preparations for artificial insemination (AI) (Rodriguez-Martinez et al., 1997; Hallap et al., 2004; Morrell, 2006). It has also been used to retrieve the best spermatozoa out of suspensions from sub-standard bull ejaculates (Rodriguez-Martinez et al., 1997), or bull spermatozoa subjected to diverse in vitro handling, including high-speed sorting (Hollinshead et al., 2004; Underwood et al., 2006), consecutive freezing–thawing, etc (Maxwell et al., 2007). The latter studies, however, used a formulation not specific for the species, being a product designed for human spermatozoa. Density

gradients with species-specific formulation have been used to prepare bovine spermatozoa for diagnostic purposes (Rodriguez-Martinez et al., 1997), or for in vitro fertilization (IVF) (Sieren and Youngs, 2001; Samardzija et al., 2006a; 2006b), and to prepare stallion spermatozoa for research purposes (Macpherson et al., 2002; Morrell and Geraghty, 2006). However, widespread application of the technique in processing animal spermatozoa has been hampered by the time-consuming protocol, the scarcity of animal-specific colloid formulations (Sieren and Youngs, 2001; Samardzija et al., 2006a; 2006b) and, particularly, by the difficulty of processing sufficient spermatozoa for the large insemination doses required in some domestic animal species.

Recently, species-specific (glycidoxypropyltrimethoxysilane, GPMS)-coated silica colloid formulations for use with animal spermatozoa have been developed at the Swedish University of Agricultural Sciences (SLU). Furthermore, a modification of the density gradient technique, centrifugation through a single layer of colloid, was compared with density gradient colloid centrifugation for preparing boar and stallion spermatozoa (JM Morrell et al., unpublished results). In both species, the spermatozoa harvested through either method showed higher motility values, better morphology and longevity than the overall initial sperm suspension, thus indicating that the simplified method could have a practical application in animal breeding. However, neither of these previous preliminary studies investigated the fertilizing ability of the spermatozoa after centrifugation through a single layer of colloid using AI because an AI trial in domestic animals would be extremely costly and time-consuming (Rodriguez-Martinez, 2007). In vitro fertilization, although not directly comparable to the in vivo situation, provides some assessment of the capacity of spermatozoa to initiate embryonic development and can thus be used to test out new methods of sperm preparation before proceeding to an AI trial.

The present study aimed to determine the fertilizing ability of frozen-thawed bull spermatozoa separated by GPMS-coated silica colloidal centrifugation in an IVF system as a model for in vivo fertilization in this species. The hypothesis tested was that centrifugation through a single layer of a silica colloid with species-specific formulation (patent applied for; treatment, T) was equally good in terms of sperm recovery and sperm functionality (sperm motility and fertilizing capacity) as controls (C). The latter included sperm preparations obtained by centrifugation through gradients of different density of either the same colloid (C1) or Percoll^{TM} (Amersham Biosciences AB, Uppsala, Sweden) (C2). The intended advantage of the simplified method is its ease of use, and gentler handling of spermatozoa, when compared with controls.

MATERIALS AND METHODS

Spermatozoa

Straws (0.25 ml plastic straws) of cryopreserved spermatozoa, originating from the same ejaculate from one individual bull with recognized in vitro fertility, were made available at the University of Ghent, Belgium, where the IVF trial was carried out. Prior to centrifugation, the straws of extended/frozen semen were thawed in water at 37°C for 30 s to 1 min. The sperm concentration was approximately 60 x 10^6 spermatozoa/ml, i.e. the standard dose for commercial AI use. The threshold of sperm motility used was 50%. Other post-thaw sperm motility parameters of the ejaculates used are described in Table 6.

Computerized sperm motility analysis

Computer-assisted sperm analysis (CASA) was carried out by an experienced operator using a Hamilton Thorne motility analyzer (Ceros-version 12.2 c; Hamilton Thorne Research, Beverly, MA, USA). Data were collected for the following parameters: velocity of the smoothed path (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head deviation (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), concentration, % motility, % progressive motility, % rapid motility, % medium motility, % slow motility and % static (Hoflack et al., 2007). The mean post-thaw sperm motility parameters of the ejaculate used in this experiment are shown in Table 6 (frozen-thawed semen).

Media

Colloid

Glycerolpropylsilane-coated silica beads were suspended in a buffered salt solution (patent applied for) and used to prepare layers for the density gradient. Two different densities were used for the gradient.

Percoll

Percoll was used to prepare two density layers: a higher density layer of 90% Percoll [1:9 (v/v) mixture of Percoll and 10x Hepes-buffered Tyrode's solution supplemented with albumin, lactate and pyruvate (TALP)] which was kept for 1 week at 4°C. Immediately before use, a lower density layer of 45% Percoll [1:1 (v/v) mixture of 90% Percoll and 10x Hepes-buffered TALP] was prepared.

Silane-coated silica colloid centrifugation

Density gradient centrifugation (C1)

A density gradient was prepared by pipetting 2 ml of the higher density layer into a 15-ml centrifuge tube and carefully layering 2 ml of the lower density layer on top. Thereafter, an aliquot (0.75 ml) of frozen thawed semen containing 60 x 10^6 spermatozoa/ml was pipetted on top of the upper layer. The gradients were centrifuged at 300 x g for 20 min, after which the semen extender and most of the gradient material were discarded. The sperm pellet was transferred to a clean centrifuge tube and was washed in a modified Tyrode's medium (Sp-TALP; Parrish et al., 1988) for IVF use.

Single-layer centrifugation (treatment)

Four millilitres of the higher density colloid material were pipetted into a 15-ml centrifuge tube and an aliquot (0.75 ml) of frozen-thawed semen containing 60 x 10^6 spermatozoa/ml was pipetted on top of the colloid column. After centrifugation, as described earlier, the sperm pellet was transferred to a clean centrifuge tube and was washed in Sp-TALP for IVF use (see previously).

Percoll gradient centrifugation

A Percoll gradient (C2) was prepared in a 15-ml conical tube with 2 ml 90% Percoll added to the tube, which was layered with 2 ml 45% Percoll. Frozen-thawed semen (three pooled straws of 0.25 ml, 60 x 10^6 spermatozoa/ml) was layered on the Percoll gradient and centrifuged at 700 x g for 30 min. The supernatant and the interface were removed, and the sperm pellet was washed in Sp-TALP by centrifugation at 100 x g for 10 min at room temperature prior to IVF use.

In vitro maturation and fertilization

The methods used for in vitro maturation and IVF have been described previously (Tanghe et al., 2002). Briefly, bovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 3-4 h after collection. Cumulus-oocyte complexes (COCs) were aspirated from 2 to 6 mm diameter follicles using an 18 G needle and a 10-ml syringe. After washing the COCs in HEPES-buffered TALP medium [10 mM HEPES, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 2 mM sodium bicarbonate, 10 µg⁄ml gentamycin sulphate, 3 mg⁄ml bovine serum albumin (BSA; Sigma-Aldrich, Bornem, Belgium)], the COCs were matured by incubation in 500 µl-droplets of a modified bicarbonate-buffered

TCM 199 medium (Gibco BRL, Merelbeke, Belgium) supplemented with 20% heat inactivated fetal calf serum (FCS; NV HyClone Europe AS, Erembodegem, Belgium), 0.2 mM sodium pyruvate (Sigma-Aldrich) and 0.4 mM glutamine (Sigma-Aldrich) at 38.5°C in an atmosphere of 5% CO_2 in air at 100% humidity for 18-26 h. After maturation, the COCs were coincubated with 1 x 10^6 spermatozoa/ml in 500 µl fertilization medium, consisting of IVF-TALP, supplemented with 6 mg/ml of fatty acid-free BSA and 10 µg⁄ml heparin (Sigma-Aldrich) at 38.5°C in an atmosphere of 5% CO_2 in air at 100% humidity for 20 h. Fertilized oocytes were then denuded from cumulus cells and spermatozoa by vortexing for 2 min in 2 ml HEPES-buffered TALP. To assess fertilization, presumed zygotes (n = 312) were fixed for at least 12 h in 2% paraformaldehyde and 2% gluteraldehyde in phosphate-buffered saline without calcium and magnesium, and stained for 10 min with 10 µg⁄ml Hoechst 33342 in ethanol (Molecular Probes, Leiden, The Netherlands), with confirmation of zygote status being characterized by the presence of two pronuclei. This rate was expressed as percentage. The remaining fertilized oocytes (n = 819) were cultured for 8 days in synthetic oviduct fluid supplemented with amino acids and FCS (SOFaa + 5% FCS) medium in 50 µl droplets under mineral oil in groups of 25 in 5% CO_2, 5% O_2 and 90% N_2, after which the percentage of development to blastocyst (blastocyst rate) and the total number of cells were assessed.

Statistics

Mean values for each of the parameters determined by CASA as well as fertilization rate, percentage ofblastocyst rate and total number of blastocyst cells were compared for the C1, C2 and T sperm preparation methods assayed using ANOVA (Campbell, 1974). The level of statistical significance was set to $p < 0.05$.

RESULTS

In total, the experiment was replicated five times. The characteristics of the post-thaw spermatozoa, the sperm concentrations following centrifugation, and the kinematics of the recovered spermatozoa (CASA assessment) are depicted in Table 1. Both controls (C1⁄C2) and treatment method (T) showed a sperm recovery rate above 50% (51.5%) when compared with the post-thaw semen (see Table 6, $p < 0.05$) and an increased percentage of motile spermatozoa. However, the spermatozoa separated by colloidal centrifugation (C1⁄T) showed a lower LIN when compared with Percoll (C2). There were also significant differences for STR between Percoll (C2) and T (single-layer colloid centrifugation; $p < 0.05$). All other sperm kinematic parameters, both absolute (e.g. velocities) and re-calculated (patterns of sperm movement, etc.) were similar, following either handling (C1⁄C2 vs T) (n.s.). Centrifugation by any of the methods (C1, C2 and T) significantly improved several of the motility parameters: LIN, percentage motile spermatozoa and proportion of rapidly motile spermatozoa ($p < 0.05$), compared with the post-thaw spermatozoa. Both ALH and the proportion of static spermatozoa significantly decreased when compared with the uncentrifuged sample. Furthermore, VAP, VSL and percentage progressively motile spermatozoa were significantly increased for both the single layer (T) and Percoll gradient centrifugation (C2) ($p < 0.05$) but not for the density gradient (C1). STR was significantly different only for Percoll gradient centrifugation (C2), while percentage slow motility was significantly different only for the single layer of colloid (T).

For the IVF trials, a summary of the results (fertilization, blastocyst rates and total blastomere cell numbers) is presented in Table 7. Although the fertilization rate for the treatment spermatozoa (T, single-layer colloid centrifugation) seemed numerically higher than the colloid control (C1, gradient density), this difference was not significant ($p > 0.2$), owing to the large SD. The results of fertilization rate did not differ (n.s.) when compared with the Percoll density gradient separation (C2) although the variation between replicates was significantly lower. The rates of fertilized oocytes that developed to the blastocyst stage and the total number of blastomere cells present in the blastocysts did not differ significantly between the different sources of spermatozoa (T or C1), when compared with Percoll (C2) (n.s.).

Table 6: Kinematics (using a Hamilton Thorne CASA instrument) of frozen-thawed bull spermatozoa before and after centrifugation through a single layer of a species-specific glycerolpropylsilane-coated silica bead colloid with species-specific formulation (patent applied for; treatment, T) or controls (C). Controls included centrifugation through gradients of different density of either the same colloid (C1) or Percoll™ (C2) (means ± SD, five replicates)

		Sperm separation method		
		Colloid centrifugation		
CASA parameter	Frozen-thawed semen	Single layer (T)	Density gradient (C1)	Percoll (C2)
VAP (μm/s)	101.0 ± 3.73	112.4 ± 9.93[a]	111.1 ± 8.87	117.7 ± 8.70[a]
VSL (μm/s)	85.2 ± 4.25	95.4 ± 8.87[a]	94.92 ± 10.58	103.5 ± 7.06[a]
VCL (μm/s)	171.7 ± 5.77	171.5 ± 10.98	167.44 ± 16.18	170.5 ± 12.17
ALH (μm)	7.9 ± 0.45	7.1 ± 0.57[a]	6.98 ± 0.71[a]	6.7 ± 12.17[a]
BCF	26.8 ± 1.35	23.1 ± 5.63	23.3 ± 5.54	25.7 ± 3.84
STR (%)	82.3 ± 2.34	82.2 ± 2.28[a,b]	83.0 ± 4.04	86.2 ± 1.64[a]
LIN (%)	49.7 ± 3.14	55.6 ± 4.05[a,b]	56.2 ± 3.70[a,b]	60.4 ± 1.67[a]
Sperm concentration ($x10^6$/ml)	61.7 ± 10.01	37.7 ± 17.69	36.96 ± 20.3	34.6 ± 8.82
Motile spermatozoa (%)	51.5 ± 4.18	75.4 ± 10.5[a]	71.4 ± 17.09[a]	71.0 ± 17.16[a]
Progressively motile (%)	39.3 ± 5.20	61.4 ± 13.3[a]	54.8 ± 16.80	58.6 ± 15.10[a]
Rapid motile (%)	46.7 ± 4.84	69.4 ± 11.04[a]	64.6 ± 17.87[a]	65.8 ± 17.16[a]
Medium motile (%)	4.7 ± 1.37	6.6 ± 1.95	6.6 ± 1.95	5.4 ± 0.54
Slow motile (%)	7.7 ± 2.80	14.0 ± 6.12[a]	15.2 ± 9.49	15.0 ± 10.19
Static motile (%)	39.8 ± 4.31	10.4 ± 6.69[a]	13.4 ± 8.53[a]	14.4 ± 9.48[a]

[a]Significantly different from uncentrifuged spermatozoa ($p < 0.05$).

[b]Significantly different from Percoll™ ($p < 0.05$). Differences between single layer and gradient were not significant.

Table 7: Fertilizing ability after IVF and embryo culture of frozen-thawed bull spermatozoa selected by centrifugation through a single layer of a species-specific glycerolpropylsilane-coated silica bead colloid with species-specific formulation (patent applied for; treatment, T) or control gradient density centrifugation (C). Controls included centrifugation through gradients of different density of either the same colloid (C1) or Percoll™ (C2) (means ± SD, five replicates)

Sperm separation method	Oocytes (nr)	Fertilization rate (%)	Blastocyst rate (%)	Total blastocyst cells (nr)
Single layer colloid centrifugation (T)	340	58.1 ± 23.3	24.5 ± 14.3	94.6 ± 23.4
Gradient density colloid centrifugation (C1)	403	56.3 ± 23.3	23.5 ± 17.4	83.2 ± 29.9
Gradient density Percoll™ centrifugation (C2)	388	55.3 ± 7.3	27.5 ± 15.6	98.6 ± 24.6

DISCUSSION

Although IVF does not offer the same challenges to spermatozoa as AI and subsequent in vivo fertilization, these preliminary results show that the newly simplified method for sperm preparation (selection of the better quality spermatozoa) by centrifugation through a single layer of GPMS silane-coated silica bead colloid did not have any adverse effect on their fertilizing ability, compared with density gradient centrifugation, either of a similar colloid (C1) or of Percoll (C2). Basically, the results, with the exception of a couple of CASAderived, re-calculated kinematic parameters, were statistically similar. Percoll density gradient centrifugation was able, however, to select spermatozoa with a higher degree of linearity, a recognized ability of Percoll, polyvinylpyrrolidone (PVP)-coated silica bead suspensions that were the first colloids used for the selection of spermatozoa. (The CASA results presented here for both frozen-thawed and centrifuged sperm samples confirm these previous observations). Yet, concern was raised that the PVP might have toxic effects on spermatozoa (Avery and Greve 1995), and in 1996, Percoll was restricted to non-clinical use by its manufacturers (Mortimer 2000). PVP-coated silica was subsequently superseded by the documented non-toxic GPMS-coated silica preparations commercially available for some species (PureSperm® for human, BoviPure™ for bull, EquiPure™ for stallion and PorciPure™ for boar semen; Nidacon International AB, Gothenburg, Sweden). These commercial products have been used inter-species (for instance bull spermatozoa on preparations designed for human), with acceptable results, but the studies were restricted to

diagnostic tests for sperm function only, e.g. consecutively frozen-thawed for diagnostic or flow-sorting purposes (Hollinshead et al., 2004; Underwood et al., 2006; Maxwell et al., 2007). In other cases, species-specific preparations (e.g. BoviPure) have been used to examine the value of the use of density gradient sperm selection procedure for IVF to, ultimately, improve the efficiency of in vitro embryo production. Thus, comparisons were made with standard sperm washing procedures (Sieren and Youngs, 2001), with Percoll (Samardzija et al., 2006a) or with swim-up (Samardzija et al., 2006b), all including recovery and selection of frozen-thawed bull spermatozoa. The results were variable, with BoviPure-recovered spermatozoa not leading to better production of bovine embryos compared with single washing in modified Brackett-Oliphant medium (Sieren and Youngs, 2001); yielding better cleavage rates but not blastocyst rates comparedwith Percoll-recovered spermatozoa (Samardzija et al., 2006a), or the opposite results when compared with swim-up sperm selection (Samardzija et al., 2006b).

Interestingly, although some of the studies mentioned earlier lacked proper controls in their experimental designs, a low (30-40%) recovery rate was consistently found. The recovery rate in the present study was higher (above 50%), even when a single-layer colloid was used. Differences in procedures (handling, layering, centrifugal force and duration, etc.) as well as differences in formulations might have contributed to these results. As such, the use of a single colloid layer (T), centrifuged at 300 g for 20 min, resulted in the harvesting of spermatozoa in similar numbers and with a similar functionality (in terms of motility and fertilizing ability) as when density gradient controls (C1 and C2) were used.

Centrifugation on a single layer of colloid has several advantages over density gradient centrifugation. As there is only one layer of colloid in the tube, preparation time is shorter and the process is less complicated than for the density gradient, which requires at least two densities of colloid to be layered in the tube. Care is required in the layering process for the gradient as mixing of different densities because of careless layering destroys the integrity of the interface between the two layers, thus reducing the efficiency of the sperm selection process. Moreover, use of the single layer facilitates scaling-up the volumes of colloid and ejaculate used, in order to process the large number of spermatozoa required for insemination doses in some animal species such as boar or stallion (Watson, 1990); it would be time-consuming and tedious to process such large volumes using only 1.5 ml aliquots of extended ejaculate on small density gradients.

In addition to non-toxicity, use of a GPMS-coated silica colloid and optimized species-specific formulations, as used in the present study, confers several advantages: it is stable in

salt solutions, thus permitting large batches of ready-made colloids of different densities and standardized formulations to be mass-produced in advance of their use, and it can be autoclaved, thus reducing endotoxin levels. The results reported hereby follow the same pattern seen in ongoing in vitro studies with ejaculated boar (motility) or stallion spermatozoa (motility, morphology; JM Morrell et al., unpublished results). Moreover, the single colloid layer centrifugation technique has also been used successfully to separate frozen-thawed bull and dog spermatozoa from cryopreservation medium (JM Morrell, unpublished data). In those studies, a low density colloid corresponding to the upper layer of the density gradient described in this report was used as a single layer. After centrifugation, the vast majority of the spermatozoa was in the pellet, while the cryopreservation medium remained in the supernatant. Furthermore, a single layer of low-density colloid has been used to separate boar spermatozoa from seminal plasma, to allow research to be carried out on its components (Jiwakanon et al., 2007).

To conclude, centrifugation on a single layer of colloid offers an alternative method to density gradient centrifugation for selection of viable, potentially fertile frozen-thawed bull spermatozoa. This single-layer technique is gentle, versatile and convenient, and the results would indicate that T-derived spermatozoa could be used for AI without expecting subsequent loss in pregnancy rate. Adopting the use of the single layer may allow the process to be scaled-up to prepare sufficient ejaculated spermatozoa for AI. As both centrifugation techniques are likely to be employed in future, we suggest use of the term ‘colloidal centrifugation’ to cover the use of either density gradient or the modification whereby aliquots of ejaculate are centrifuged on a single layer of colloid.

REFERENCES

Avery B, Greve T. 1995. Impact of Percoll on bovine spermatozoa used for in vitro insemination. *Theriogenology* **44** 871–878.

Campbell RC. 1974. Statistics for Biologists. Cambridge University Press, London.

Hallap T, Håård M, Jaakma U, Larsson B, Rodriguez-Martinez H. 2004. Does cleansing of frozen-thawed bull semen before assessment provide samples that relate better to potential fertility? *Theriogenology* **62** 702–713.

Hoflack G, Opsomer G, Rijsselarer T, Van Soom A, Maes D, de Kruif A, Duchateau L. 2007. Comparison of computerassisted sperm motility analysis parameters in semen from Belgian Blue and Holstein-Friesian bulls. *Reprod Domest Anim* **42** 153–161.

Hollinshead FK, O'Brien JK, Maxwell WMC, Evans G. 2004. Assessment of in vitro sperm characteristics after flow cytometric sorting of frozen-thawed bull spermatozoa. *Theriogenology* **62** 958–968.

Jiwakanon J, Fossum C, Persson E, Morrell JM, Dalin A-M. 2007. Influence of seminal plasma on the distribution of T-lymphocytes in the pig oviduct. *Reprod Domest Anim* **42** Suppl.2 77-143.

Macpherson M, Blanchard TL, Love CC, Brinsko SP, Thompson JA, Varner DD. 2002. Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. *Theriogenology* **58** 317–320.

Maxwell WMC, Parrilla I, Caballero I, Garcia E, Roca J, Martinez EA, Vazquez JM, Rath D. 2007. Retained functional integrity of bull spermatozoa after double freezing and thawing using PureSperm® density gradient centrifugation. *Reprod Domest Anim* **42** 489–494.

Morrell JM. 2006. Update on semen technologies for animal breeding. *Reprod Domest Anim* **40** 1-5.

Morrell JM, Geraghty RJ. 2006. Effective removal of equine arteritis virus from stallion semen. *Equine Vet J* **38** 224–229.

Mortimer D. 2000. Sperm preparation methods. *J Androl* **21** 357–366.

Parrish JJ, Susko-Parrish J, Winter MA, First NL. 1988. Capacitation of bovine sperm by heparin. *Biol Reprod* **38** 1171–1180.

Rodriguez-Martinez H. 2007. State of the art in farm animal sperm evaluation. *Reprod Fertil Dev* **19** 91–101.

Rodriguez-Martinez H, Larsson B, Pertoft H. 1997. Evaluation of sperm damage and techniques for sperm clean-up. *Reprod Fertil Dev* **9** 297–308.

Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J, Karadjole T. 2006a. A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. *Anim Reprod Sci* **91** 237–247.

Samardzija M, Karadjole M, Getz I, Makek Z, Cergolj M, Dobranic T. 2006b. Effects of bovine spermatozoa preparation on embryonic development *in vitro*. *Reprod Biol Endocrinol* **4** 58.

Sieren KR, Youngs CR. 2001. Evaluation of Bovipure™ for *in vitro* production of bovine embryos. *Theriogenology* **55** 438.

Tanghe S, Van Soom A, Sterckx V, Maes D, de Kruif A. 2002. Assessment of different sperm quality parameters to predict in vitro fertility of bulls. *Reprod Domest Anim* **37** 127–132.

Underwood SL, Bathgate R, Gillan L, Maxwell WMC, Evans G. 2006. A comparison of staining media for sex-sorting cooled dairy bull spermatozoa. *Reprod Domest Anim* **41** 378–382.

Watson PF. 1990. Artificial insemination and the preservation of semen. In: Lamming GE (ed.), Marshall's Physiology of Reproduction. Churchill Livingstone, London, 785–825.

World Health Organisation. 1999. WHO laboratory manual for the analysis of human semen and sperm–cervical mucus interaction, 4th edn. Cambridge University Press, Cambridge.

CHAPTER 6

GENERAL DISCUSSION

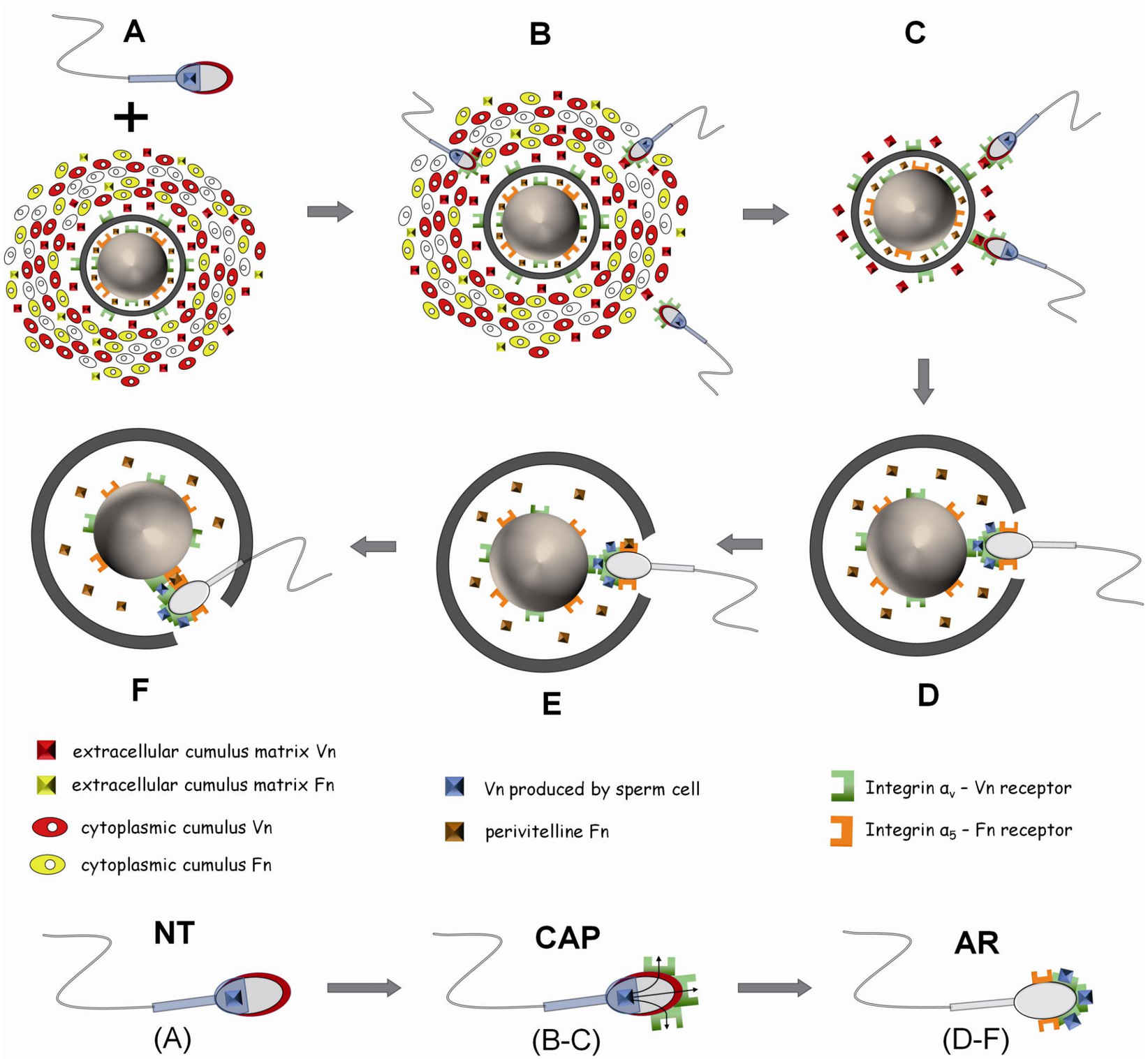
This research was essentially carried out to explore the molecular basis of bovine fertilization in order to get a better understanding and eventually be able to further optimize the *in vitro* embryo production system in this species. Since a previous study – investigating the effect of several glycoconjugates on bovine IVF – revealed that fibronectin and vitronectin (both ECM glycoproteins) inhibited sperm penetration of *in vitro* matured cumulus-oocyte-complexes to a certain extent, these glycomolecules represent interesting candidates to investigate which fertilization step(s) are exactly affected by their presence and to locate the carbohydrate receptor sites and corresponding ligands on male and female gametes. In this final chapter the results of this work are discussed. To scale-up the *in vitro* sperm preparation procedure required for AI and IVF a new – technically simplified – sperm selection technique has recently been suggested. The new technique (based on a single layer of a species-specific colloid suspension) would allow faster selection of large numbers of viable sperm. In this thesis the technique was thoroughly assessed.

THE ROLE OF FIBRONECTIN AND VITRONECTIN DURING BOVINE IVF

To elucidate the function of these two glycoproteins during bovine sperm-egg interaction, we made use of both inhibition studies (identifying the fertilization step(s) influenced by these molecules) and localization studies by immunofluorescence (identifying the localization of these molecules and their corresponding receptors). Incubation of sperm and/or oocytes with possible ligands in order to inhibit fertilization has been shown to be a convenient way to study receptor-ligand interactions.

Combining all the presented results concerning Fn and Vn, the following hypothesis can be put forward concerning the interaction of both glycoproteins during bovine fertilization (Fig.25).

Figure 25: Hypothetical model concerning the role of fibronectin (Fn) and vitronectin (Vn) during bovine sperm-egg interaction. (A): Non-treated (NT) sperm cells, comprising intracellular Vn in the postacrosomal region, are co-incubated with *in vitro* matured COCs. On approaching the cumulus oophorus, the spermatozoa become fully capacitated (CAP), thereby exhibiting integrin subunit α_v on their rostral sperm head. (B): By means of reversible interactions between the exposed Vn integrin receptor and the Vn in the extracellular cumulus matrix, capacitated sperm cells are able to migrate through the cumulus oophorus. (C): After reaching the zona pellucida (ZP), sperm-ZP binding is initiated through attachment of Vn to both corresponding Vn integrin receptors on sperm head and exterior side of the ZP. Subsequently, the acrosome reaction (AR) takes place, exposing the intracellular sperm Vn rostrally (in connection with its integrin receptor), and the Fn integrin receptor at the equatorial segment. (D): After sperm-ZP penetration, primary sperm-oolemma binding occurs through sperm Vn, acting as a reversible dual binding molecule between the corresponding integrin receptors on both sperm and oolemma. (E): Consequently, the sperm Fn integrin receptor is able to connect to the perivitelline Fn, (F): allowing secondary sperm-oolemma binding through attachment of Fn to its matching oolemmal integrin receptor.



Unlike the situation in human, no Fn expression could be observed in bovine spermatozoa, irrespective of their functional state. In human, Fn (Fusi and Bronson, 1992) – and its receptor, integrin $\alpha_5\beta_1$ (Fusi et al., 1996a) – emerge on the surface of spermatozoa after capacitation. In bovine, integrin subunit α_5 was exposed at the equatorial segment, but only after acrosome reaction (Fig.25D-F). With respect to Vn, our results were analogous to the human situation: Vn also appears to be an intrinsic protein of bovine sperm cells which is released during the acrosome reaction (Fusi et al., 1994). Since the α_v subunit of the Vn integrin receptor was also detected on ZP and oolemma, a reversible dual binding function connecting both male and female gamete – as suggested in human by Fusi et al. (1996b) – can be expected. Such a role is even more plausible in the bovine species, since ruminant Vn apparently displays two integrin binding RGD sequences, in contrast to only RGD site in human Vn (Suzuki et al., 1985; Mahawar and Joshi, 2008). Furthermore, the presumed reversible dual binding function could additionally be exerted through spontaneous multimerization of several Vn molecules as described by Stockmann et al. (1993). The C-terminal half of the molecule comprises two hemopexin-like domains, able to interact with the acidic residues of the connecting segment of the same molecule, consequently allowing intramolecular and intermolecular linking to form vitronectin polymers (Royce and Steinmann, 2002). The α_v subunit of the Vn-receptor was present on the rostral sperm head after capacitation (Fig.25B-C), yet to a lesser extent than after acrosomal exocytosis (Fig.25D-F). Possibly, sperm cells undergoing capacitation are assisted in traversing the cumulus oophorus by reversible interactions between the α_v integrin on the sperm cell surface and Vn present in the cumulus ECM (Fig.25B). Both uncapacitated and acrosome reacted spermatozoa may – hence – be unable to efficiently penetrate this vast egg vestment respectively due to a lack of the requisite integrin receptors or due to the endogenous sperm Vn prematurely occupying the sperm integrin receptors. Consequently, this ligand-receptor mechanism may play a role in sperm selection allowing only spermatozoa in the appropriate functional state to enter the COC. Fibronectin was also observed at the cumulus oophorus (in the cytoplasm of cumulus cells as well as in the ECM, Fig.25A-B). However, no integrin subunit α_5 was identified on the sperm cell surface (prior to acrosome reaction). Therefore, it seems reasonable to assume that Fn is not involved in sperm penetration of the cumulus oophorus. Nevertheless, the possibility of a specific positive reaction of the anti-integrin subunit α_5 polyclonal antibody - hidden underneath the observed non-specific fluorescence - might not be completely excluded. However, our hypothesis is supported by the finding that supplementation of exogenous Fn induced inhibition of sperm penetration independent of the

presence of the cumulus cells. Furthermore, cumulus Fn possesses only one integrin binding RGD-sequence, in contrast to the splice variant present underneath the ZP which is containing two RGD-sequences (Goossens et al., 2009).

As such this Fn variant cannot function as a connecting molecule between sperm cell and COC. Since supplementation of exogenous Vn to the fertilization medium resulted in an increased sperm-ZP binding and since endogenous Vn as well as integrin subunit α_v seemed to be abundantly present at the exterior side of the ZP, a function for the ligand-receptor mechanism in sperm-ZP interaction could be expected (Fig.25C). The superior sperm-ZP binding observed in the presence of Vn could explain the favorable effect of low concentrations of Vn on bovine IVF. The fact that high Vn concentrations resulted in high sperm-ZP binding, but decreased sperm penetration, is most likely due to the observed substantial negative effect on (progressive) sperm motility. Successful ZP penetration appears to be dependent on the sperm hyperactivation (Bedford, 1998).

After ZP penetration and acrosomal exocytosis, bovine sperm cells display endogenous Vn on their rostral surface and integrin subunit α_5 at their equatorial segment (Fig.25D). Concurrent expression of integrin subunit α_v on the oolemma suggests – again – a reversible dual binding function for sperm Vn connecting both corresponding integrin receptors on sperm and egg. This interaction might accomplish primary sperm-oolemma binding and modification of the Fn integrin receptor on the oolemma (enabling the receptor to interact with the Fn at the perivitelline space). Secondary sperm-oolemma binding could – then – occur through binding of Fn to the matching integrin receptors on both gametes (Fig.25E-F). Presumed involvement of Fn in sperm-oolemma binding was also supported by our observation that the main inhibitory effect of exogenously supplemented Fn on bovine IVF was located at the level of sperm-oolemma binding and (concurrently) sperm-egg fusion. Probably the Fn-integrin $\alpha_5\beta_1$ ligand-receptor interaction is not involved in the actual fusion process, but brings about the prerequisite initial attachment of both concerning membranes.

Membrane fusion – which involves merging of the membranes and cytoplasm of two different cells – is a rather exceptional (and relatively poorly explored) event, restricted to a limited number of cell types, but critical for fertilization (Stein et al., 2004). Membrane fusion has been described in three different frameworks: 1) virus-cell fusion (essential for infection of the host cell); 2) intracellular vesicle fusion (indispensable for protein trafficking and exocytosis); and 3) cell-cell fusion (as is happening during fertilization). Both the oocyte and

the sperm cell possess lipid bilayer membranes, which do not fuse spontaneously: the energy required to induce biological membrane fusion is provided by specific fusion proteins (Tamm et al., 2003). In fact, the actual fusion process consists of three consecutive key events (Stein et al., 2004). First, initial – target specific – membrane contact/attachment is accomplished through protein-protein mediated or protein-carbohydrate mediated binding of the concerning membranes (which is the putative function of the Fn-integrin $\alpha_5\beta_1$ ligand-receptor interaction during fertilization). Subsequently, fusion proteins bring both membranes in close apposition, frequently by spanning the intermembrane space and physically linking of the membranes. Consequently, the molecule undergoes an irreversible conformational change withdrawing the two membrane-inserted ends of the protein (Smith and Helenius, 2004). Finally, lipid mixing will take place resulting in cytoplasmic continuity between the two merging cells (Stein et al., 2004).

Besides being associated with sperm-oocyte interaction, glycoproteins are also key components in the host cell invasion process of herpesviruses (Tanghe et al., 2005). To infect a cell, viruses are obliged to transfer their genome and accessory proteins into the host cell, bypassing or modifying the barrier represented by the plasma membrane. The required virus-induced membrane fusion has been shown to be mediated by specific viral surface glycoproteins (Da Poian et al., 2005). Viral fusion proteins must undergo structural reorganization to be converted from the non-fusogenic to the fusogenic conformation (Da Poian et al., 2005; Teissier and Pécheur, 2007). These changes are triggered either by receptor binding on the plasma membrane for viruses fusing at neutral pH, or by protonation of specific residues in the endosome for viruses that penetrate their host cells via endocytosis (Teissier and Pécheur, 2007). Various integrins appear to recognize RGD-sequences displayed on the exterior loops of viral capsid proteins, analogous to the interaction of both Fn and Vn with their respective integrin receptor during sperm-oolemma binding. Similar to binding of these ECM glycoprotein ligands, virus binding also induces clustering and/or conformational alterations in the quaternary structure of the integrin receptor. These integrin modifications initiate cell-signaling events (increasing ligand affinity/avidity) as well as cytoskeletal rearrangement and virus internalization (Stewart and Nemerow, 2007).

In contrast to our prior hypothesis stating that the Vn-integrin $\alpha_v\beta_3$ interaction is initiating the sperm-oolemma binding, we could merely detect a slight – statistically insignificant – decrease in sperm-oolemma binding during bovine IVF when 500 nM

exogenous Vn was supplemented to the fertilization medium. On the other hand, the physiological relevance of the number of sperm cells bound to a ZP-free oocyte is debatable (Talbot et al., 2003). The underlying assumption is that spermatozoa are bound to the oolemma by a mechanism that can result in sperm-egg fusion. This *in vitro* assay may – though – include a heterogeneous population of bound sperm cells, including non-physiologically bound sperm, besides the specific population of physiologically relevant sperm that are tethered or docked before fusion. Some of the sperm cells may well be bound via interactions that will not result into fusion. Acrosome-intact sperm are – for instance – established to be able to bind to ZP-free oocytes, but not to fuse. If the non-specifically bound sperm fraction outnumbers the specifically bound fraction, variations in the number of physiologically relevant bound sperm will not be measured (Talbot et al., 2003). A high concentration (500 nM) of exogenously supplemented Vn – however – did have a significant inhibitory effect on sperm penetration of ZP-free oocytes. To check whether this is due to competition between the exogenous Vn and the Vn liberated from the sperm cell following the acrosome reaction, additional experiments are necessary investigating whether supplementation of low Vn concentrations to an IVF system with ZP-free oocytes and acrosome reacted sperm effectively inhibit sperm penetration.

Prospects for further research

Different from the linear dose-dependent inhibitory effect of Fn on bovine IVF, a dual effect of exogenous Vn supplementation was observed. Low Vn concentrations (100 nM) appeared to enhance sperm penetration, whereas a negative influence was noted in the presence of high concentrations (≥ 500 nM). This inhibitory effect may well be – at least partially – due to compromised membrane integrity. Furthermore, the observed substantial twofold decrease in progressive motility in presence of 500 nM Vn (compared to the control) should most likely be considered as an important factor contributing to the inhibitory effect of Vn supplementation on sperm penetration during IVF. Combining these results with the explicit head-to-head agglutination noted when incubating sperm with 500 nM of Vn, high concentrations of Vn should obviously be regarded as detrimental for successful fertilization. More research is required to distinguish this dual effect of low versus high concentrations of exogenous Vn during bovine IVF. To verify our model, additional experiments are necessary investigating whether supplementation of low Vn concentrations to an IVF system with ZP-free oocytes and acrosome reacted sperm effectively inhibits sperm penetration.

Actual involvement of the RGD sequence in sperm-egg interaction was assessed to verify whether the inhibitory effect of exogenously supplemented Fn was indeed due to binding to the α_5 integrin. A Fn-specific RGD-containing peptide (Fusi et al., 1996b) negatively affected sperm penetration at all tested concentrations, whereas a non-RGD control peptide did not seem to have any effect. Analogous research applying a Vn-specific RGD-containing oligopeptide, would allow confirmation whether Vn truly interacts with bovine fertilization through integrin binding.

Further research (ideally making use of the specific Fn splice variant present underneath the ZP displaying two integrin-binding sites), identifying the effect of Fn/Vn binding to its corresponding integrin receptor on the intracellular signal transduction in male and female gametes, is indispensable to elucidate the exact underlying mechanism of interaction in order to validate our model and to create a non-hormonal topical contraceptive – based on the glycoprotein – in the future. Supplementing both glycomolecules simultaneously during bovine IVF might provide a convenient means to efficiently block fertilization (without making use of hormonal products). *In vivo* application of a vaginal gel based on these glycomolecules should however be addressed carefully, since topical administration will very likely result in a substantially diluted (hence uneffective) medium at the site of fertilization.

The involvement of Fn and Vn in bovine fertilization has been unequivocally established by our research. The fact that Vn was detected within the sperm cell prior to acrosome reaction, raised the question whether this characteristic could be used to assess bull fertility. Up till now, no single sperm trait has been established to accurately predict bull fertility, indicated by the modest – and highly variable – correlations between results obtained *in vitro* and field fertility (Rodríguez-Martínez, 2003). Statistically significant correlations ($r = 0.35\text{-}0.59$) between IVF-fertility and fertility *in vivo* have occasionally (but not always) been observed. The present laboratory assays are obviously not always able to provide a prediction (merely an estimation) of the fertility of a given semen sample of the sire following bovine AI (Rodríguez-Martínez, 2003). Currently, DNA-denaturation is regarded to be the most useful and sensitive indicator of male fertility potential in bovine (Januskauskas et al., 2003). Correct transmission of information coded in the sperm genome is critical for the pre- and post-natal development of the offspring (Shamsi et al., 2009). External sperm membrane markers for fertility are controversial: with respect to the expression of seminal plasma

proteins, the variation between ejaculates from one bull is higher than the variation between different bulls (Fischer, 2008). Moreover, molecules present on the sperm cell surface prior to capacitation and acrosome reaction might not be present anymore at the time of fertilization, as both processes induce several substantial modifications of the sperm membranes. However, since Vn is an intrinsic sperm protein (liberated after acrosome reaction), this glycoprotein could represent a possible marker for fertility. Comparing semen from bulls with different *in vivo* fertility (and from different ejaculates of these bulls) for Vn expression is naturally required to confirm this assumption. In human, Vn content of motile spermatozoa seems to vary between different men, even though no correlation was detected between sperm morphology within the ejaculate and the observed sperm content of Vn (Bronson and Preissner, 1997). Thus, detection or absence of Vn might be reflecting differences in sperm viability, as suggested in human by Bronson et al. (2000). Nevertheless, even if Vn were correlated with sperm viability, sorting of a semen sample by means of MACS® Technology (magnetic labeling by MACS MicroBeads coupled to highly specific antibodies or proteins) would not be possible (unlike for molecules expressed at the sperm cell surface). Accordingly, the intrinsic sperm protein cannot be used for selection of viable spermatozoa from an ejaculate, unless the intrinsic Vn could be labeled without prior fixation and/or permeabilization (causing membrane damage). Bull semen is currently being sex-sorted flowcytometrically after DNA-staining with Hoechst 33342. This stain is able to penetrate without pre-treating the sperm. If Vn were found to be associated with sperm viability, assessment of endogenous Vn expression could – however – be readily used to select potentially fertile bulls or ejaculates for AI or IVF.

IN VITRO SPERM SELECTION THROUGH SINGLE LAYER (GLYCIDOXYPROPYL-TRIMETHOXYSILANE) SILANE-COATED SILICA COLLOIDAL CENTRIFUGATION

Percoll®, consisting of PVP-coated silica bead suspensions, was the first colloid used for the selection of spermatozoa. Our data – resulting from the comparison of Percoll density gradient centrifugation with colloidal centrifugation based on a species-specific GPMS silane-coated silica bead colloid confirmed the commonly recognized ability of Percoll density gradient centrifugation to select spermatozoa with a higher degree of linearity. However, concern was raised some time ago that the PVP might have toxic effects on spermatozoa (Avery and Greve, 1995), and in 1996, Percoll was limited to non-clinical use by its manufacturers (Mortimer 2000). PVP-coated silica was progressively superseded by the documented non-toxic GPMS-coated silica preparations commercially available for some species (PureSperm® for human, BoviPure® for bull, EquiPure® for stallion and PorciPure® for boar semen; Nidacon International AB, Gothenburg, Sweden). Our presented results (although preliminary) clearly show that the newly simplified method for sperm preparation by centrifugation through a single layer of GPMS silane-coated silica bead colloid did not have any adverse effect on their fertilizing ability compared with density gradient centrifugation, either of a similar colloid or with Percoll. Basically, the results, with the exception of a couple of CASA-derived, re-calculated kinematic parameters, were statistically similar. Species specific preparations (e.g. BoviPure®) have been used previously to evaluate the value of the use of density gradient sperm selection procedure for IVF. Therefore, comparisons were made with standard sperm washing procedures (Sieren and Youngs, 2001), with Percoll (Samardzija et al., 2006a) or with swim-up (Samardzija et al., 2006b), all including recovery and selection of frozen-thawed bull spermatozoa. The results were variable, with BoviPure®-recovered spermatozoa not leading to better production of bovine embryos compared with single washing in modified Brackett-Oliphant medium (Sieren and Youngs, 2001); yielding better cleavage rates but not blastocyst rates compared with Percoll-recovered spermatozoa (Samardzija et al., 2006a), or the opposite results when compared with swim-up sperm selection (Samardzija et al., 2006b). Interestingly, a rather low (30-40%) recovery rate was consistently found. The recovery rate in the present study was considerably higher (> 50%), even after single layer colloid centrifugation. Differences in procedures (handling, layering, centrifugal force and duration, etc.) as well as differences in formulations might have contributed to these results. As such, the use of a single colloid layer (centrifuged at 300 g for 20 min) resulted in the harvest of similar numbers of sperm cells with a similar

functionality (in terms of motility and fertilizing ability) as when density gradient controls were used.

Centrifugation on a single layer of colloid has various practical advantages over density gradient centrifugation. Requiring only one layer of colloid in the tube, the preparation time is shorter and the process is less complicated than for the density gradient, which involves at least two densities of colloid to be layered in the tube. Mixing of different densities (because of careless layering) destroys the integrity of the interface between the two layers, consequently reducing the efficiency of the sperm selection process. Additionally, single layer centrifugation facilitates scaling-up the volumes of colloid and ejaculate used, allowing proper handling of the large number of spermatozoa required for insemination doses in some animal species (Watson, 1990; Morrel et al., 2009). Other than non-toxicity, use of a GPMS-coated silica colloid and optimized species-specific formulations – as used in the present study – offers several advantages: it is stable in salt solutions (permitting large batches of ready-made colloids of different densities and standardized formulations to be mass-produced) and it can be autoclaved (hence reducing endotoxin levels).

In conclusion, centrifugation on a single layer of colloid provides an alternative method to density gradient centrifugation for selection of viable – potentially fertile – frozen-thawed bull spermatozoa. This single layer technique is gentle, versatile and convenient, and our results indicate that spermatozoa selected by the technique could be used for AI without expecting subsequent loss in pregnancy rate. Adopting the use of the single layer may allow the process to be scaled-up to prepare sufficient numbers of ejaculated spermatozoa for AI. As both centrifugation techniques are likely to be employed in future, we suggest use of the term *colloidal centrifugation* to cover the use of either density gradient or the modification whereby aliquots of ejaculate are centrifuged on a single layer of colloid.

Prospects for further research

In addition to the fact that IVF does not offer the same challenges to spermatozoa as AI and subsequent *in vivo* fertilization, the correlation between *in vivo* and *in vitro* fertility is still controversial. Despite the development of many new techniques, laboratory assays still do not predict male fertility accurately (Braundmeier et al., 2002). No significant correlations between any of the single *in vitro* results (various bull sperm traits, including the ability to bind to homologous ZP and to fertilize oocytes *in vitro*) and the *in vivo* fertility of frozen-thawed bull sperm were found (Zhang et al., 1999; Braundmeier et al., 2002; Puglisi et al.,

2004). Only after combination of several measures of sperm characteristics a correlation with observed 56d-NRRs was detected (Zhang et al., 1999). Very recently, models were produced combining the results of the performance of spermatozoa from a number of *in vitro* assays (including various sperm characteristics, such as sperm motility parameters, sperm morphology, chromatin integrity, etc.) showing a high correlation with fertility ($R^2 > 0.9$; Gillan et al., 2008). The tests used are claimed to be relatively simple, to require limited equipment and to be practicable in an AI centre. The authors suggest that such models may be a valuable tool to assess the potential fertility of a young bull in a future prospective study. However, in this study the performance of spermatozoa in the *in vitro* diagnostic assays was correlated with a statistically derived fertility value, in contrast to most other studies using non-return or pregnancies rates as a measure of *in vivo* fertility. The high correlations between sperm parameters and fertility observed in this study may very well be biased by the calculation of this statistically derived value where irrelevant sources of variation have been accounted for (Gillan et al., 2008). However, since most *in vitro* results are based on a limited number of observations, it would be premature to state that the approach can be used to determine the life-time fertility of a breeding sire. The production of normal sperm cells alters with age, and pathologies affecting spermatogenesis or the genital function potentially occur during the reproductive lifetime of the sires at an AI centre (Zhang et al., 1999). Fertility results obtained in a sperm assay only apply to the sample analyzed, and it hence seems unlikely that male fertility could be accurately predicted using sporadic *in vitro* tests (considering the intrinsic variation among ejaculates and the tendency for male fertility to vary with time; Larsson and Rodríguez-Martínez, 2000). From the results obtained in this thesis it is obvious that fertilization is a very complicated process, leaving little if any room for the development of one single marker for appropriate assessment of fertility. Until now, no method reliably mimics the complex and essential interactions between the sperm cells and the female genital tract which occur during sperm transport towards the site of fertilization. Used singly or in combination with other tests, the different assays based on IVF techniques allow screening of potential semen donors, as well as assessment of insemination doses or methods of semen preservation. They can be used to make a first screening of the young bulls, thereby allowing semen with a lower fertility to be excluded from test inseminations (Larsson and Rodríguez-Martínez, 2000). One could speculate that the selection of high quality spermatozoa prior to freezing would increase sperm survival after cryopreservation and – hence – lead to higher fertility in the field, since it is known that for some bulls, fertility is dependent on the insemination doses used (Den Daas et al., 1998).

In order to evaluate whether the commercial AI industry benefits from application of the single layer colloidal centrifugation technique, field trials are required. Sperm of different bulls should be included: conventionally processed insemination doses as well as doses processed after single layer colloidal centrifugation (both originating from the same ejaculates) should be administered at random in the field. A major drawback of such large field trials is the fact that the AIs have to be performed in different herds in order to obtain sufficient observations. As a result, a herd-effect cannot be excluded. Additional care must be taken with respect to the parity of the inseminated animals and whether or not include more than one inseminator, since these variables often significantly affect the final outcome. Finally, the practicability of the single layer colloidal centrifugation technique during collection and production of frozen-thawed bull semen in commercial settings should be assessed. Currently, whole ejaculates are evaluated (without prior sperm preparation or selection) for sperm concentration and motility in order to determine whether they will be diluted and frozen. When an ejaculate does not meet the minimum criteria, it is destroyed. As already mentioned above, selecting for viable sperm cells prior to freezing, could enhance the quality of the insemination dose. The activation of an aromatic amino acid oxidase occuring after the death of spermatozoa has been identified as a chief source of reactive oxygen species (ROS) production in bovine semen (Bailey et al., 2000). The release of this enzyme from dead sperm cells in egg yolk extender negatively affects the motility and viability of the remaining living bull sperm. In human, ROS levels are inversely correlated with fertilization rates following IVF (Agarwal et al., 2005). Increased generation of ROS in semen affects sperm function, particularly fusion events associated with fertilization leading to infertility (Agarwal et al., 2006). Removal of dead sperm cells (being a major source of ROS production) prior to cryopreservation may - hence - be beneficial for the sperm quality after thawing.

GENERAL CONCLUSIONS

Based on the results presented in this thesis, the following conclusions can be put forward:

1. Fertilization involves a complex series of molecular interactions. To ensure the reproduction of a species, nature created a multitude of involved ligand-receptor interaction mechanisms. Such redundancy implies that blocking of one ligand-receptor system will never be sufficient to inhibit fertilization completely, which has been observed frequently in the past (as well as in the presented experiments).
2. Exogenously supplemented Fn negatively influences bovine IVF by inhibiting sperm-oolemma binding and (concurrently) sperm-egg fusion. The presence of endogenous Fn underneath the ZP together with integrin subunit α_5 expression on the oolemma and the acrosome reacted sperm cell surface, suggests a reversible dual binding interaction between the endogenous Fn ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating sperm-egg binding.
3. Detection of endogenous Vn and integrin subunit α_v at the exterior side of the ZP and integrin subunit α_v on the sperm cell surface, combined with an increased sperm-ZP binding in presence of exogenous Vn, implies at least some intervention of this glycoprotein in initial sperm-ZP interaction. Since integrin subunit α_v was identified on the oolemma, and spermatozoa appeared to express both integrin subunit α_v and Vn after acrosomal reaction, this receptor-ligand mechanism may play a role in sperm-oocyte interaction. The inhibitory effect of exogenously supplemented Vn on sperm penetration of ZP-free oocytes could consequently be explained by the competition between the exogenous Vn and the Vn liberated from the sperm cell following the acrosome reaction.
4. Centrifugation on a single layer of species-specific GPMS-coated silica colloid offers an alternative – technically simplified, faster – method to density gradient centrifugation for selection of viable, potentially fertile frozen-thawed bull spermatozoa. Adopting the use of this single layer technique may allow the process to be scaled-up to prepare sufficient ejaculated spermatozoa for AI.

REFERENCES

Agarwal A, Allamaneni SS, Nallella KP, George AT, Mascha E. 2005. Correlation of reactive oxygen species levels with the fertilization rate after *in vitro* fertilization: a qualified meta-analysis. *Fertil Steril* **84** 228-231.

Agarwal A, Gupta S, Sikka S. 2006. The role of free radicals and antioxidants in reproduction. *Curr Opin Obstet Gynecol* **18** 325-332.

Avery B, Greve T. 1995. Impact of Percoll on bovine spermatozoa used for *in vitro* insemination. *Theriogenology* **44** 871-878.

Bailey JL, Bilodeau JF, Cormier N. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J Androl* **21** 1-7.

Bedford JM. 1998. Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. *Biol Reprod* **59** 1275-1287.

Braundmeier AG, Demers JM, Shanks RD, Saacke RG, Miller DJ. 2002. Examination of the binding ability of bovine spermatozoa to the zona pellucida as an indicator of fertility. *J Androl* **23** 645-651.

Bronson RA, Preissner KT. 1997. Measurement of vitronectin content of human spermatozoa and vitronectin concentration within seminal fluid. *Fertil Steril* **68** 709-713.

Da Poian AT, Carneiro FA, Stauffer F. 2005. Viral membrane fusion: is glycoprotein G of rhabdoviruses a representative of a new class of viral fusion proteins? *Braz J Med Biol Res* **38** 813-823.

Den Daas JH, De Jong G, Lansbergen LM, Van Wagtendonk-De Leeuw AM. 1998. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. *J Dairy Sci* **81** 1714-1723.

Elmore RG. 1994. Focus on bovine reproductive disorders: Evaluating bulls for breeding soundness. *Vet Med* **89** 372-378.

Fischer A. 2008. Untersuchungen zur Kryokonservierbarkeit von Bullensperma unter besonderer Berücksichtigung des Verdünnungsgrades und des Seminalplasmas. PhD thesis, Hannover, Germany, pp 80-81.

Fusi FM, Bronson RA. 1992. Sperm surface fibronectin. Expression following capacitation. *J Androl* **13** 28–35.

Fusi FM, Lorenzetti I, Mangili F, Herr JC, Freemerman AJ, Gailit J, Bronson RA. 1994. Vitronectin is an intrinsic protein of human spermatozoa released during the acrosome reaction. *Mol Reprod Dev* **39** 337-343.

Fusi FM, Tamburini C, Mangili F, Montesano M, Ferrari A, Bronson RA. 1996a. The expression of alpha v, alpha 5, beta 1, and beta 3 integrin chains on ejaculated human spermatozoa varies with their functional state. *Mol Hum Reprod* **2** 169–175.

Fusi FM, Bernocchi N, Ferrari A, Bronson RA. 1996b. Is vitronectin the velcro that binds the gametes together? *Mol Hum Reprod* **2** 859-866.

Gillan L, Kroetsch T, Maxwell WM, Evans G. 2008. Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. *Anim Reprod Sci* **103** 201-214.

Goossens K, Van Soom A, Van Zeveren A, Favoreel H, Peelman LJ. 2009. Quantification of fibronectin 1 (FN1) splice variants, including two novel ones, and analysis of integrins as candidate FN1 receptors in bovine preimplantation embryos. *BMC Dev Biol* **9** 1.

Januskauskas A, Johannisson A, Rodriguez-Martinez H. 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology* **60** 743-758.

Larsson B, Rodríguez-Martínez H. 2000. Can we use *in vitro* fertilization tests to predict semen fertility? *Anim Reprod Sci* **60-61** 327-336.

Mahawar M, Joshi P. 2008. Goat vitronectin: characterization and binding to Staphylococcus aureus. *Comp Biochem Physiol B Biochem Mol Biol* **149** 410-418.

Morrell JM, Johannisson A, Dalin AM, Rodriguez-Martinez H. 2009. Single-layer centrifugation with Androcoll-E can be scaled up to allow large volumes of stallion ejaculate to be processed easily. *Theriogenology* **72** 879-884.

Mortimer D. 2000. Sperm preparation methods. *J Androl* **21** 357-366.

Puglisi R, Balduzzi D, Galli A. 2004. *In vitro* sperm penetration speed and its relationship with *in vivo* bull fertility. *Reprod Domest Anim* **39** 424-428.

Rodríguez-Martínez H. 2003. Laboratory semen assessment and prediction of fertility: still utopia? *Reprod Domest Anim* **38** 312-318.

Royce PM, Steinmann B. 2002. Connective tissue and its heritable disorders. Molecular, Genetic, and Medical Aspects. Second Edition. Wiley, New York, USA, p 305.

Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J, Karadjole T. 2006a. A comparison of BoviPure and Percoll on bull sperm separation protocols for IVF. *Anim Reprod Sci* **91** 237–247.

Samardzija M, Karadjole M, Getz I, Makek Z, Cergolj M, Dobranic T. 2006b. Effects of bovine spermatozoa preparation on embryonic development *in vitro*. *Reprod Biol Endocrinol* **4** 58.

Shamsi MB, Venkatesh S, Tanwar M, Talwar P, Sharma RK, Dhawan A, Kumar R, Gupta NP, Malhotra N, Singh N, Mittal S, Dada R. 2009. DNA integrity and semen quality in men with low seminal antioxidant levels. *Mutat Res* **665** 29-36.

Sieren KR, Youngs CR. 2001. Evaluation of Bovipure™ for *in vitro* production of bovine embryos. *Theriogenology* **55** 438.

Smith AE, Helenius A. 2004. How viruses enter animal cells. *Science* **304** (5668) 237-242.

Stein KK, Primakoff P, Myles D. 2004. Sperm-egg fusion: events at the plasma membrane. *J Cell Sci* **117** 6269-6274.

Stewart PL, Nemerow GR. 2007. Cell integrins: commonly used receptors for diverse viral pathogens. *Trends Microbiol* **15** 500-507.

Stockmann A, Hess S, Declerck P, Timpl R, Preissner KT. 1993. Multimeric vitronectin. Identification and characterization of conformation-dependent self-association of the adhesive protein. *J Biol Chem* **268** 22874-22882.

Suzuki S, Oldberg A, Hayman EG, Pierschbacher MD, Ruoslahti E. 1985. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. *EMBO J* **4** 2519-2524.

Talbot P, Shur BD, Myles DG. 2003. Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion. *Biol Reprod* **68** 1-9.

Tamm LK, Crane J, Kiessling V. 2003. Membrane fusion: a structural perspective on the interplay of lipids and proteins. *Curr Opin Struct Biol* **13** 453-466.

Tanghe S, Vanroose G, Van Soom A, Duchateau L, Ysebaert MT, Kerkhofs P, Thiry E, van Drunen Littel-van den Hurk S, Van Oostveldt P, Nauwynck H. 2005. Inhibition of bovine sperm-zona binding by bovine herpesvirus-1. *Reproduction* **130** 251-259.

Teissier E, Pécheur EI. 2007. Lipids as modulators of membrane fusion mediated by viral fusion proteins. *Eur Biophys J* **36** 887-899.

Watson PF. 1990. Artificial insemination and the preservation of semen. In: Lamming GE (ed.), Marshall's Physiology of Reproduction. Churchill Livingstone, London, UK, p.785–825.

Zhang BR, Larsson B, Lundeheim N, Håård MG, Rodriguez-Martinez H. 1999. Prediction of bull fertility by combined in vitro assessments of frozen-thawed semen from young dairy bulls entering an AI-programme. *Int J Androl* **22** 253-260.

CHAPTER 7

SUMMARY

In order to successfully fertilize an oocyte, sperm cells must overcome several barriers in the bovine female genital tract. Besides escaping mechanical entrapment in the cervical folds, spermatozoa have to reversibly bind the oviductal epithelium (resulting in the formation of a functional sperm reservoir) ensuring that fit sperm cells reach the oocyte at the right time. At the site of fertilization, spermatozoa need to traverse different egg vestments to finally reach and penetrate the oocyte. In cattle, cumulus cells are shed from the oocyte in the oviduct within a few hours to 10 h after ovulation, but it is not entirely clear yet whether the cumulus cells or the matrix are necessary for bovine fertilization *in vivo*: sperm cells probably need to penetrate the cumulus matrix first, before they can pass through the ZP and subsequently fuse with the oolemma. Involvement of various carbohydrates and glycoproteins in adhesion and binding events during these reproductive processes has been described in ruminants, ranging from roles in sperm-oviduct adhesion or sperm-oocyte interactions to embryo implantation. An overview of the current knowledge concerning the role of glycomolecules in bovine reproduction is given **Chapter 1.1**. In cattle, fertilization takes place at the ampulla of the oviduct where both male and female gametes gather. During transport towards the fertilization site, the sperm cells pass several natural selection mechanisms. As the cervix represents the primary barrier for sperm transport, cervical mucus is thought to provide some means of sperm selection, since it seems to generate a more distinct barrier to abnormal spermatozoa unable to swim efficiently or displaying a poor hydrodynamic profile than it does to morphologically normal and motile spermatozoa. The next (anatomical, physiological and/or mucus) barrier to sperm passage in the female reproductive tract is the uterotubal junction (UTJ). Even though the UTJ expands during estrus or when stimulated by coitus, spermatozoa may not be able to pass it without exposing certain proteins on their cell surface (possibly allowing sperm to gain footholds on the wall lining the UTJ and so to progress forward by attaching reversibly to the epithelium). After passage through the UTJ, a number of sperm cells will reversibly bind to the epithelium of the caudal segment of the oviducal isthmus, resulting in formation of a functional storage reservoir (serving to guarantee successful fertilization by providing suitable numbers of sperm cells in the appropriate physiological state at the ampulla soon after ovulation). Upon mating, uncapacitated sperm cell are bound and stored in the reservoir. Endocrine signals associated with imminent ovulation are assumed to initiate release of sperm cells from the reservoir through induction of capacitation and hyperactivation. Accordingly, capacitated spermatozoa will finally reach the oocyte. In order to obtain adequate results *in vitro*, it is required to select for viable (potentially fertile) sperm cells prior to *in vitro* fertilization (IVF), since thorough sperm selection normally occurs in the

female reproductive tract. The ideal *in vitro* sperm selection procedure should therefore be able to considerably improve the sperm quality with higher rates of progressive motility and morphologically normal spermatozoa. In **Chapter 1.2** the four basic approaches for sperm preparation are discussed: 1) dilution and washing (centrifugation and resuspension); 2) sperm migration (swim-up procedures); 3) adherence methods (differential filtration through glass wool, glass beads or polysaccharide beads); and 4) density gradient centrifugation (selective fractionating subpopulations).

The general aim of the present thesis was to further optimize the bovine *in vitro* embryo production system by 1) elucidating the molecular basis underlying the previously observed inhibitory effect of fibronectin (Fn) and vitronectin (Vn) on bovine IVF, and by 2) simplifying the requisite *in vitro* sperm selection procedure **(Chapter 2)**.

In **Chapter 3**, potential endogenous expression of Fn and its integrin receptor ($\alpha_5\beta_1$) on male and female bovine gametes was evaluated using indirect immunofluorescence and the putative function of Fn during bovine fertilization was determined. Endogenous Fn was detected underneath the ZP and integrin subunit α_5 on the oolemma of cumulus-denuded oocytes. Bovine spermatozoa displayed integrin subunit α_5 at their equatorial segment after acrosome reaction. The main inhibitory effect of exogenously supplemented Fn appeared to be located at the sperm-oolemma binding, with a (concurrent) effect on fusion, and this can probably be attributed to the binding of Fn to spermatozoa at the equatorial segment, as shown by means of AlexaFluor®488-conjugated Fn. Combining these results, the inhibitory effect of exogenously supplemented Fn seemed to be exerted on the male gamete by binding to the exposed integrin $\alpha_5\beta_1$ receptor after acrosome reaction. The presence of endogenous Fn underneath the ZP together with integrin subunit α_5 expression on oolemma and acrosome reacted sperm cell surface, suggests a reversible dual binding interaction between the endogenous Fn ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating sperm-egg binding.

Analogously, the expression of Vn and its integrin receptor ($\alpha_v\beta_3$) on male and female bovine gametes and their function during IVF was investigated **(Chapter 4)**. Vitronectin was identified as an intrinsic sperm protein (exposed during acrosome reaction) and was also observed at the cumulus oophorus (especially near the exterior side of the ZP). Integrin subunit α_v appeared to be expressed on the anterior sperm cell surface (to some extent after

capacitation and substantially more pronounced after acrosome reaction). Integrin subunit α_v was also present at the oolemma of all sampled oocytes, and at the exterior side of the ZP in the majority of the female gametes. A fairly low dose of exogenous Vn (100 nM) enhanced sperm penetration of bovine cumulus-oocyte complexes, whereas 500 nM had a significant inhibitory effect. Sperm-zona binding was slightly increased, while sperm-oolemma binding was not significantly affected by the presence of 500 nM Vn. Compared to the respective control group, sperm-oocyte fusion was significantly inhibited by supplementing 500 nM Vn to the medium. This high concentration of Vn appeared to compromise sperm membrane integrity. However, the inhibitory effect of 500 nM Vn seemed to be exerted mainly through its twofold reduction of the progressive sperm motility. Low doses of Vn may well improve sperm penetration through boosting initial sperm-ZP binding. The presence of endogenous Vn at the sperm cell surface after acrosome reaction together with integrin subunit a_v expression on the oolemma suggests a reversible dual binding interaction between the endogenous Vn ligand and corresponding receptors on both (AR) sperm cell and oolemma, mediating sperm–oocyte interaction.

Recently, a new – technically simplified – sperm selection technique has been suggested (based on a single layer of a species-specific colloid suspension). **Chapter 5** describes our investigation of the fertilizing ability during IVF of frozen-thawed bovine spermatozoa following centrifugation through a single layer of this glycidoxypropyltrimethoxysilane (GPMS)-coated silica colloid. The presented data (although preliminary) clearly show that the newly simplified method for sperm preparation (selection of the better quality spermatozoa) by centrifugation through a single layer of GPMS-coated silica bead colloid did not have any adverse effect on their fertilizing ability (compared with density gradient centrifugation, either of a similar colloid or of Percoll). Basically, sperm characteristics, with the exception of a couple of CASA-derived, re-calculated kinematic parameters, were statistically similar. Fertilization rate, blastocyst development rate and the total number of blastomeres did not differ from both controls. In contrast to former studies, the sperm recovery rate in the present study was considerably high (> 50%), even after single layer colloid centrifugation. As such, the use of a single colloid layer (centrifuged at 300g for 20 min) resulted in yielding of similar numbers sperm cells with a similar functionality (in terms of motility and fertilizing ability) as when density gradient controls were used.

Finally, in **Chapter 6**, the main results of this thesis are summarized and discussed. A hypothetical model concerning the role of Fn and Vn during bovine sperm-egg interaction is put forward (based on the combined results).

In conclusion, fertilization involves a complex series of molecular interactions. To ensure the reproduction of a species, nature created a multitude of involved ligand-receptor interaction mechanisms. The presence of endogenous Fn underneath the ZP together with integrin subunit α_5 expression on the oolemma and the acrosome reacted sperm cell surface, suggests a reversible dual binding interaction between the Fn ligand and corresponding receptors on both (acrosome reacted) sperm cell and oolemma, initiating sperm-egg binding. Detection of Vn and integrin subunit α_v at the exterior side of the ZP and integrin subunit α_v on the sperm cell surface, combined with an increased sperm-ZP binding in presence of exogenous Vn, implies some intervention of this glycoprotein in initial sperm-ZP interaction. Since integrin subunit α_v was identified on the oolemma, and spermatozoa appeared to express both integrin subunit α_v and Vn after acrosomal reaction, this receptor-ligand mechanism may play a role in sperm-oocyte interaction as well. The inhibitory effect of exogenously supplemented Vn on sperm penetration of ZP-free oocytes could consequently be explained by the competition between the exogenous Vn and the Vn liberated from the sperm cell following the acrosome reaction. Single layer centrifugation using species-specific GPMS-coated silica colloid offers an alternative – technically simplified – method to density gradient centrifugation for selection of viable frozen-thawed bull spermatozoa. Adopting the use of this technique may allow the process to be scaled-up to prepare sufficient numbers of ejaculated spermatozoa for AI.

SAMENVATTING

Om een eicel succesvol te kunnen bevruchten, dienen zaadcellen verscheidene barrières te overwinnen in de vrouwelijke geslachtstractus van het rund. Naast ontsnappen aan verstrikking in de cervicale plooien, moeten de spermacellen ook in staat zijn om zich op reversiebele manier te binden aan het oviductepitheel (waardoor een functioneel spermareservoir wordt gevormd dat verzekert dat geschikte spermatozoa de eicel bereiken op het juiste moment). Op de plaats van de bevruchting dienen de zaadcellen doorheen de verschillende lagen rondom de eicel te penetreren om uiteindelijk effectief met de vrouwelijke gameet te kunnen fusioneren. Bij het rund worden de cumuluscellen afgestoten van het cumulus-eicel-complex in de eileider binnen het uur tot 10 uur na ovulatie. Het is echter nog niet duidelijk of deze cumuluscellen of hun extracellulaire matrix noodzakelijk zijn voor de bevruchting *in vivo* bij het rund: zaadcellen moeten waarschijnlijk eerst deze matrix passeren vooraleer de zona pellucida (ZP) te bereiken en penetreren en vervolgens te versmelten met het oölemma. Betrokkenheid van diverse koolhydraten en glycoproteïnen in adhesie- en bindingsprocessen in het voortplantingsgebeuren is aangetoond bij herkauwers, gaande van een rol bij de sperma-oviduct-adhesie, sperma-eicelinteracties tot embryo-implantatie. In **Hoofdstuk 1.1** wordt een overzicht gegeven van de recente kennis betreffende de rol van glycomoleculen in de voortplanting bij het rund.

Bij koeien vindt de bevruchting plaats ter hoogte van de ampulla van het oviduct waar de mannelijke en vrouwelijke gameet elkaar ontmoeten. Tijdens het transport naar deze bevruchtingsplaats ondergaan de zaadcellen verschillende natuurlijke selectiemechanismen. De cervix vormt de primaire barrière voor het spermatransport. Het aanwezige cervicale slijm wordt verondersteld enigszins spermacellen te selecteren, aangezien het een meer uitgesproken barrière lijkt te vormen voor abnormale spermatozoa (met inefficiënte motiliteit of een ontoereikend hydrodynamisch profiel) dan voor de morfologisch normale en motiele zaadcellen. De volgende (anatomische, fysiologische en/of slijmerige) barrière voor spermapassage in de vrouwelijke geslachtstractus is de uterotubale junctie (UTJ). Hoewel deze UTJ verwijdt tijdens de oestrus of bij stimulatie door de coïtus, zouden spermatozoa zonder expressie van bepaalde proteïnen op hun celoppervlak niet kunnen passeren (dergelijke moleculen zouden de zaadcellen mogelijkerwijs toelaten grip op de wand van de UTJ te krijgen waardoor ze door reversiebele bindingen aan het epitheel hun voorwaartse beweging kunnen verder zetten). Na passage doorheen de UTJ, zullen een aantal spermacellen zich tijdelijk binden aan het epitheel van het caudale segment van de isthmus, waardoor een functioneel spermareservoir tot stand komt (dat er voor zorgt dat voldoende aantallen

geschikte spermacellen kort na ovulatie de ampulla zullen bereiken zodat een succesvolle bevruchting verzekerd wordt). Na dekking worden niet-gecapaciteerde spermacellen door binding *opgeslagen* in het reservoir. Endocriene signalen geassocieerd met nakende ovulatie worden verondersteld het loslaten van de gebonden zaadcellen te mediëren via inductie van capacitatie en hyperactivatie. Bijgevolg zijn de spermatozoa die uiteindelijk de eicel zullen bereiken reeds gecapaciteerd. Teneinde goede resultaten te bekomen na *in vitro* fertilisatie (IVF) dient het spermastaal vooraf geselecteerd te worden op vitaliteit en potentieel bevruchtend vermogen, daar de grondige spermaselectiemechanismen in de vrouwelijke geslachtstractus worden omzeild. De ideale *in vitro* spermaselectieprocedure moet in staat zijn om de spermakwaliteit van het staal aanzienlijk te verbeteren door zaadcellen met een normale morfologie en voldoende progressieve motiliteit te selecteren. In **Hoofdstuk 1.2** worden de vier basistechnieken voor spermaselectie toegelicht: 1) verdunnen en wassen van het staal (door centrifugatie en resuspensie); 2) spermamigratie (swim-up techniek); 3) adhesietechnieken (differentiële filtratie door glaswol, glazen kralen of polysaccharide kralen); en 4) gradiëntcentrifugatie op basis van verschillen in densiteit (selectieve fractionering van subpopulaties).

De algemene doelstelling van deze thesis was het verder optimaliseren van het *in vitro* embryoproductiesysteem bij het rund door 1) ophelderen van de moleculaire basis verantwoordelijk voor het eerder beschreven inhiberend effect van fibronectine (Fn) en vitronectine (Vn) op runder-IVF, en door 2) het vereenvoudigen van de noodzakelijke *in vitro* spermaselectieprocedure **(Hoofdstuk 2)**.

In **Hoofdstuk 3** werd de potentiële expressie van endogeen Fn en zijn receptor (integrine $\alpha_5\beta_1$) op mannelijke en vrouwelijke gameten nagegaan met behulp van indirecte immunofluorescentie en werd de vermoedelijke functie van Fn tijdens de bevruchting bij het rund onderzocht. Endogeen Fn werd teruggevonden onder de ZP en integrine subunit α_5 op het oölemma van cumulusvrije eicellen. Spermacellen bleken integrine subunit α_5 tot expressie te brengen ter hoogte van hun equatoriaal segment na acrosoomreactie. Het belangrijkste inhiberende effect van exogeen Fn was gelokaliseerd op het niveau van de sperma-oölemmabinding, met een (gelijktijdig) effect op het fusieproces. Dit is hoogstwaarschijnlijk toe te schrijven aan de binding van het exogene Fn aan de spermatozoa ter hoogte van het equatoriaal segment, hetgeen waargenomen werd met behulp van AlexaFluor®488-gelabeld Fn. Op basis van al deze resultaten lijkt het inhiberende effect van

exogeen Fn te worden uitgeoefend op de mannelijke gameet door binding aan het integrine $\alpha_5\beta_1$ (tot expressie gebracht na acrosoomreactie). De aanwezigheid van endogeen Fn onder de ZP samen met de integrine subunit α_5-expressie op het oölemma en het acrosoomgereageerde spermaceloppervlak, suggereert een reversibele dubbele bindingsinteractie tussen het endogene Fn ligand en de corresponderende receptoren op de (acrosoomgereageerde) spermacel en het oölemma, die de sperma-eicelbinding zou kunnen initiëren.

Analoog werd de expressie van Vn en de overeenkomstige integrine-receptor ($\alpha_v\beta_3$) op de mannelijke en vrouwelijke gameten beoordeeld en werd hun functie tijdens het IVF-proces nagegaan **(Hoofdstuk 4)**. Vitronectine werd enerzijds geïdentificeerd als een intrinsiek spermaproteïne (dat tot expressie wordt gebracht op het celoppervlak tijdens de acrosoomreactie) en anderzijds gedetecteerd in de cumulus oöphorus (vooral aan de buitenzijde van de ZP). Integrine subunit α_v bleek aanwezig te zijn op het rostrale spermaceloppervlak (in beperkte mate na capacitatie en duidelijk meer uitgesproken na acrosoomreactie). Integrine subunit α_v kwam ook tot expressie op het oölemma van alle bemonsterde eicellen, en aan de buitenzijde van de ZP in het merendeel van de vrouwelijke gameten. Een lage dosis exogeen Vn (100 nM) werkte de spermapenetratie van cumulus-eicel-complexen in de hand, terwijl 500 nM er een significant inhiberend effect op uitoefende. De sperma-ZP-binding was lichtjes toegenomen en de sperma-oölemmabinding werd niet beïnvloed in aanwezigheid van 500 nM Vn. Vergeleken met de controlegroep werd de sperma-eicelfusie significant geïnhibeerd door supplementatie van 500 nM Vn aan het fertilisatiemedium. Deze hoge Vn-concentratie bleek de membraanintegriteit van de zaadcellen te compromitteren. Het inhiberende effect van 500 nM Vn bleek echter voornamelijk toe te schrijven aan de tweevoudige reductie van de progressieve spermamotiliteit. Lage doses Vn zouden de spermapenetratie mogelijkerwijs kunnen bevorderen door extra ondersteuning van de initiële sperma-ZP-binding. De aanwezigheid van het endogene Vn op het spermaceloppervlak na acrosoomreactie samen met de integrine subunit α_v-expressie op het oölemma impliceert een reversibele dubbele bindingsinteractie tussen het endogene Vn-ligand en de corresponderende receptoren op de (acrosoomgereageerde) spermacel én het oölemma, die een rol zou kunnen spelen bij het tot stand komen van de sperma-eicelinteractie.

Enige tijd geleden werd een nieuwe – technisch vereenvoudigde – spermaselectietechniek voorgesteld (op basis van één enkele laag van een speciës-specifieke colloïdsuspensie). **Hoofdstuk 5** beschrijft ons onderzoek naar het *in vitro* bevruchtend vermogen van ingevroren stierensperma na centrifugatie met dit

glycidoxypropyltrimethoxysilaan (GPMS)-gecoat silica colloid. De voorgestelde data tonen eenduidig aan dat de nieuwe – vereenvoudigde – spermaselectiemethode op basis van één laag van dit GPMS-gecoat silica colloïd geen enkel nadelig effect had op de bevruchtingscapaciteit van de spermatozoa (vergeleken met densiteit-gradiënt-centrifugatie van hetzelfde colloïd of van Percoll). Samengevat verschilden de spermakenmerken – met uitzondering van enkele onrechtstreeks berekende kinetische (CASA) parameters – niet. Het bevruchtingspercentage, het percentage blastocysten en het totale aantal blastomeren waren eveneens vergelijkbaar met die van beide controles. In tegenstelling tot de resultaten van voorafgaande studies bleek het spermarecuperatiepercentage in dit onderzoek behoorlijk hoog (> 50%), zelfs na centrifugatie (bij 300g gedurende 20 min) op één enkele laag colloïd. Bijgevolg resulteerde deze vereenvoudigde spermaselectietechniek in vergelijkbare aantallen spermatozoa met vergelijkbare functionaliteit (op het gebied van motiliteit en bevruchtend vermogen) als na beide densiteit-gradiënt-centrifugatietechnieken.

Tot slot worden de belangrijkste resultaten van deze thesis op een rijtje gezet in **Hoofdstuk 6**. Op basis van de gecombineerde resultaten, wordt een hypothetisch model betreffende de rol van Fn en Vn tijdens de sperma-eicel-interactie bij het rund naar voor geschoven.

Uit de data verzameld in dit werk kunnen de volgende besluiten getrokken worden:

1. Fertilisatie komt tot stand door een complexe opeenvolging van moleculaire interacties. Om de voortplanting van een diersoort te verzekeren, creëerde de natuur een veelvuldigheid aan betrokken ligand-receptor-interactiemechanismen. Dergelijke overvloed aan mechanismen impliceert dat het bevruchtingsproces nooit volledig kan geblokkeerd worden door uitschakeling van één enkel ligand-receptor-systeem, hetgeen al vaak waargenomen werd in het verleden (evenals in dit werk).
2. Exogene supplementatie van Fn heeft een negatieve invloed op IVF door inhibitie van de sperma-oölemma-binding en (bijgevolg) de sperma-eicelfusie bij het rund. De aanwezigheid van endogeen Fn onder de ZP samen met de integrine subunit α_5-expressie op het oölemma en het acrosoomgereageerde spermaceloppervlak, suggereert een reversibele dubbele bindingsinteractie tussen het endogene Fn-

ligand en de corresponderende receptoren op de (acrosoomgereageerde) spermacel en het oölemma, die de sperma-eicelbinding zou kunnen initiëren.

3. Detectie van endogeen Vn en integrine subunit α_v op de buitenzijde van de ZP en integrine subunit α_v op het spermaceloppervlak in combinatie met een toegenomen sperma-ZP-binding in aanwezigheid van exogeen Vn impliceert alleszins enige betrokkenheid van dit glycoproteïne in de initiële sperma-ZP-interactie. Aangezien integrine subunit α_v geïdentificeerd was ter hoogte van het oölemma, en spermatozoa zowel integrine subunit α_v als Vn tot expressie bleken te brengen na acrosoomreactie, zou dit receptor-ligand-mechanisme een rol kunnen spelen in de sperma-eicel-interactie. Het inhiberende effect van exogeen Vn op de spermapenetratie van ZP-vrije eicellen zou dan verklaard kunnen worden door competitie tussen het exogene Vn en het Vn vrijgesteld vanuit de spermacel bij acrosoomreactie.
4. Centrifugatie op één enkele laag van speciës-specifiek GPMS-gecoat silica colloïd biedt een alternatieve – technische vereenvoudigde en bijgevolg snellere – methode voor densiteit-gradiënt-centrifugatie voor selectie van vitale, potentieel fertiele ingevroren spermatozoa bij de stier. De toepassing van deze nieuwe techniek zou het proces nodig om voldoende spermacellen te selecteren uit ejaculaten voor kunstmatige inseminatie, aanzienlijk kunnen versnellen.

DANKWOORD

All's Well That Ends Well... (W. Shakespeare). En gelijk heeft ie: bij het bereiken van de eindmeet vergeet een mens - gelukkig - al gauw het zweet en de tranen, de mislukte experimenten, de commentaren van de reviewers en de gebruikelijke frustraties die een doctoraat blijkbaar onvermijdelijk met zich meebrengt. Het was dan natuurlijk ook niet allemáál kommer en kwel, want we hebben het goed gehad (de pintjes en babbels in het Boerderijtje en op de terrasjes, de middagpauzes in het zonnetje op het gras, de internationale congressen, de assistentenfuiven en de onnavolgbare fopjes in en rond ons bureau).

Een klein woordje van dank is dan ook op zijn plaats...

Ere wie ere toekomt, het was Steven V die me in mijn laatste studiejaar vroeg of ik geïnteresseerd was om zijn project over diepe intra-uteriene inseminatie af te werken. Ook Prof. Ann Van Soom en Prof. Aart de Kruif waren bereid mij die kans te geven en zo begon ik als wetenschappelijk medewerker aan onze vakgroep die gedurende vijf jaar mijn bakermat werd.

Ann, bedankt om me de gelegenheid te geven om als onderzoeker aan de slag te gaan en vooral om me hierbij de nodige vrijheid en zelfstandigheid te geven zonder me uit het oog te verliezen. Je stond altijd klaar voor kleine en minder kleine vraagstukken en gunde me ook mijn praktijkuurtjes en de cursus vakdierenarts rund ('zolang mijn onderzoek maar niet in het gedrang kwam')! Je bent - terecht - veeleisend en kordaat als het moet, maar hebt me altijd veel vertrouwen gegeven en bent me blijven motiveren ook als het soms wat tegen sloeg. Ik heb veel van je geleerd...

Prof. Hans Nauwynck werd gaandeweg mijn co-promotor en heeft er meer dan eens voor gezorgd dat de publicaties, de foto's en figuren nog net ietsje beter werden (ook al wilde ik dat niet altijd direct aannemen). De discussies waren soms nogal levendig, maar ik moet toegeven dat ze ervoor zorgden dat ik mezelf nog harder wilde bewijzen. Bedankt voor het kritisch nalezen van mijn publicaties en het helpen denken over ons 'IVF model'. Het mag er zijn!

Prof. de Kruif, als voorzitter runde je onze vakgroep als een echte pater familias. We waren met velen en toch maakte je er een punt van met ieder van ons regelmatig een informeel gesprek te hebben om te checken hoe het met onszelf en ons onderzoek gesteld was.

Diezelfde betrokkenheid reflecteerde zich in je consciëntieuze opvolging van patiënten én studenten tijdens de dagelijkse stalronde.

Voorts gaat mijn dank uit naar de leden van de examen- en leescommissie. Prof. Fazeli, I greatly appreciate that you were willing to accept the invitation for the exam committee. Prof. De Sutter, bedankt voor het nalezen van en meedenken over de inhoud en vorm van mijn laatste publicatie en thesis. Prof. Favoreel, Herman, jij verdient een welgemeende dankjewel voor al je hulp en geduld bij de microscopische opnames en flowcytometrische analyses, de praktische tips bij de fluorescente kleuringen en het nauwgezet nalezen van mijn artikels.

Dries (wij noemden je *Farmacie-Dries* om alle verwarring uit te sluiten), je hebt me geweldig geholpen met je expertise én geduld bij de confocale en DIC opnames voor het fibronectine-artikel. Als doctoraatsstudent was je bereid om mij te helpen op je vrije dagen en avonden (hetgeen ik dan ook heel erg weet te appreciëren). Ik wens je veel succes met je eigen doctoraat en de rest van je carrière.

Ik wil ook Dr. Nele Festjens (Vakgroep Biochemie, Fysiologie en Microbiologie - Faculteit Wetenschappen - UGent) bedanken voor de hulp bij (en het lenen van de infrastructuur voor) de FITC-conjugatie van het fibronectine. Het was een vruchtbare samenwerking.

De mensen van CRV (het vroegere CR-Delta en VRV) wil ik zeker niet vergeten voor het ter beschikking stellen van het sperma en het uitvoeren van de inseminaties voor het project over diepe intra-uteriene inseminatie. Ook een woordje van dank voor Betsie Grotentraast en haar ploeg in de KI-stal te Harfsen (Nederland) voor de bereidwilligheid en enorme gastvrijheid. Vervolgens eveneens dank aan de directie, het personeel en de dierenartsen van de slachthuizen van Rekkem (LAR) en Brugge voor het verzamelen van eierstokken.

Prof. Maes en Prof. Dewulf, Dominiek en Jeroen, bedankt voor alle hulp bij de statistische verwerking van mijn data en de antwoorden op al mijn vragen hieromtrent. Statistiek is en blijft immers iets aparts.

Dhr. Marc Pensaert (Business Unit Manager Gynaecology & Endocrinology) van de farmaceutische firma Ferring wil ik bedanken voor de toestemming en het aanleveren van de foto voor de kaft van mijn doctoraatsthesis.

Ook een dikke kus aan An Van Loo voor het ontwerpen van de kaft in haar vrije tijd. Het resultaat mag gezien worden...

Aiai, weer een dierenarts in het labo! was het antwoord op mijn vraag of je *17,5 keer verdunnen* mocht afronden naar 17 of 18. Gelukkig werd ik snel en vakkundig ingewijd in de wondere wereld van verdunningen, steriel werken, pipetteren, puncteren, opzoeken, bevruchten en op cultuur zetten van eicellen. Alle lof gaat hier naar de laboranten van het IVF-labo: Joke, Griet, Nele, Sofie, Isabel en Petra! Ook bedankt om - tijdens de laatste loodjes - extra bij te springen door voor mij naar het slachthuis te rijden en zonder veel hulp mijn proeven op te starten.

Dan rest er natuurlijk nog mijn andere collega's van de IVF-groep te bedanken. Hierbij denk ik - in eerste instantie - aan Bart die me vanaf het begin met raad en daad heeft bijgestaan en er met veel toewijding voor heeft gezorgd dat ik me al snel thuis voelde op onze vakgroep! Al gauw belandde ik op het bureau beneden en ook daar heb ik vriendschapsbanden gesmeed. Sofie T, we hebben niet zolang samen gewerkt, maar lang genoeg om je nauwkeurigheid en inzet voor je werk te leren waarderen. Het was dan ook een eer om jouw werk te mogen verder zetten. Leen, wij hebben vijf jaar samen gevaren op het IVF-schip. Ik denk dat we trots mogen zeggen dat we elkaar gesteund en geholpen hebben waar het kon en dat we het beiden tot een goed einde hebben gebracht. Ik ben er ook zeker van dat je je in de toekomst zal blijven inzetten voor je werk en dat je zal blijven streven om van je werkomgeving een aangename plek te maken. Jo B, de bio-ingenieur tussen de veeartsen, je hebt je mannetje goed gestaan. Ik apprecieer je rechttoe rechtaan aanpak en de manier waarop je iedereen onbevooroordeeld benadert. We hebben alle fasen van het doctoraat samen doorlopen en hebben dan ook veel aan elkaar gehad (niet alleen professioneel, maar ook daarbuiten). Ik hoop dat we elkaar niet uit het oog verliezen (ons paardrij-uurtje is al een goed begin). En toen kwam... Muriel. Daar kunnen we een boek over schrijven. Onder alle grappen en grollen schuilt er een degelijke, intelligente doctoraatsstudente die met veel passie student én collega bijstaat in de reproductieve geneeskunde van hond en kat. Ik twijfel er niet aan dat je de rol van bureau-oudste met glans zal vervullen. Gelukkig waakt ook het oog van Josine (de jongste telg in ons bureau). Je had al snel je vaste plek in onze *hiërarchie* (en hartjes) veroverd. We hebben je leren kennen als gemotiveerde, verantwoordelijke, maar ook goedlachse meid, die nooit te beroerd is om de handen uit de mouwen te steken. We waren een goed team...

Karen, ook voor jou een dankjewel voor de hulp bij het op punt stellen van de kleuringsprotocols en het gewillig delen van zinvolle informatie voor mijn onderzoek/publicatie. Het congres in Polen samen op de kamer was ook heel plezant. Nog veel succes aan de *overkant*.

Naast het onderzoekswerk heb ik ook altijd graag gewerkt op de kliniek verloskunde rund en de buitenpraktijk. Ik bewaar er vele mooie herinneringen aan en heb er veel van geleerd zowel op diergeneeskundig als op menselijke vlak. Bedankt aan alle mensen met wie ik heb mogen samenwerken en van wie ik heb mogen leren: Jef, Dirk B, Marcel, Geert H, Bart M, Steven V, Tom VH, Jo L, Boudewijn, Sarne, Leen VD, Stefaan, Iris K, Hans, Philippe, Steven C, Tim, Karlien, Sofie P, Miel, Emily DB, Nele G, Leen M, Jan, Maarten, Catharina, Tom T, Tom M, Jo V, Nathalie N, Kristof VD, Bianca, Liesbeth, Sebastiaan, Katrien S, Davy, Emilie VH, Kim, Josine, Ellen, Vanessa en Alfonso. Iris K, nog veel succes met de laatste loodjes van jouw doctoraat - je laatste deadline - en tot volgende week dinsdag, op ons paard! Bedankt ook aan de jongere generatie om tijdens de eindspurt enkele diensten van mij over te nemen.

Alle andere mensen (die niet onmiddellijk in één vakje thuishoren, maar daarom niet minder belangrijk zijn) waarmee ik de afgelopen vijf jaar op onze vakgroep heb gewerkt, wil ik bedanken voor de ondersteuning, de leuke gesprekken en/of het oplossen van allerlei praktische problemen: Steven B (van onschatbare waarde voor al het computergebeuren op onze vakgroep, een dikke merci voor alles), Nadine (ik ben je laatste doctoraat), Ria, Els, Leïla, Marnik, Véronique, Willy, Dirk, Wilfried, Tom R, Mohammed, Caroline, Sarah, Lotte, Inès, Nele E, Margit, Katrien M, Iris V, Nicole en haar team, Roger V, Marc C en Prof. Marc Verdonck.

Het leven is natuurlijk ook meer dan werken alleen. Naast de vrienden die ik tijdens mijn doctoraat heb leren kennen, zijn er uiteraard - en gelukkig - ook vrienden te bedanken die ik eerder (tijdens mijn schooltijd en studie diergeneeskunde) heb gemaakt. Gezien de afstand tot het thuisfront en de nieuwe horizonten die eenieder heeft opgezocht, zien we elkaar niet vaak, maar het is en blijft altijd leuk om nog eens goed bij te babbelen en te lachen zoals vroeger!

Jos en Inge, bedankt voor alle taalkundige hulp bij het schrijven én voor het toffe knulletje Emile :-). Jullie zijn altijd welkom en als ik jullie eens met iets kan helpen, laat het maar weten.

Papa, ik had nooit gedacht dat je dit niet meer zou kunnen meemaken. Als er iemand trots was op zijn jongste telg als doctoraatsstudente, dan was jij het wel. Als ik al eens twijfelde aan mijn onderzoek en het halen van deadlines, bleef jij er altijd rotsvast van overtuigd dat ik de eindmeet zou halen. Wel, ik ben er en ik weet dat je er hoe dan ook blij mee bent.

Mama, bedankt voor alles. Jij staat steeds met je nooit aflatend enthousiasme aan de zijlijn, terwijl ik mijn eigen weg baan (zonder je te willen opdringen of er iets voor in de plaats te willen). Bedankt voor al de goede zorgen, je luisterende oor en al de kansen die je me gegeven hebt.

Hans, er zijn heel veel dingen waarvoor ik je zou willen bedanken, maar je hebt gevraagd *maak het vooral niet te lang, he*. Bedankt om in mij te geloven en me zo nu en dan met mijn twee voetjes op de grond te zetten als ik al eens doordraaf. *Iemand door en door compleet verstaan - dat lukt niet - maar daar wen je aan - en voor je 't weet - word je samen oud (Bart Peeters).* We doen dat goed met ons tweetjes. Laat maar komen dat vervolg...

Nogmaals dank aan iedereen die mij ooit op gelijk welke manier heeft geholpen bij het realiseren van dit proefschrift.

Mirjan

CURRICULUM VITAE

Mirjan Thys werd geboren op 26 april 1980 te Tongeren. Na het behalen van het diploma hoger secundair onderwijs aan het Koninklijk Atheneum te Tongeren (Wetenschappen-Wiskunde), begon zij in 1998 met de studie Diergeneeskunde aan de Universiteit Antwerpen. In 2004 behaalde ze aan de Universiteit Gent haar diploma van dierenarts (optie herkauwers) met grote onderscheiding.

Op 1 augustus 2004 trad zij in dienst van de Vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde, waar zij als wetenschappelijk medewerker gedurende een jaar meewerkte aan het project diepe intra-uteriene inseminatie met een nieuwe inseminatiepipet bij het rund. In oktober 2005 startte ze – als BOF-bursaal – haar doctoraatsonderzoek naar de rol van glycoproteïnen tijdens de *in vitro* fertilisatie bij het rund. Naast haar onderzoek was Mirjan Thys ook werkzaam in de kliniek verloskunde rund en de buitenpraktijk rund, waar ze participeerde in de nacht- en weekenddiensten. In 2009 voltooide zij de Doctoraatsopleiding in de Diergeneeskundige Wetenschappen en behaalde ze het diploma vakdierenarts rund met grote onderscheiding.

Mirjan Thys is auteur of mede-auteur van verschillende publicaties in internationale en nationale wetenschappelijke tijdschriften en nam actief deel aan diverse internationale en nationale congressen.

BIBLIOGRAPHY

PUBLICATIONS

Verberckmoes S, Van Soom A, **Thys M**, de Kruif A. 2004. Intra-uteriene inseminatie bij mens en dier. *Vlaams Diergeneeskundig Tijdschrift* **73** 317-324.

Verberckmoes S, Van Soom A, Dewulf J, **Thys M**, de Kruif A. 2005. Low dose insemination in cattle with the Ghent device. *Theriogenology* **64** 1716-1728.

Thys M, Vandaele L, Morrell JM, Mestach J, Van Soom A, Hoogewijs M, Rodriguez-Martinez H. 2009. *In vitro* fertilising capacity of frozen-thawed bull spermatozoa selected by single-layer (glycerolpropylsilane, GS) silane-coated silica colloidal centrifugation. *Reproduction in Domestic Animals* **44** 390-394.

Thys M, Nauwynck H, Maes D, Hoogewijs M, Vercauteren D, Rijsselaere T, Favoreel H, Van Soom A. 2009. Expression and putative function of fibronectin and its receptor (integrin $\alpha_5\beta_1$) in male and female gametes during bovine fertilization *in vitro*. *Reproduction* **138** 471-482.

Vandaele L, **Thys M**, Bijttebier J, Van Langendonckt A, Donnay I, Maes D, Meyer E, Van Soom A. Short-Term Exposure to Hydrogen Peroxide during Oocyte Maturation Improves Bovine Embryo Development. *Reproduction,* accepted.

Thys M, Nauwynck H, Maes D, Favoreel H, Van Soom A. Vitronectin and its receptor (integrin $\alpha_v\beta_3$) during bovine fertilization *in vitro. Reproduction, Fertility and Development*, submitted.

Hoogewijs M, Rijsselaere T, De Vliegher S, Vanhaesebrouck E, De Schauwer C, Govaere J, **Thys M**, Hoflack G, Van Soom A, de Kruif A. Influence of different centrifugation protocols on equine semen preservation. *Theriogenology*, submitted.

ORAL PRESENTATIONS AT NATIONAL AND INTERNATIONAL CONFERENCES

Thys M., Van Soom A., Dewulf J., Rijsselaere T., de Kruif A. Effect of sperm coating on the quality of bovine frozen-thawed spermatozoa. In: Proceedings of the 4th –FNRS contact group 'Competence to development of the mammalian oocyte and embryo quality. Mons, 13th May 2005.

Thys M., Van Soom A., Maes D., de Kruif A. Effect of fibronectin and vitronectin on sperm penetration of bovine *in vitro* matured oocytes. Proceedings of the 5th meeting of the FNRS contact group 'Competence to development of the mammalian oocyte and embryo quality. Louvain-la-Neuve, 2nd June 2006.

Thys M., Azevedo S., Van Soom A. Expressie van fibronectine op de vrouwelijke en mannelijke gameet bij het rund. Voorjaarsvergadering Vereniging voor Fertiliteitsstudie. Ghent, 11th May 2007.

Thys M., Azevedo S., Nauwynck H., Van Soom A. Expression of vitronectin on male gamete in cattle. Proceedings of the 6th meeting of the FNRS contact group 'Competence to development of the mammalian oocyte and embryo quality. Liège, 14th September 2007.

Thys M., Nauwynck H., Maes D., Favoreel H., Van Soom A. Expression and function of fibronectin in male and female gametes during bovine fertilization *in vitro*. Proceedings of the British Fertility Society (BFS) Summer College 2008. Liverpool, UK, 2nd-5th September 2008. Published online in *Human Fertility* 2008; 11(Suppl 1): 7.

AWARD FOR SCIENTIFIC PRESENTATION

'British Fertility Society Exchange Award' granted on 23th November 2007 by the 'Vereniging voor Fertiliteitsstudie' (VFS) and sponsored by Organon / Schering-Plough, for the best scientific presentation in 2007 (Expression of fibronectin on female and male gamete in cattle).

ABSTRACTS AND POSTERS AT AN INTERNATIONAL CONFERENCE

Thys M., Van Soom A., Dewulf J., Rijsselaere T., de Kruif A. Effect of sperm coating on the quality of bovine frozen-thawed spermatozoa. Proceedings of the Annual Conference of the International Embryo Transfer Society (IETS). Orlando, FL, USA, 7th-11th January 2006. *Reproduction, Fertility and Development* 2006; 18(1,2): 164-165.

Thys M., Van Soom A., Maes D., de Kruif A. Effect of fibronectin and vitronectin on sperm penetration of bovine *in vitro* matured oocytes. Proceedings of the 10th International Symposium on Spermatology. Madrid, Spain, 17th-22th September 2006, p96.

Thys M., Azevedo S., Nauwynck H., Van Soom A. Expression of fibronectin on male and female gametes in bovine. Proceedings of the 23rd Scientific Meeting of the European Embryo Transfer Association (AETE). Alghero, Sardinia, Italy, 7th-8th September 2007, p242.

Thys M., Nauwynck H., Maes D., Van Soom A. Effect of pre-incubation of male and female gametes with fibronectin prior to fertilization *in vitro* in cattle. Proceedings of the 16th International Congress on Animal Reproduction (ICAR). Budapest, Hungary, 13th-17th July 2008. *Reproduction in Domestic Animals* 2008; 43 (Suppl 3): 151.

Vandaele L., **Thys M**., Bijttebier J., Van Soom A. Hydrogen peroxide supplementation before fertilization of bovine oocytes has no effect on *in vitro* fertilization or glutathione content. Proceedings of the First Cost Action - Gemini General Conference 'Maternal Interaction with Gametes and Embryos'. Volos, Greece, 9th-11th October 2008.

Thys M., Nauwynck H., Favoreel H.,Van Soom A. Presence of integrin alpha5 in bovine gametes. Proceedings of the 12th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR). Utrecht, the Netherlands, 20th-23th November 2008. *Reproduction in Domestic Animals* 2008; 43 (Suppl 5): 85.

Thys M., Nauwynck H., Maes D., Van Soom A. Evidence that an Arg-Gly-Asp (RGD) adhesion sequence plays a role in bovine fertilization. Proceedings of the Cost Action - Gemini 'Maternal Interaction with Gametes and Embryos' - Workshop Working Group 1: Experimental models - 'Experimental models in animal reproduction: Theoretical and practical approaches'. Lansk, Poland, 7th-8th May 2009.

ADDENDUM

MATERIALS AND METHODS

Media and Chemicals

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and Invitrogen TM life technologies (Merelbeke, Belgium). Media and chemicals were analogous to those used by Tanghe et al. (2004)[*].

A modified HEPES-buffered Tyrode's balanced salt solution, termed **HEPES-TALP**, consisted of 114 mM NaCl, 3.1 mM KCl, 2 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 10 mM HEPES, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 3 mg/ml BSA, and 10 µg/ml gentamycin sulphate.

Maturation medium contained modified bicarbonate-buffered TCM-199 medium supplemented with 20% heat-inactivated fetal calf serum (FCS) (N.V. HyClone Europe S.A., Erembodegem, Belgium), 0.2 mM sodium pyruvate, 50 µg/ml gentamycin sulphate, and 0.4 mM glutamine.

Fertilization medium consisted of Tyrode's balanced salt solution supplemented with 25 mM $NaHCO_3$, 10 mM sodium lactate, 0.2 mM sodium pyruvate, 6 mg/ml fatty acidfree BSA, 10 µg/ml gentamycin sulphate, and 20 µg/ml heparin.

[*] Tanghe S, Van Soom A, Duchateau L, de Kruif A. 2004. Inhibition of bovine sperm-oocyte fusion by the p-aminophenyl derivative of D-mannose. *Mol Reprod Dev* **67** 224-232.