REPRODUCTION
RESEARCH

Expression and putative function of fibronectin and its receptor (integrin $\alpha_5\beta_1$) in male and female gametes during bovine fertilization *in vitro*

Mirjan Thys[1], Hans Nauwynck[2], Dominiek Maes[1], Maarten Hoogewijs[1], Dries Vercauteren[3], Tom Rijsselaere[1], Herman Favoreel[2] and Ann Van Soom[1]

Departments of [1]Reproduction, Obstetrics and Herd Health and [2]Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium and [3]Ghent Research Group on Nanomedicines, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, 9000 Gent, Belgium

Correspondence should be addressed to M Thys; Email: mirjan.thys@ugent.be

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 IVF – strongly inhibits sperm penetration. The present study was conducted firstly to evaluate the expression of Fn and its integrin receptor ($\alpha_5\beta_1$) on male and female bovine gametes using indirect immunofluorescence and secondly, to determine the function of Fn during bovine IVF. Endogenous Fn was detected underneath the zona pellucida (ZP) and integrin α_5 on the oolemma of cumulus-denuded oocytes. Bovine spermatozoa displayed integrin 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 Alexa Fluor 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 α_5 expression on oolemma and acrosome-reacted (AR) sperm cell surface suggests a 'velcro' interaction between the endogenous Fn ligand and corresponding receptors on both (AR) sperm cell and oolemma, initiating sperm–egg binding.

Reproduction (2009) **138** 471–482

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 & Fusi 1996, Fusi *et al*. 1996*a*, 1996*b*, Nixon *et al*. 2007, Vjugina & Evans 2008), the exact mechanisms of 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 & 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 & 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 (Fn), and collagen type IV (Diaz *et al*. 2007).

Fn is a dimeric filament-forming 440 kDa glycoprotein consisting of two similar 200–250 kDa subunits connected by disulfide bridges (McKeown-Longo & Mosher 1984, Fusi & 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 & Mosher 1984,

© 2009 Society for Reproduction and Fertility
ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-09-0094
Online version via www.reproduction-online.org
Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

Fusi & 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). Fn 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 & Pierschbacher 1986, Fusi & 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 & Pierschbacher 1987, Fusi & Bronson 1992, Fusi *et al.* 1992).

Involvement of Fn and its $\alpha_5\beta_1$ integrin receptor in 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 & Fusi 1996). Furthermore, human spermatozoa express Fn on their surface following capacitation (Bronson & 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 & 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.

Results

Localization of Fn on female and male bovine gametes

Immature and matured cumulus–oocyte complexes

Immature and matured bovine cumulus–oocyte complexes (COCs) expressed Fn at the level of the cytoplasm in 42.3 and 58.2% of the cumulus cells respectively (Fig. 1). 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.

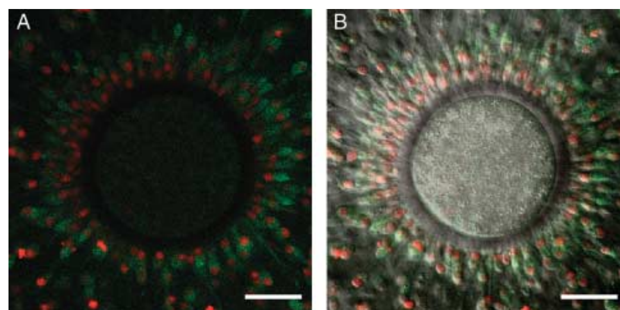

Figure 1 *In vitro* matured bovine COC labeled with mouse monoclonal to Fn and goat anti-mouse FITC combined with propidium iodide staining after fixation and permeabilization. (A) Confocal fluorescent image and (B) overlay with DIC image (original magnification ×400; bar=50 μm).

Cumulus-denuded and zona pellucida-free oocytes

The cumulus-denuded (CD) oocytes that were not treated with protease showed a fluorescent band underneath the zona pellucida (ZP) at the perivitelline space (Fig. 2), 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

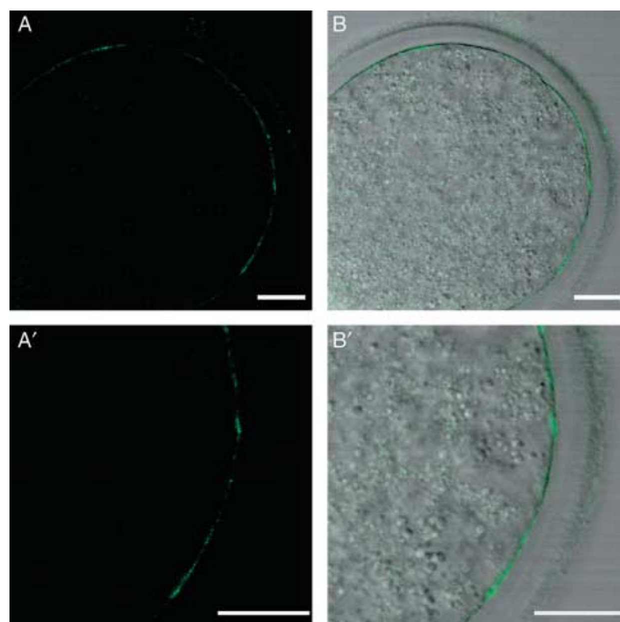

Figure 2 Cumulus-denuded ZP-intact bovine oocyte labeled with mouse monoclonal to Fn and goat anti-mouse FITC combined with propidium iodide staining after fixation. (A) and (A′) Confocal fluorescent image and (B) and (B′) overlay with DIC image (original magnification ×400; bar=20 μm).

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

exterior side of the ZP should likely be considered as remnants of Fn molecules present at the extracellular corona radiata matrix.

Sperm cells

Using indirect immunofluorescence, we could not detect Fn expression on the surface of non-treated (NT), capacitated (CAP), or 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 CD oocytes (Fig. 3). 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).

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. 4A–D), while a fluorescent band at the equatorial segment was noted in the AR spermatozoa (Fig. 4E and 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 α_5), this fluorescent signal was due to non-specific binding of rabbit serum to the sperm. Combining these results, integrin α_5 appears to be only expressed at the equatorial segment of sperm cells after acrosome reaction.

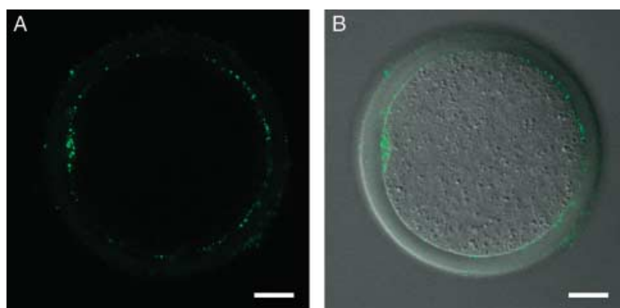

Figure 3 Cumulus-denuded ZP-intact bovine oocyte labeled with rabbit anti-human polyclonal to CD49e/integrin α_5 and goat anti-rabbit FITC combined with propidium iodide staining after fixation. (A) Confocal fluorescent image and (B) overlay with DIC image (original magnification ×400; bar=25 μm).

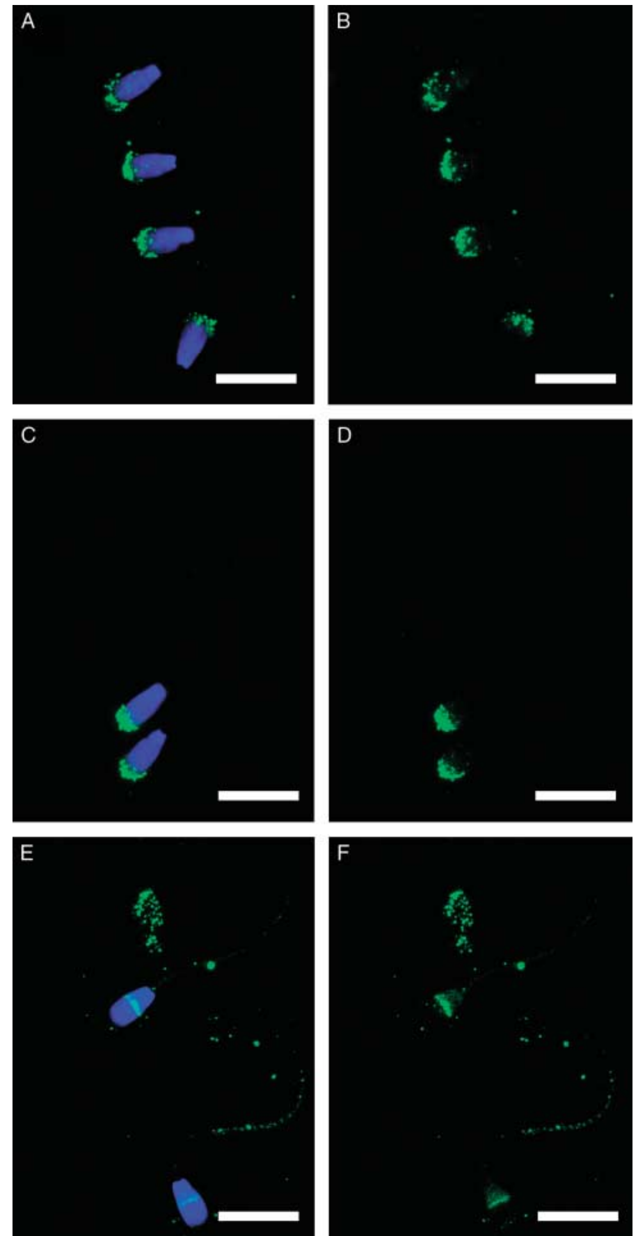

Figure 4 Fluorescent images of frozen–thawed bovine semen labeled with rabbit anti-human polyclonal to CD49e/integrin α_5 and goat anti-rabbit FITC (B, D, and F) combined with Hoechst 33342 staining (A, C, and E) after fixation (original magnification ×600; bar=10 μm). (A and B) Non-treated sperm; (C and D) capacitated sperm; (E and F) acrosome-reacted sperm.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

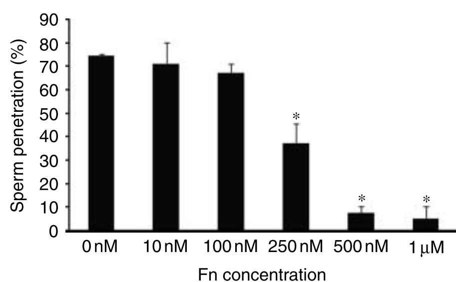

Figure 5 Dose–response effect of fibronectin (Fn) on sperm penetration after bovine IVF. Data represent mean ± S.E.M. *values significantly different from control with 0 nM Fn ($P<0.01$).

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, and the dose–response curve displayed a linear aspect (Fig. 5). Further increase in the Fn concentration to 1 μM did not result in a significantly different sperm penetration percentage ($P=0.20$) compared with the 500 nM Fn group.

Effect of Fn on sperm penetration of the cumulus oophorus

The sperm penetration and fertilization percentages in both cumulus-enclosed (CE) and CD 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.

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 fourfold 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 COCs with Fn prior to IVF.

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

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	$15.0^{*} \pm 2.07$	$0.4^{*} \pm 0.43$	$15.4^{*} \pm 1.86$	–
	500	258	$1.5^{\dagger} \pm 0.30$	$2.3^{*} \pm 1.22$	$3.8^{\dagger} \pm 1.47$	75.3
CE	0	296	$62.9^{*} \pm 7.19$	$7.7^{*} \pm 1.04$	$70.6^{*} \pm 7.72$	–
	500	290	$5.8^{\dagger} \pm 1.19$	$2.8^{\dagger} \pm 1.26$	$8.6^{\dagger} \pm 0.60$	87.8

Data represent mean ± S.E.M. *,†values with a different superscript in the same column within the CD and the CE groups differ significantly ($P<0.05$).

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

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*±6.53	2.6*±1.47	28.0*±7.82	–
	500	116	$6.1^{\dagger}$±1.01	5.0*±2.27	$11.1^{\dagger}$±1.79	60.4

Data represent mean±S.E.M. $*^{,\dagger}$values with a different superscript in the same column differ significantly ($P<0.05$). ZP, zona pellucida.

not show a decreased sperm penetration (42.0 vs 46.9%) nor fertilization percentage (37.1 vs 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. 6). Since the Fn receptor (integrin α_5) was also observed at the level of the equatorial segment of AR sperm cells, binding of exogenously supplemented Fn to the AR 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.

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 ($P>0.05$; Figs 7 and 8). Total and progressive motility were significantly lower in the presence of 500 nM Fn (Fig. 9). However, the rather small decrease in motility cannot account for the strong inhibition of sperm penetration (as described in Table 1).

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.* 1996*b*). 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. 10).

Discussion

In the present study, we confirmed our earlier findings that exogenously supplemented Fn 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 ZP (at the level of the perivitelline space) and α_5 (subunit of Fn-receptor

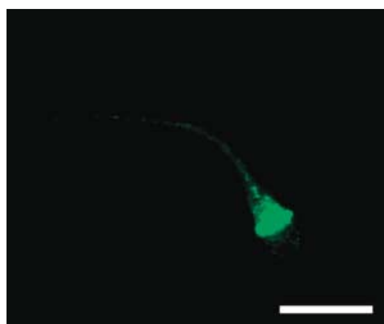

Figure 6 Fluorescent image of frozen–thawed bovine sperm cell (incubated for 20 h with 500 nM of Alexa Fluor 488-conjugated fibronectin) displaying fluorescence at the equatorial segment (original magnification ×600; bar=10 μm).

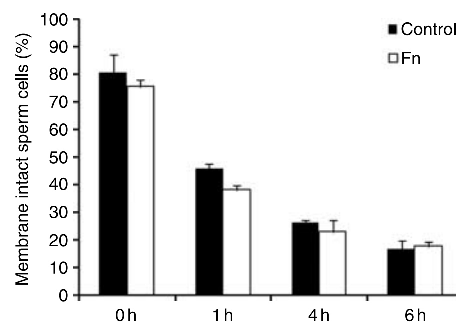

Figure 7 Effect of 500 nM fibronectin on membrane integrity of bovine frozen–thawed spermatozoa during incubation (evaluated by means of SYBR14–PI staining). Data represent mean±S.E.M.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

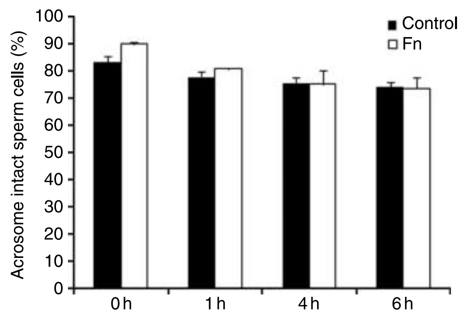

Figure 8 Effect of 500 nM fibronectin on the acrosome reaction of bovine frozen–thawed spermatozoa during incubation (evaluated by means of PSA staining). Data represent mean±S.E.M.

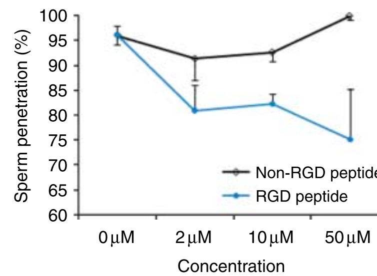

Figure 10 Effect of an Arg–Gly–Asp (RGD)-containing oligopeptide and a control (non-RGD) oligopeptide on sperm penetration of bovine cumulus–oocyte complexes. Data represent mean±S.E.M.

integrin $\alpha_5\beta_1$) on the oolemma of CD bovine oocytes. In addition, bovine spermatozoa displayed integrin 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 ZP. Plasma Fn (or exogenous Fn for that matter) lacks the IIICS segment that displays a second RGD sequence, and as such cannot function as a connecting molecule between sperm cell and oocyte. To exert this type of velcro function, 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 & 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 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 AR sperm cells to bind the ZP (in this case in a biologically irrelevant manner). In human, experimental models have demonstrated that acrosome-reacting sperm is 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 (fourfold) decreased sperm–oolemma binding.

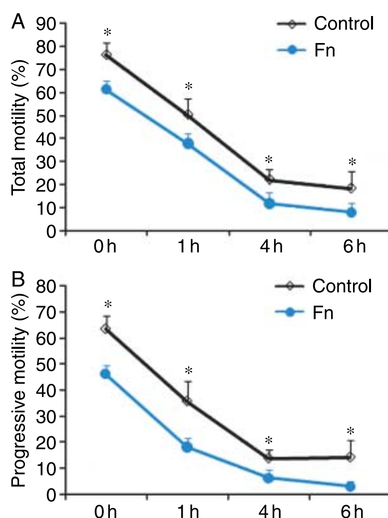

Figure 9 Effect of 500 nM fibronectin on total motility (A) and progressive motility (B) of bovine frozen–thawed spermatozoa during incubation (evaluated by means of CASA). Data represent mean±S.E.M.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

The presence of endogenous Fn at the perivitelline space of CD oocytes combined with integrin α_5 expression on the oolemma and on the surface of AR spermatozoa implies a potential 'velcro' interaction between the endogenous Fn ligand and corresponding receptors on both (AR) sperm cell and oolemma, initiating sperm–egg binding (Fig. 11). 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 Fn velcro hypothesis during bovine IVF. Since there was no effect observed on sperm penetration/fertilization when CE 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 α_5 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 AR 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 AR 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 α_5. 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 CD ZP-intact oocytes did not display an exact co-localization of Fn and integrin 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 α_5 on their oolemma (but no Fn), indicating that the receptor was present on the membrane (data not shown).

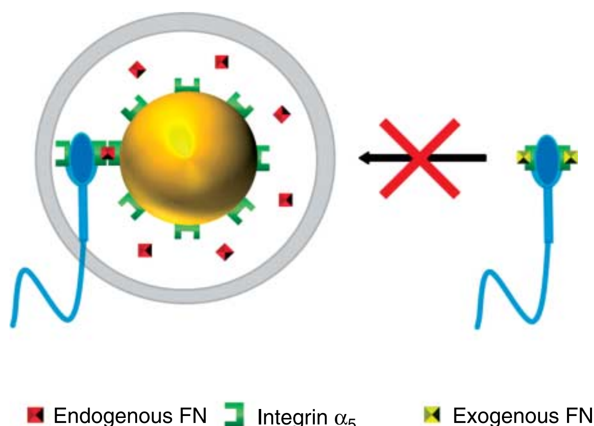

Figure 11 Hypothetical model of function of endogenous fibronectin during bovine sperm–oocyte interaction and interference of exogenously supplemented fibronectin during bovine IVF.

Nonetheless, the diffuse pattern of Alexa Fluor 488-conjugated Fn at the equatorial and posterior sperm head (Fig. 6) differs from that of the discrete equatorial immunolabeling of integrin α_5 (Fig. 4E and 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 ~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. An Fn-specific RGD-containing peptide (Fusi *et al.* 1996*b*) 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 Fn negatively influences bovine IVF by inhibiting sperm–oolemma binding and (concurrently) sperm–egg fusion. The presence of

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

endogenous Fn underneath the ZP together with integrin α_5 expression on the oolemma and the AR sperm cell surface suggests a 'velcro' interaction between the endogenous Fn ligand and corresponding receptors on both (AR) 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 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.

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) as described by Thys *et al*. 2009.

Media and chemicals were analogous to those used by Tanghe *et al*. (2004*a*). Fn from bovine plasma (F4759) used in all experiments was purchased from Sigma–Aldrich.

Localization of Fn on female and male bovine gametes

With respect to the female bovine gamete, immature COCs, *in vitro* matured COCs, CD 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 ZP was dissolved by incubation of the CD oocytes in 0.1% (w/v) protease (P5147, Sigma–Aldrich) in PBS for 5–15 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) 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 CD oocytes (which were processed without permeabilization). Subsequently, they were incubated with 10% (v/v) goat serum (16210-064, Invitrogen) in polyvinyl pyrrolidone (PVP, 0.1% (w/v) in PBS) solution for 30 min (37 °C), with mouse MAB (A17) to Fn (Abcam, Cambridge, UK; 1/100) for 1–2 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) 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) 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 40× 0.95 NA air objective and suitable optical elements to acquire differential interference contrast 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 three fractions. Fraction 1 was diluted to a concentration of 10×10^6 spermatozoa (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, and 10 μg/ml gentamycin sulfate) prior to indirect immunofluorescence, and represented NT sperm. Fraction 2 and 3 were diluted to a concentration of 5×10^6 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 sulfate, 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 CAP sperm) was processed in the same way as fraction 1. Fraction 3 was supplemented with 100 μg/ml lysophosphatidylcholine (LPC; L5004, Sigma–Aldrich) and incubated for 15 min (39 °C; 5% CO_2) in order to induce the acrosome reaction (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 MAB (A17) to Fn (Abcam; 1/100) for 1–2 h (37 °C), and with goat anti-mouse FITC antibody (Molecular Probes; 1/100) for 1 h (37 °C). To stain the nuclei, all sperm fractions were treated with 10 μg/ml Hoechst 33342 (Molecular Probes) for 10 min (RT). Between each treatment, the sperm fractions were centrifuged (10 min, 200 g) and resuspended 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 whether 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 MAB (A17) to Fn (Abcam), two controls were included: 1) a sample processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody, and 2) a sample incubated with an isotype-matched mouse IgG_1 antibody prior to the FITC-labeled secondary antibody treatment.

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 CD oocytes were sampled (as described above) and fixed with 2% (w/v) paraformaldehyde in PBS for 30 min (4 °C) prior to indirect immunofluorescence.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

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 10×10^6 sp/ml. Subsequently, the sample was split into three fractions. Each fraction was processed as described previously, resulting in a NT, CAP, and 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 α_5 (USBioLogical, Swampscott, MA, USA; 1/100), which was fluorescently labeled with goat anti-rabbit FITC antibody (Molecular Probes; 1/100). To evaluate the specificity of the rabbit anti-human polyclonal antibody to CD49e/integrin α_5 (USBioLogical), two controls were included: 1) a sample processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody, and 2) a sample incubated with heat-inactivated rabbit serum prior to the FITC-labeled secondary antibody treatment.

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*. (2004*b*) was the most appropriate, a preliminary experiment was conducted to assess the dose-response effect of Fn on sperm penetration after bovine IVF. *In vitro* matured COCs were randomly assigned to six different fertilization media (Tanghe *et al*. 2004*a*): fertilization medium supplemented with 0, 10, 100, 250, 500 nM, and 1 μM Fn. The oocytes were co-incubated with sperm at a final concentration of 10^6 sp/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). 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 four groups (three replicates). Half of the oocytes were denuded by vortexing for 8 min (CD) and the other half was kept 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 co-incubated 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 – 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. Zygotes were mounted in glycerol with 25 mg/ml DABCO and sperm penetration was evaluated using a Leica DMR fluorescence microscope (Leica Microsystems).

Effect of Fn on sperm–zona binding

In vitro matured COCs were denuded by vortexing and randomly assigned to two groups (four replicates). The first group was fertilized under control conditions and the second group in the presence of 500 nM Fn. The oocytes were co-incubated with sperm at a final concentration of 10^5 sp/ml. After 4 h of co-incubation (Fazeli *et al*. 1993), the oocytes were washed thrice 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 at a concentration of 5×10^5 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 CD oocytes were incubated in 0.1% (w/v) protease in PBS for 5–15 min in order to dissolve their ZP. The ZP-free oocytes (three replicates) were randomly assigned to two different media: standard fertilization medium or fertilization medium supplemented with 500 nM Fn. The female gametes were co-incubated with sperm at a final concentration of 2.5×10^5 sp/ml in 50 μl droplets of medium (ten oocytes/droplet) covered with paraffin oil. One hour after insemination, half of the oocytes from each group were washed thrice 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 co-incubation. 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 (three replicates). The first group was inseminated with spermatozoa (1×10^6 sp/ml) previously incubated with 500 nM Fn for 30 min. The second group was fertilized with spermatozoa (from the same ejaculate) that 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.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

Pre-incubation of female gametes with Fn prior to fertilization

After *in vitro* maturation, about 200 COCs were divided into two groups (three 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 set-up 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

Fn was labeled with Alexa Fluor 488 using the Alexa Fluor 488 Protein Labeling Kit (A10235; Molecular Probes) 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 (three replicates) was centrifuged on a discontinuous Percoll gradient and diluted to a concentration of 60×10^6 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 sulfate, 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 two 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, 1, 4, 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). 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 were 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 FITC-conjugated *Pisum sativum* agglutinin (PSA–FITC; L0770; Sigma–Aldrich), whereas total and progressive motility were determined by means of computer-assisted sperm analysis (Hamilton-Thorne CEROS 12.3; Tanghe *et al.* 2004*a*).

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

In vitro matured COCs were randomly assigned to seven different fertilization media: standard fertilization medium, fertilization medium supplemented with 2, 10, and 50 μM of a custom made GRGDdSP oligopeptide (Thermo Fisher Scientific, Ulm, Germany), and fertilization medium supplemented with 2, 10, and 50 μM of a non-RGD containing (GRGES) oligopeptide (Thermo Fisher Scientific, Germany) according to Fusi *et al.* (1996*b*). The oocytes were co-incubated with sperm at a final concentration of 10^6 sp/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, SPSS, Heverlee, Belgium). Data were reported as mean ± s.e.m.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

Funding

This work was supported by the 'Bijzonder Onderzoeksfonds – Universiteit Gent' (Belgium; number 01D03305). The authors are members of the EU-COST Action FA0702 – GEMINI.

Acknowledgements

The authors would like to thank Johanna Mestach, Griet Spaepen, Isabel Lemahieu, and Petra Van Damme for their excellent technical assistance in the laboratory; Sofia Azevedo for the help with the immunofluorescent fibronectin stainings; and Nele Festjens (Department of Biochemistry, Physiology and Microbiology; Faculty of Sciences; Ghent University) for the supervision during the labeling procedure.

References

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

Almeida EA, Huovila AP, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG *et al.* 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. *Molecular Reproduction and Development* **59** 221–226.

Benoff S 1997 Carbohydrates and fertilization: an overview. *Molecular Human Reproduction* **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 Anatomica* **160** 159–171.

Bronson RA & Fusi FM 1996 Integrins and human reproduction. *Molecular Human Reproduction* **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. *Human Reproduction* **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. *Veterinary Record* **32** 14–16.

Fraser LR, Umar G & Sayed S 1993 Na^{+}-requiring mechanisms modulate capacitation and acrosomal exocytosis in mouse spermatozoa. *Journal of Reproduction and Fertility* **97** 539–549.

Fusi FM & Bronson RA 1992 Sperm surface fibronectin. Expression following capacitation. *Journal of Andrology* **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. *Journal of Andrology* **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. *Molecular Reproduction and Development* **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. *Molecular Human Reproduction* **2** 169–175.

Fusi FM, Bernocchi N, Ferrari A & Bronson RA 1996*b* Is vitronectin the velcro that binds the gametes together? *Molecular Human Reproduction* **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 Developmental Biology* **9** 1.

Gougoulidis T, Trounson A & Dowsing A 1999 Inhibition of bovine sperm–oocyte fusion by the carbohydrate GalNAc. *Molecular Reproduction and Development* **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. *Biology of Reproduction* **77** 906–913.

Lefebvre R, Lo MC & Suarez SS 1997 Bovine sperm binding to oviductal epithelium involves fucose recognition. *Biology of Reproduction* **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. *Journal of Biological Chemistry* **275** 4599–4606.

Mattioli M, Lucidi P & Barboni B 1998 Expanded cumuli induce acrosome reaction in boar sperm. *Molecular Reproduction and Development* **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. *Journal of Biological Chemistry* **259** 12210–12215.

Midwood KS, Williams LV & Schwarzbauer JE 2004 Tissue repair and the dynamics of the extracellular matrix. *International Journal of Biochemistry & Cell Biology* **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. *Developmental Biology* **133** 385–392.

Mosher DF 1984 Physiology of fibronectin. *Annual Review of Medicine* **35** 561–575.

Myles DG 1993 Molecular mechanisms of sperm–egg membrane binding and fusion in mammals. *Developmental Biology* **158** 35–45.

Nixon B, Aitken RJ & McLaughlin EA 2007 New insights into the molecular mechanisms of sperm–egg interaction. *Cellular and Molecular Life Sciences* **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. *Italian Journal of Anatomy and Embryology* **110** 219–224.

Revah I, Gadella BM, Flesch FM, Colenbrander B & Suarez SS 2000 Physiological state of bull sperm affects fucose- and mannose-binding properties. *Biology of Reproduction* **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. *Mechanisms of Ageing and Development* **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. *Biology of Reproduction* **72** 346–357.

Suarez SS, Revah I, Lo M & Kolle S 1998 Bull sperm binding to oviductal epithelium is mediated by a Ca^{2+}-dependent lectin on sperm that recognizes Lewis – a trisaccharide. *Biology of Reproduction* **59** 39–44.

Sutovsky P, Flechon JE & Pavlok A 1995 F-actin is involved in control of bovine cumulus expansion. *Molecular Reproduction and Development* **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*. *Biology of Reproduction* **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. *Molecular Reproduction and Development* **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. *Molecular Reproduction and Development* **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*. *Molecular Reproduction and Development* **68** 492–499.

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access

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.

Tulsiani DR, Yoshida-Komiya H & Araki Y 1997 Mammalian fertilization: a carbohydrate-mediated event. *Biology of Reproduction* **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. *Frontiers in Bioscience* **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.

Received 10 March 2009
First decision 15 April 2009
Revised manuscript received 19 May 2009
Accepted 8 June 2009

Downloaded from Bioscientifica.com at 08/25/2022 05:48:47PM
via free access