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Vitronectin and Its Receptor (Integrin $\alpha_v\beta_3$) During Bovine Fertilization *In Vitro*

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1. Introduction

The complex series of molecular interactions between male and female gametes required for successful fertilization has captured the interest of several research groups for many years (Benoff 1997). Even though substantial insights into these interactions have been elucidated in particular in human and mouse (Fusi *et al.* 1992; 1996*a*; 1996*b*; Almeida *et al.* 1995; Evans *et al.* 1995; Bronson & 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 & 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) inhibited 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 & 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 & Fusi 1996), Vn 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.

2. Materials and methods

2.1 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*).

Media and chemicals were analogous to those used by Tanghe *et al.* (2004*a*). Vitronectin from bovine plasma (V9881) used in all experiments was purchased from Sigma-Aldrich (Bornem, Belgium).

2.2 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*).

2.3 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.

2.4 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-response 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.

2.5 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.* 2004*b*). The oocytes were co-incubated 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.

2.6 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 co-incubated 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 co-incubation, 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.

2.7 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 co-incubated 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.

2.8 Effect of Vn on sperm-oocyte fusion

The experimental setup was identical to the one described in the previous experiment, 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).

2.9 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 by 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 Vn (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 Vn (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 Vn, since there is no evidence for cross reactivity with other connective tissue proteins (fibronectin, elastin, collagen, laminin). Nevertheless, two negative controls were additionally included: 1) a sample processed without primary antibody prior to the incubation with the secondary FITC-labeled goat-anti-mouse antibody, and 2) a sample incubated with an isotype-matched mouse IgG1 antibody prior to the FITC-labeled secondary antibody treatment.

2.10 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 processed without primary antibody prior to the incubation with the FITC-labeled secondary antibody was included as negative control.

2.11 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*).

2.12 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).

3. Results

3.1 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.1). 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.

3.2 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 1). 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.

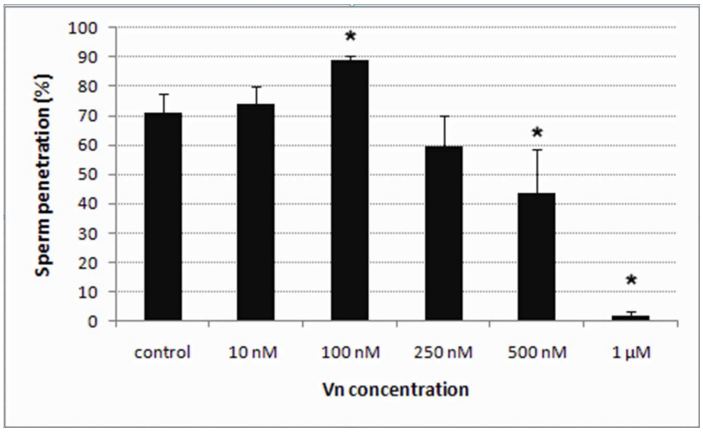

Fig. 1. Dose-response effect of vitronectin (Vn) on sperm penetration after bovine IVF. Data represent mean ± SEM. *Values significantly different from control with 0 nM Vn ($P < 0.05$).

Oocytes	Vn (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

Table 1. 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 (Vn). ^{a,b} Values with a different superscript in the same column within the CD and the CE groups differ significantly ($P<0.05$).

3.3 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; $P<0.05$).

3.4 Effect of Vn on sperm-oolemma binding

Vitronectin supplementation did not significantly influence the sperm-oolemma binding. 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 ($P>0.05$).

3.5 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%.

3.6 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 2) and the predominant fluorescent pattern observed in the AR fraction (Fig.2*e* – fluorescence at the acrosomal region and midpiece) also differed from the one mainly observed in the NT and CAP groups (Fig.2*a* and 2*c* – 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.2*f*). No fluorescence could be visualized in the NT and CAP sperm fractions (Fig.2*b* and *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 2).

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

Table 2. 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. NT: non-treated spermatozoa; CAP: capacitated spermatozoa; AR: acrosome-reacted spermatozoa.

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.3*a*). 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.3*b*). Cumulus-denuded oocytes appeared to express Vn on the surface of the ZP, including fluorescent spurs penetrating the ZP (Fig.4). Protease treated (ZP-free) oocytes did not show membrane expression.

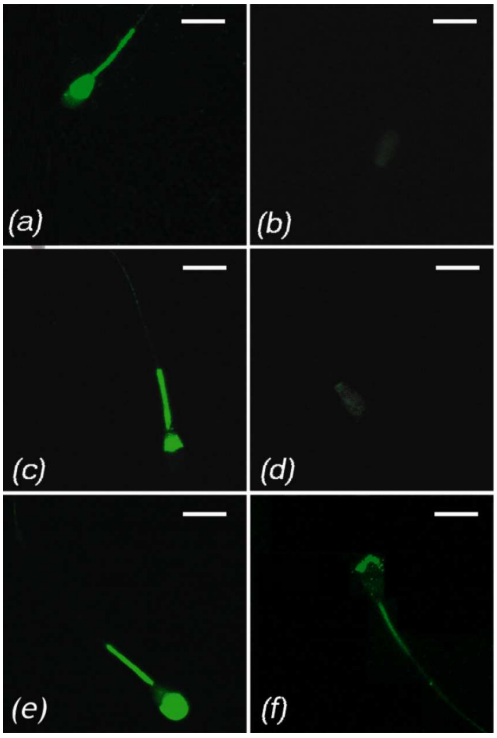

Fig. 2. Fluorescent images of frozen-thawed bovine semen labeled with mouse monoclonal to vitronectin and goat-anti-mouse FITC after fixation and permeabilization (*a*, *c* and *e*) and without prior fixation and permeabilization (*b*, *d* and *f*) (Original Magnification x600; Bar = 10 µm) (*a*-*b*) non-treated sperm; (*c*-*d*) capacitated sperm; (*e*-*f*) acrosome reacted sperm.

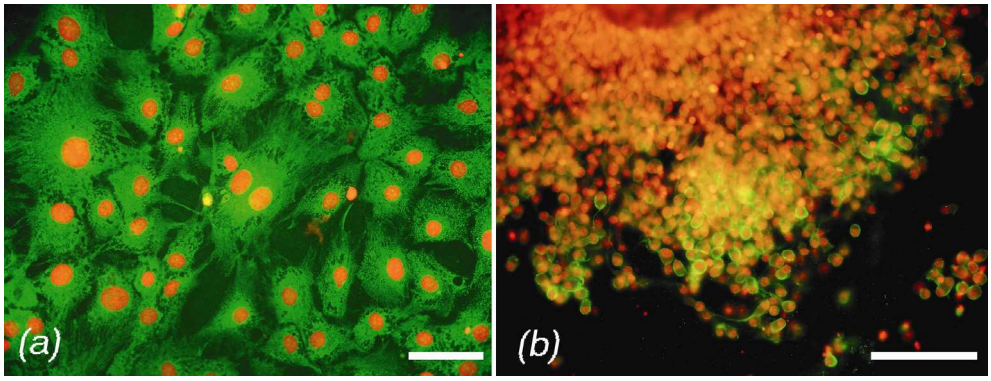

Fig. 3. Indirect immunofluorescent staining with mouse monoclonal to vitronectin and goat-anti-mouse FITC combined with Propidium Iodide staining after fixation and permeabilization (*a*) cumulus cell monolayer (Original Magnification x600; Bar = 25 µm), (*b*) *in vitro* matured COC (Original Magnification x400; Bar = 50 µm).

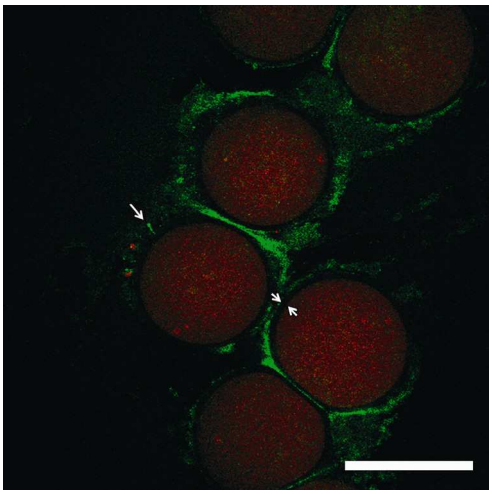

Fig. 4. Confocal fluorescent image of cumulus-denuded ZP intact bovine oocytes labeled with mouse monoclonal to vitronectin and goat-anti-mouse FITC combined with Propidium Iodide staining after fixation and permeabilization (Original Magnification x200; Bar = 100 µm). ⇨ fluorescent spur penetrating the ZP; ⇨ ⇦ ZP

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

After fixation and permeabilization, all sampled sperm cells were positive for integrin subunit α_v, irrespective of their functional state (NT, CAP or AR sperm). All spermatozoa displayed the same fluorescent pattern (Fig.5*a*, 5*c* and 5*e*). When staining NT, CAP and AR sperm without prior fixation and permeabilization, no integrin expression was visually observed in the NT group (Fig.5*b*). The CAP sperm fraction showed faint fluorescence (Fig.5*d*), whereas a bright signal was detected in the AR sperm cells (Fig.5*f*). Flow cytometric evaluation of unfixed sperm cells confirmed these subjective observations (Table 3). 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.

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

Table 3. 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. NT: non-treated spermatozoa; CAP: capacitated spermatozoa; AR: acrosome-reacted spermatozoa.

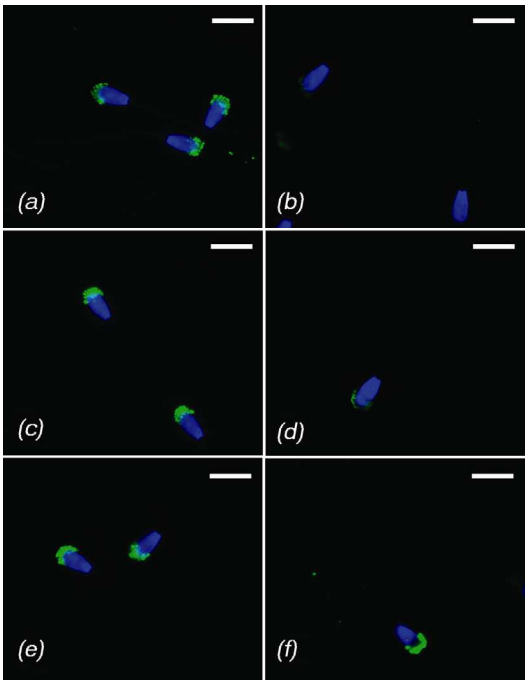

Fig. 5. Fluorescent images of frozen-thawed bovine semen labeled with rabbit polyclonal antibody to integrin subunit α_v and goat-anti-rabbit FITC combined with Hoechst 33342 staining after fixation (*a-c-e*) and without prior fixation (*b-d-f*) (Original Magnification x600; Bar = 10 μm) (*a-b*) non-treated sperm; (*c-d*) capacitated sperm; (*e-f*) acrosome reacted sperm.

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).

Integrin subunit α_v was detected in *in vitro* matured CD bovine oocytes (Fig.6). The fluorescent pattern varied between the sampled cells: some oocytes (12.5%) expressed integrin subunit α_v only at their oolemma (Fig.6*a*), whereas the greater part (87.5%) appeared to express the receptor molecule also at (the exterior side of) the ZP, including fluorescent spurs penetrating the ZP (Fig.6*b*). ZP-free bovine oocytes displayed green fluorescent spots at their surface.

3.8 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.7). Total and progressive motility differed significantly ($P < 0.05$) between all sampled groups (control, 100 nM Vn and 500 nM Vn; Fig.8). 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.

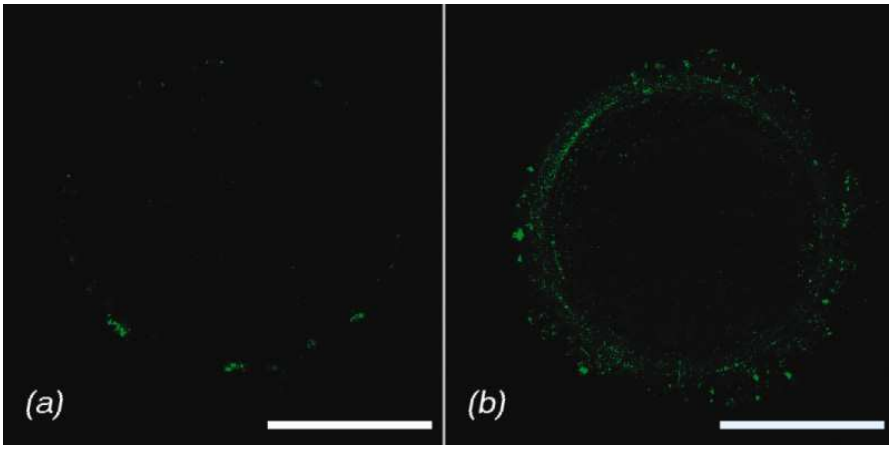

Fig. 6. Confocal fluorescent images of cumulus-denuded ZP intact bovine oocytes labeled with rabbit polyclonal antibody to integrin subunit α_v and goat-anti-rabbit FITC after fixation (*a*) fluorescent signal confined to the oolemma, (*b*) fluorescent signal at the level of the oolemma, the ZP and exterior side of the ZP (Original Magnification x400; bar = 50 µm).

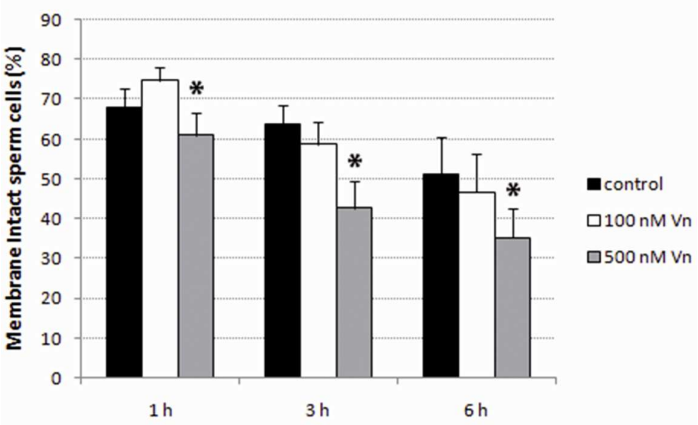

Fig. 7. Effect of 100 nM and 500 nM Vn on membrane integrity of bovine frozen-thawed spermatozoa during incubation (evaluated by means of SYBR14-PI staining). Data represent mean ± SD. *Values significantly different from control with 0 nM Vn ($P < 0.05$).

4. Discussion

In cattle, cumulus cells are shed from the oocyte in the oviduct within a few hours (Lorton & 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

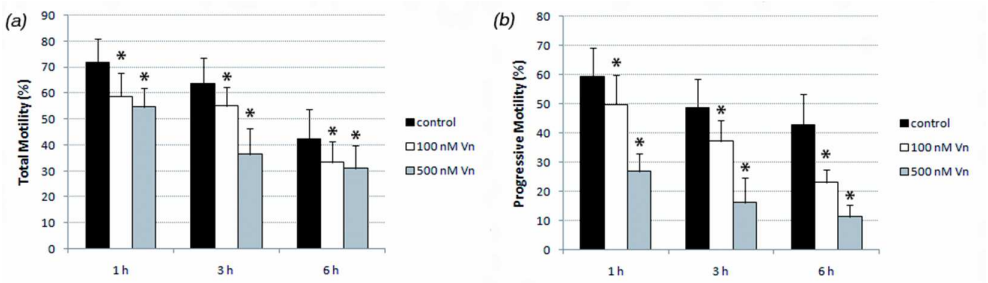

Fig. 8. Effect of 100 nM and 500 nM vitronectin (Vn) on total motility (*a*) and progressive motility (*b*) of bovine frozen-thawed spermatozoa during incubation (evaluated by means of CASA). Data represent mean ± SD. *Values significantly different from control with 0 nM Vn ($P < 0.05$).

binding requires a multitude of receptor-ligand interactions (Lyng & 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 strongly reduced sperm penetration in CD oocytes, but did 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 integrin α_v at the sperm cell surface to the integrin α_v 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 & 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 & 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 Vn-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), Vn also appears to be an intrinsic protein of bovine sperm cells (Fig.9*a-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 Vn sequestered inside the sperm head is exposed at the sperm cell surface after acrosomal reaction (Fig.9*c*). 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 secondary FITC-labeled antibody 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

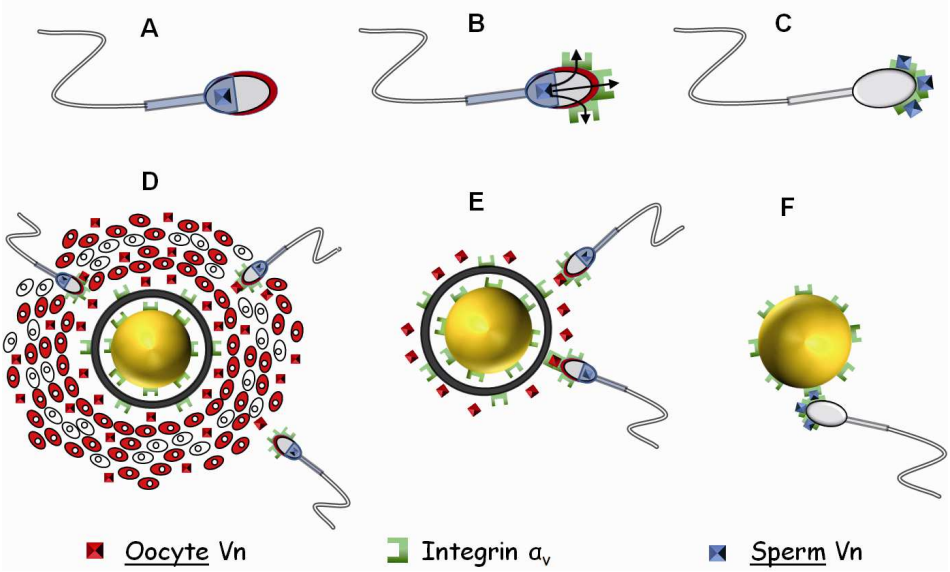

Fig. 9. Hypothetical model concerning the function of endogenous vitronectin (Vn) during bovine IVF. (A) non-treated sperm cell, (B) capacitated sperm cell, (C) acrosome-reacted sperm cell, (D) capacitated spermatozoa traversing the cumulus oophorus, (E) spermatozoa binding the zona pellucida through endogenous oocyte Vn, (F) sperm-oocyte interaction through endogenous sperm Vn liberated after acrosome reaction.

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 dual 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.9*d*). In order to elucidate the beneficial effect of low Vn concentrations on sperm penetration, further studies are required.

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 after 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.9*e*). 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.9*f*). 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.

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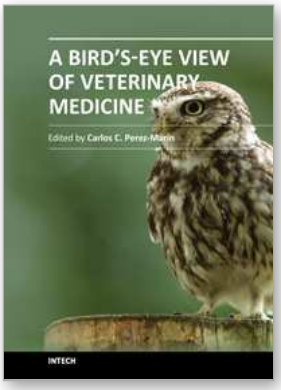

A Bird's-Eye View of Veterinary Medicine

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