Article

"Fibronectin from oviductal cells fluctuates during the estrous cycle and contributes to sperm-oviduct interaction in cattle" †

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Running head: Fibronectin contributes to sperm-oviduct interaction.

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Abstract

During the passage of sperm through the oviduct, spermatozoa bind to the oviductal epithelium and form the oviductal reservoir. This interaction keeps the fertilizing capacity of sperm until ovulation-associated signals induce sperm release from the oviductal epithelium, allowing the transit of spermatozoa to the fertilization site.

Fibronectin is a glycoprotein from the extracellular matrix that binds to α5β1 receptors. Fibronectin has been found to be expressed in the oviduct, whereas α5β1 has been found to be expressed in the sperm of different species. Fibronectin is involved through α5β1 in sperm functions.

The aim of this work was to study the participation of oviductal fibronectin in the regulation of the sperm-oviduct interaction in cattle. We found that oviductal epithelial cells differentially expressed all mRNA splice variants of fibronectin during the estrous cycle. Fibronectin was localized in the apical region of oviductal epithelial cells and fibronectin levels in the oviductal fluid fluctuated during the estrous cycle. Also, bovine spermatozoa expressed α5β1. Using *in vitro* sperm-oviduct co-cultures, we found that spermatozoa were attached to the oviductal epithelium through α5β1. The incubation of co-cultures with fibronectin induced sperm release from the oviductal cells through α5β1. The sperm population released from oviductal cells by fibronectin was enriched in motile and capacitated spermatozoa. Based on our *in vitro* culture system results, we propose that fibronectin and α5β1 are involved in the sperm-oviduct interaction. Also, an increase in fibronectin levels in the oviductal fluid during the pre-ovulatory period may promote sperm release from the oviductal epithelium in cattle.

Key Words: Fibronectin, α5β1, sperm-oviduct interaction, bovines.

Introduction

Mammalian spermatozoa are not able to fertilize an oocyte after they leave the testis. They acquire this ability during their transit through the female genital tract in a process known as capacitation, during which they undergo a large number of membrane and metabolic modifications that result in the acquisition of their fertilizing capacity (Suarez, 2015; Visconti et al., 2011). The female reproductive tract interacts with spermatozoa to facilitate their transport to the site of fertilization, to select the motile and live sperm, and to keep them alive until ovulation occurs (Suarez, 2015).

The oviduct is not only a structure where the gametes or embryos are transported; it also plays an essential role in the physiology of reproduction. This organ provides a beneficial environment for gamete maturation and transport as well as for fertilization and early embryo development. The oviduct also acts as a functional reservoir of sperm. Spermatozoa bind to the oviductal epithelium in a process mediated by specific interactions between carbohydrate moieties on the epithelial surface of the oviduct and lectins on the sperm head surface (Miller, 2015; Suarez, 2015).

During estrus, sperm detachment is mainly modulated by changes in the sperm plasma membrane that occur through the capacitation process and/or by sperm hyperactivation (Gervasi et al., 2015, 2009; Osycka-Salut et al., 2012; Suarez, 2008). Sperm selection by the female reproductive tract is fundamental to ensure successful fertilization.

Proteins, glycosaminoglycans and lipid molecules present in the oviductal fluid are involved in the regulation of the sperm-oviduct interaction and mammalian fertilization. Conditioned media from whole oviduct (Anderson and Killian, 1994) or monolayers of oviductal epithelial cells (Chian et al., 1995) have a capacitating activity that peaks at estrus and declines during the luteal phase, suggesting that some molecules contained in the oviductal fluid act as capacitating agents. In cattle, heparin or heparin-like glycosaminoglycans present

in the oviductal fluid are considered potential *in vivo* capacitating agents (Parrish et al., 1988; Talevi and Gualtieri, 2001). The oviductal fluid consists of different components, including secretions from oviductal epithelial cells and components from blood plasma or follicular fluid (Leese, 1988; Mondéjar et al., 2012; Paula-Lopes et al., 1999). It is well known that the composition of the oviductal fluid varies along the female cycle. Molecules that increase their concentration near ovulation are proposed to participate in the interaction and/or release of spermatozoa from the oviductal epithelium (Coy et al., 2012; Gervasi et al., 2013; Grippo et al., 1994; Hunter, 2012). For example, the expression of most of the components of the extracellular matrix in the human oviduct show changes during the menstrual cycle (Diaz et al., 2012), specifically an increase in fibronectin mRNA levels around ovulatory period is observed (Diaz et al., 2012).

Fibronectin (Fn) is a high molecular weight glycoprotein that consists of two similar subunits of 250 kDa, linked by two disulfide bonds near their carboxy terminal region. It is found mainly as a soluble dimer (sFn) and as an insoluble multimer of high molecular weight (cFn), maintained by covalent bonds and arranged as a fibrillar extracellular matrix component (Bradshaw and Smith, 2014). Each monomer has multiple Fn domains, through which it can bind to surface receptors (integrins), molecules found in the extracellular space such as glycosaminoglycans, fibrin, collagen, and laminin, and structures on the cell surface. This suggests the importance of Fn participation in different biological functions such as cell adhesion, cell growth, differentiation process and cell migration. Fibronectin is synthesized from a single gene of approximately 70 Kb but alternative splicing of its mRNA within three specific regions, the extra domains (ED) A and B and the variable (V) or IIICS region, results in the production of different isoforms (De Candia and Rodgers, 1999). The sFn isoform lacks both the EDA and EDB regions, but may include or not the V region (De Candia and

Rodgers, 1999), whereas the cFn isoform includes several variants that may include or not the EDA, EDB or the V regions (Magnusson and Mosher, 1998).

Fibronectin binds to the cell surface through integrin receptors, specifically α5β1. Both Fn and integrins are essential in cell adhesion, proliferation and migration, as well as in differentiation processes, and participate in multiple reproductive process as oocyte uptake by fimbriae of the oviduct (Talbot et al., 2003), gamete interaction (Thys et al., 2009), fertilized oocyte activation (Yue et al., 2004), and embryo development (Chen et al., 2015).

In the human oviduct, Fn is localized to the luminal surface of ciliated cells and on the apex of cilia. The location of the glycoprotein in the isthmus and ampulla is similar and does not vary throughout the cycle (Inan et al., 2004; Makrigiannakis et al., 2009). Notably, the presence of Fn in the oviduct or oviductal fluid of other species has not yet been described.

On the other hand, it is known that integrins are expressed in mammalian spermatozoa (Fusi et al., 1996; Glander and Schaller, 1993; Rohwedder et al., 1996; Thys et al., 2009). Specifically, integrin α5β1, Fn receptor, is present in human and bovine sperm (Fusi et al., 1996; Thys et al., 2009). Fibronectin binds to α5β1 through the RGD (Arg-Gly-Asp) domain. Binding of integrins to their ligands activates different signal transduction pathways, such as an increase in intracellular Ca^{2+}, or activation of kinases, such as cAMP/PKA, inositol triphosphate/PKC and Src, which are involved in the regulation of sperm function (Diaz et al., 2007; Suh and Han, 2013). More specifically, through α5β1, Fn induces the acrosome reaction and sperm capacitation by activating the cAMP/PKA pathway in humans (Diaz et al., 2007; Martínez-León et al., 2015).

Since the mentioned evidence indicates that **i)** Fn and integrins initiate molecular signaling pathways that regulate sperm functions**, ii)** Fn induces sperm capacitation in humans, **iii)** Fn is present on the apex of cilia in the mammalian oviduct epithelium, **iv)** Fn mRNA levels fluctuate during the menstrual cycle in humans, **v)** and that α5β1 is present in bovine sperm,

we hypothesized that oviductal Fn might participate in the regulation of the sperm–oviduct interaction during the bovine estrous cycle. Thus, the aims of the present study were: 1) to characterize Fn expression in bovine oviductal epithelial cells during the estrous cycle; 2) to study α5β1 expression in bovine sperm, and 3) to investigate the participation of oviductal Fn and sperm integrins in the sperm–oviduct interaction.

Materials and Methods

Chemicals

Gentamicin and fungizone were from GIBCO (Life Technologies, NY, USA). Fibronectin, M199 medium, chlortetracycline (CTC), *Pisum sativum* agglutinin-FITC staining (PSA-FITC), Hoechst 33258 (H258), Hoechst 33242, and Bovine Serum Albumin (BSA; V fraction) were from Sigma Chemicals (St. Louis, MI, USA). Fetal Bovine Serum (FBS) was purchased from (Natocor, Argentina). Glass wool columns for sperm selection were from MicroFiber Manville. Salts used to prepare sperm-Tyrode's albumin lactate pyruvate (sp-TALP) medium were purchased from MERK (Germany). All PCR reagents were from Biodynamics and Genbiotech (Argentina). Integrin α5-FITC antibody (ab25076), isotype control-FITC antibody (ab18446), Fn antibody (ab2413) and Fn ELISA kit (ab181419) were from Abcam Inc. (Cambridge, MA, USA). Integrin α5β1 antibody (clone JBS5) and α5 antibody (clone SAM-1) were from Millipore (Darmstadt, Germany). All the other chemicals were of analytical grade and obtained from standard sources.

Culture media

Oviduct handling and development of monolayer cultures were performed with M199 medium, as described previously (Osycka-Salut et al., 2012). Sperm handling and co-culture

experiments were performed with Tyrode bicarbonate buffered medium (sp-TALP (Parrish et al., 1989a)) without heparin or BSA (BSA-free sp-TALP: 99 mM NaCl; 3.1 mM KCl; 0.4 mM NaH_2PO_4; 0.4 mM $MgCl_2.6H_2O$; 21.6 mM Na-Lactate; 10 mM HEPES; 2 mM $CaCl_2.H_2O$; 25 mM $NaHCO_3$; 1 mM Na-pyruvate; pH=7.4). The sperm viability was not affected by the lack of BSA because the gametes were washed and immediately co-incubated with oviductal cells.

Sperm preparation

Cryopreserved semen from 10 different bulls (15×10^6 spermatozoa/0.5 ml straw) was obtained from Cabaña LAS LILAS (Buenos Aires, Argentina), ALTA CIALE (Center of Artificial Insemination La Elisa, Buenos Aires, Argentina) and CIAVT (Artificial Insemination Center Venado Tuerto, Santa Fe, Argentina). Straws were thawed in a water bath (37 °C for 30 s). Spermatozoa were subjected to sperm selection using glass wool columns and washed by centrifugation at 800 g with BSA-free sp-TALP (without heparin). Pellets were resuspended in BSA-free sp-TALP (without heparin) and assessed for sperm concentration and motility using a hemocytometer mounted on a microscope stage heated at 38.5 °C.

Oviducts

Bovine oviducts were obtained as a donation from the Río de la Plata slaughterhouse (Buenos Aires, Argentina). The slaughterhouse gave the necessary permissions for the collection of the organs. Briefly, oviducts were collected at the time of slaughter, and transported to the laboratory at 4 °C.

Oviduct collection and classification

According to the ovarian and corpus luteum morphology, the oviducts were classified into one of four different stages of the estrous cycle: post-ovulatory (days 1-5), early-to-mid luteal (days 6-12), late luteal (days 13-18), and pre-ovulatory (days 19-21) phases (Ireland et al., 1980). The oviducts were cleaned of surrounding tissues and the oviductal content was collected by squeezing (applying pressure) with tweezers. The oviducts were not immersed in the liquid after classification. We processed the oviducts individually and immediately to obtain epithelial cells and oviductal fluid for qPCR and ELISA determinations. After that, bovine oviductal epithelial cells (BOEC) were separated from the oviductal fluid by centrifugation at 800 g for 5 min at 4 °C. The supernatants from the oviductal fluid (between 10 and 30 µl) were immediately used for Fn determination while the pellets were stored at -80°C until further PCR and qPCR analysis. In addition, bovine follicular fluids from pre-ovulatory ovaries were collected by aspiration with a syringe. The follicular fluid from each follicle was thereafter collected for measurement of Fn levels.

Measurement of mRNA expression of each Fn splice variant in oviductal tissues

To characterize the Fn variants in the bovine oviduct, we evaluated the presence of the different Fn variants by RT-PCR, using specific primers that allow detecting the absence or presence of the known exons of Fn (EDA, EDB and V).

Total RNA was isolated from BOEC using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. The concentration of RNA was determined by spectrophotometry by measuring the absorbance at 260 nm. Only samples with a 260nm/280nm ratio greater than 1.7 were used for further analysis. cDNA was synthesized from 1 µg total mRNA by using Maloney Murine Leukemia Virus Reverse Transcriptase (M-

MLVRT) and random primers (Invitrogen), according to the manufacturer's instructions in the presence of recombinant RNase inhibitor. After first-strand synthesis, PCR was performed with the following oligonucleotide primers as described previously (Goossens et al., 2009): Fn EDA+ splice variant transcript: 5'-GGACCATCGAAAACGAAAAC-3' (sense), 5'- GGAATCGACATCCACATCAG-3' (antisense); Fn EDB+ splice variant transcript: 5'-CATGCCGATCAGAGTTCCT-3' (sense), 5'-AAGAGTTTAGCGGGGTCCA-3' (antisense); Fn V+ splice variant transcript: 5'-AGGGGAGACGTAGACCATCA-3' (sense), 5'-GGCACTAGCAGAGGTTCCAG-3' (antisense); Fn EDA- splice variant transcript: 5'-GGTAACCACCATTCCTGCAC-3' (sense), 5'-CCTGATACAACCACGGATGA-3' (antisense); Fn EDB- splice variant transcript: 5'-CATGCCGATCAGAGTTCCTG-3' (sense), 5'-GAGGGACAGCTGGGATGATG-3' (antisense); Fn V- splice variant transcript: 5'-GGAGGAAAAAGACAGGCCAAG-3' (sense), 5'-GGCACTAGCAGAGGTTCCAG-3' (antisense); and GAPDH transcript: 5'-CAAGTTCAACGGCACAGTCA-3' (sense), 5'-GGTGCAGAGATGATGACCCT-3' (antisense).

Amplifications were performed using Taq DNA polymerase enzyme (Invitrogen). PCR was performed as follows: 95 °C for 5 min (initial denaturation) and 35 cycles at 95°C for 30 s, 59 °C for 30 s, and 72°C for 30 s and finally 72 °C for 5 min. Negative controls were performed without cDNA template. PCR products were separated on a 2% (w/v) agarose gel, stained with GelRed and recorded under u.v. light with a digital camera (Olympus C5060).

Real-time RT-PCR analyses

Real-time RT-PCR (qPCR) was performed on RG6000 (Corvette) using Master Mix (Biodynamics, Argentina), gene specific primers and 2.5 µl of cDNA as a template. The

qPCR conditions in all cases started with a denaturation step at 94 °C for 5 min and followed by up to 40 cycles of denaturation (94 °C), annealing (59 °C) and primer extension (72 °C). The mRNA levels of Fn splice variants were corrected to the bovine GAPDH levels by using the $2^{-\Delta\Delta Ct}$ method. The mRNA levels of the Fn isoforms were normalized to the ratio produced in EDA+, EDB+ or V+ to compare the expression levels of the different splice variants (See Supplementary Figure 1b). The mRNA levels of the Fn isoforms were normalized to the ratio produced in the post-ovulatory phase to compare the expression levels of each isoform during the estrous cycle (See Figure 1).

Determination of the Fn levels in bovine oviductal fluid

The Fn levels in oviductal fluid were measured by ELISA. Measurements were made in fluids from oviducts previously selected according to the stage of the estrous cycle. The oviductal fluids were diluted 1:10 in the diluent provided by the ELISA kit and all the manufacturer's instructions for collection and analysis of data were followed.

Immunohistochemistry

The procedures for immunodetection of Fn were performed as previously described (Higa et al., 2011). Slides containing tissue sections were deparaffinized and hydrated in xylene and a series of graded ethanol solutions, followed by three washes in phosphate buffered saline (PBS) and PBS buffer with 0.05% Tween-20 (PBS-T) and by heat-induced, epitope retrieval performed in pH 6.0 citrate buffer. Incubation in 0.3% H_2O_2 in PBS to block endogenous peroxidase was performed. Oviductal sections were incubated with blocking solution (10% v/v normal goat serum) for 20 min at room temperature and incubated in a humidified

chamber overnight with anti-Fn (1:100) primary antibody diluted in PBS-T with 1% BSA. Negative controls were performed by omitting the primary antibody or by the replacement of the specific primary antibody by serum from non-immunized rabbits at the same concentration. Biotinylated goat anti-rabbit (Vector Laboratories) (1:200 in PBS) was applied, preceded by incubation with an avidin–biotin complex for 60 min (Vector Laboratories). Staining was visualized by adding 40% 3,3 -diaminobenzidine tetrahydrochloride chromogen-buffer plus 0.02% (v/v) H_2O_2 in 0.05 M Tris (pH 7.6) for 10 min. Slides were then washed in distilled water for 5 min and dehydrated in graded alcohols for 10 min each, cleared through xylene twice for 10 min each and mounted using Entellan mounting medium (Merck, Germany). In negative controls, after 3,3'-diaminobenzidine tetrahydrochloride chromogen-buffer plus 0.02% (v/v) incubation, slides were washed in distilled water for 5 min before counterstaining in Mayer's hematoxylin for 30 s, dehydrated in graded alcohols for 10 min each, cleared through xylene twice for 10 min each, and mounted using Entellan mounting medium.

Oviductal cell cultures

The bovine oviductal epithelium used for the development of BOEC monolayers was obtained by squeezing the entire oviduct. As our previous results showed that the binding ability of BOEC from the ampulla is similar to the isthmus (data not shown), entire oviducts were used to perform the oviductal cultures.

Laminae of BOEC from the ampulla and isthmus were recovered from different animals and pools of epithelial cells from six oviducts were collected together. Different pools of BOEC were washed by centrifugation at 1500 g for 5 min and incubated in M199 medium supplemented with 10% of FBS (M199 medium + FBS), gentamicin (0.1 mg/ml) and

fungizone (1 µg/ml) at 38.5 °C in a 5% CO_2 atmosphere. Incubations were performed in six-well tissue culture dishes with 12 mm round cover slips on the well bottom. After 48 h, BOEC were washed by centrifugation (1500 g for 5 min) and replaced in the tissue dishes. The medium was changed every 48 h.

BOEC cells were grown to confluence (at Day 5-7 of culture) on glass coverslips. Epithelial cell purity of the culture was confirmed by immunocytochemical analysis using anti-cytokeratin antibodies (data not shown).

On the day of the co-culture procedures, coverslips with confluent cell monolayers were transferred to 24-well dishes, washed three times with BSA-free sp-TALP medium, and left in the same medium for stabilization for 1 h before sperm addition.

Immunocytochemistry

α5 integrin localization on sperm cells

Spermatozoa were fixed (30 min, room temperature, 0.2% w/v paraformaldehyde) and immobilized on slides. Non-specific binding sites were blocked (180 min, room temperature, PBS Tween 0.1% BSA 0.01%) and slides were treated with α5-FITC antibody (1 µg/10^6 cells). The specificity of the immunodetection was assessed by the replacement of specific primary antibody with the isotype control (Supplementary Figure 6).

Fn localization on BOEC

BOEC were incubated with Hoechst and then fixed (10 min, 4 °C, 0.2% w/v paraformaldehyde). Non-specific binding sites were blocked (60 min, 40 mg/ml BSA–PBS) and cells were incubated with Fn antibody (1:2000). Then, slides were incubated with anti-rabbit CY5-conjugate

(1:1000). The specificity of the immunodetection was assessed by a) omitting the specific primary antibody; and b) IgG control: by the replacement of the specific primary antibody with serum from non-immunized rabbits at the same concentration (Supplementary Figure S2).

Localization of α5 integrin on sperm bound to BOEC.

To evaluate the localization of α5 in spermatozoa attached to BOEC, co-cultures (on cover slides) were washed and fixed in 0.4% w/v paraformaldehyde in PBS for 1 h at room temperature. Non-specific binding sites were blocked (180 min, room temperature, PBS Tween 0.1% BSA 0.01%) and slides were treated with α5-FITC antibody (1 μg/10^6 cells). The specificity of the immunodetection was assessed by the replacement of specific primary antibody with the isotype control.

Sperm cells, BOEC and sperm-BOEC co-cultures were mounted and examined under a fluorescence microscope (Nikon E200, Japan) coupled to a digital camera.

Western blotting analysis

Fn protein expression was determined by western blotting. Proteins from oviducts at different stages of the estrous cycle were extracted in RIPA buffer, containing 10 μg/ml leupeptin, 2 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 1 mM EDTA, 1 mg/ml benzamidine, 10 μg/ml dithiothreitol, 1 mg/ml caproic acid, and 1 mM sodium orthovanadate. Oviductal cells were sonicated for 30 s and then centrifuged at 2000 g for 10 min. Protein determination was assayed by the Bradford method using BSA as standard. BOEC proteins (80 μg/ml) were separated using 8 % SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membrane non-specific binding sites were blocked with [2% (w/v) of fat-free milk powder] and incubated with primary anti-Fn (1:500) antibody followed by incubations with goat anti-

rabbit HRP-conjugated IgG (1:10000). The immunoreactive specificity was assessed by omitting the first antibody. Bands were visualized using chemiluminescence detection reagents and an Image Quant system (GE Healthcare, Buckinghamshire, UK).

Evaluation of the presence of integrin α5β1 in bovine sperm by flow cytometry

Spermatozoa were incubated with or without Fn 100 µg/ml for 0, 15 and 45 min and fixed with 4% paraformaldehyde in PBS. Nonspecific sites were blocked with blocking solution (PBS Tween 0.1% BSA 0.01%) for 4 h at room temperature under gentle agitation. Subsequently, spermatozoa were incubated with blocking buffer alone or with the α5-FITC antibody or the isotype control-FITC in blocking buffer (1 µg/10^6 sperm) for 18 h at 4° C. Finally, samples were resuspended in PBS and fluorescence was assessed by flow cytometry (FacsCalibur Becton Dickinson).

Data were analyzed by determining the percentage of the population of spermatozoa that had a positive staining for α5. The fluorescence intensity of the population with positive staining for α5 was calculated using the Cyflogic 1.2.1 software (Perttu Terho Mika Korkeamäki, CyFlo Ltd).

Co-cultures of BOEC and spermatozoa

The co-cultures were performed as described previously (Gervasi et al., 2009). Within each experiment, confluent BOEC monolayers from different pools of oviducts were inseminated with different sperm suspensions (1 x10^6 sperm/ml of BSA-free sp-TALP/well) for 60 min at 38.5 °C in a 5% CO_2 atmosphere.

α5β1 integrin participation in the sperm-oviduct interaction

Evaluation of sperm-oviduct binding

This experiment was performed to investigate whether the effect of pre-treatment of BOEC or spermatozoa with an anti-α5 antibody was able to affect sperm binding. To test whether the effect of Fn was mediated by its receptor, we used an antibody against integrin α5β1 (α5 antibody, clone SAM-1; Millipore), which recognizes the active site of the receptor.

Two experiments were performed. In the first experiment, we studied whether the pre-treatment of BOEC with an anti-α5 antibody was able to influence sperm binding. BOEC cultures were pre-incubated with BSA-free sp-TALP alone (control) or with 2 µg/ml of anti–α5 antibody or purified IgG2a from normal mouse serum (isotype control) diluted 1:100 in BSA-free sp-TALP for 15 min. The cultures were washed twice with sp-TALP. After that, pre-incubated BOEC monolayers were inseminated with different sperm suspensions (1 $x10^6$ sperm/ml of BSA-free sp-TALP/well) for 60 min at 38.5 °C in a 5% CO_2 atmosphere. Unbound spermatozoa were removed by washing three times with BSA-free sp-TALP, fixed in glutaraldehyde 2.5% v/v for 60 min at room temperature, extensively washed and the round cover slips containing the co-cultures were mounted on a glass slide.

In the second experiment, we studied whether pre-treatment of sperm with an anti–α5 antibody influenced sperm binding to BOEC. Spermatozoa were pre-incubated with BSA-free sp-TALP alone (control), or with 2 µg/ml of anti–α5 antibody or purified IgG2a from normal mouse serum (isotype control) in BSA-free sp-TALP. After 15 min, the cells were washed in BSA-free sperm-TALP by centrifugation at 800 g for 5 min and co-cultured with BOEC at 1 $x10^6$ sperm/ml for 60 min. Unbound spermatozoa were washed and monolayers were fixed for sperm counting.

The number of bound sperm was determined analyzing 20 fields of 0.11 mm^2/cover slip under a phase contrast microscope (Olympus) in blind experiments.

Results were expressed as the mean of the average of bound spermatozoa in a 0.11 mm^2 area. A replicate (n) in these experiments was defined by the co-culture of a pool of BOEC inseminated with sperm from one bull. All the treatments (including the control) were performed for each replicate. The number of spermatozoa bound to the BOEC in the controls depended on the replicate and ranged between 30 and 80 per 0.11 mm^2 area.

Evaluation of sperm release from BOEC

The co-cultures were washed three times with BSA-free sp-TALP to remove unbound sperm. After that, they were incubated with or without Fn or sp-TALP alone (control) for 15 min.

When anti-α5 was used, co-cultures were pre-incubated with or without it for 5 min. After that, the co-cultures were washed and incubated with or without Fn or sp-TALP alone (control) and washed three times with BSA-free sp-TALP to remove released sperm, fixed in glutaraldehyde 2.5% v/v for 60 min at room temperature, extensively washed, and the round cover slips containing the co-cultures were mounted on a glass slide. The number of bound sperm was determined and the results were expressed as mentioned above.

In another set of experiments, laminae of epithelia from bovine oviducts (explants) were co-cultured with spermatozoa (Supplementary Figure S3). After 1 h, co-cultures were incubated with control medium or Fn (100 µg/ml) for 15 min. Data are expressed as the mean of the number of spermatozoa bound to a 1-mm^2 explant, $a \neq b$; $p<0.05$ (n=5).

Analysis of different sperm parameters in Fn-induced released sperm

Sperm-BOEC cocultures were performed as described previously. Then, either BSA-free sp-TALP (control) or Fn 100 µg/ml were added for 15 min. After that, the medium was collected, and control and treated wells were washed three times with BSA-free sp-TALP to obtain the released sperm population. Finally, we performed different studies to evaluate the number of sperm released, and the viability, motility, and capacitation status of these spermatozoa. To assess their viability, spermatozoa were incubated with H258 (2 µg/ml) for 5 min, fixed (1% w/v paraformaldehyde) for 8 min at room temperature and washed with PBS. An aliquot was air-dried onto slides. At least 200 stained cells/treatment were scored in an epifluorescence microscope. Two aliquots were assessed for sperm number and motility using a hemocytometer or slice mounted on a microscope stage heated at 38 °C. Finally, we studied the capacitation status by the chlortetracycline assay (CTC) described by Ward and Storey (Ward and Storey, 1984). Sperm capacitation was evaluated by the ability of spermatozoa to display CTC fluorescence pattern B, indicative of the capacitating status.

Results

Characterization of the Fn variants present in the bovine oviduct

As mentioned in the introduction, different isoforms of Fn are synthesized by alternative splicing and/or post-translational modifications from a single gene producing two isoforms: sFn and cFn.

The largest difference between both splice variants lies on the fact that the mRNA of sFn does not express EDA and EDB exons.

Results indicated that the bovine oviduct presents all the variants of Fn mRNA (EDA+; EDA-; EDB+; EDB-; V+; V-) (Supplementary Figure S1a). The quantification of the different isoforms by real-time PCR indicated that the Fn variants are differentially expressed in the bovine oviduct, and that EDA-, EDB- and V+ showed the highest expression (Supplementary Figure S1b).

To study whether bovine oviductal Fn fluctuates during the estrous cycle, we quantified the different Fn isoforms in the oviduct at different stages of the cycle. Figure 1 shows that oviductal Fn variants are expressed differentially during the estrous cycle. The mRNA levels of EDA-, EDB-, EDB+ and V+ were significantly higher in the pre-ovulatory period (days 19-21) than in the post-ovulatory period (days 1-5), whereas those of EDA+ decreased at the end of the estrous cycle (pre-ovulatory period) and lower concentrations of V- were detected at days 13-28 than at all the other stages of the cycle (Figure 1).

Localization of Fn in the bovine oviduct

By immunohistochemistry, we evaluated the localization of the glycoprotein in the isthmus and the ampulla of different oviducts along the stages of the estrous cycle. Fn was found to be expressed in the muscle layer, basal laminae and connective tissue in both regions (Figure 2). Furthermore, Fn appeared in the apical zone of the epithelium, both in the ampulla and the isthmus. No differences were found in the localization and labeling intensity of the oviductal Fn at the different stages of the estrous cycle (data not shown). In addition, no differences in oviductal Fn expression were found at the different stages of the estrous cycle by Western Blotting (Supplementary Figure S4), supporting the results observed by immunohistochemistry.

Fn levels in the oviductal fluid during the estrous cycle

Taking into account that the bovine oviduct expresses sFn mRNA, the levels of this isoform were quantified along the estrous cycle. Results showed that the bovine oviductal fluid contained sFn and that the levels of this isoform were higher at the luteal (6-12 days) and pre-ovulatory (19-21 days) phases (Figure 3). The increase in sFn levels in the pre-ovulatory period in the oviductal fluid was concomitant with the increase in the mRNA expression of variants EDA- and EDB- observed by real-time PCR. The levels of sFn detected in the oviductal fluid might also be due to the follicular fluid entering the oviduct with the oocyte. Thus, sFn levels were determined in the follicular fluid from ovulatory follicles with diameter equal to or greater than 10 mm. The content of sFn measured in the follicular fluid was 7.60 ± 0.96 ng/ml, suggesting that the Fn content from follicular fluid might contribute, after ovulation, to the total Fn amount of the oviductal fluid.

Participation of Fn in the sperm-oviduct interaction

The results described above indicated that the bovine oviduct expresses both sFn and cFn and that both forms are regulated during the estrous cycle. The presence of Fn in the apical region of epithelial cells suggests a possible participation of Fn in the binding to spermatozoa during the formation of the sperm reservoir. In addition, the increase in Fn during the pre-ovulatory stage indicates a possible participation of this glycoprotein in the regulation of the sperm release from oviductal cells. Thus, we studied the role of Fn and its receptor α5β1 in the binding and release of sperm from the oviductal epithelium. These studies were performed using an *in vitro* model to evaluate the sperm-oviduct interaction developed previously in our laboratory (Gervasi et al., 2009; Osycka et al. 2012; Gervasi et al. 2015).

Fn expression in BOEC

Supplementary Figure S5a shows that BOEC cultures expressed Fn in the cytoplasm and extracellular matrix. We also evaluated the expression of EDA- and EDB- variants (sFn) in BOEC and oviduct explants to determine whether these cells produce the soluble form of the glycoprotein. mRNA expression of sFn was determined in oviduct explants, BOEC cultures and co-cultures of BOEC-spermatozoa (this experimental condition was included to evaluate the influence of spermatozoa on the soluble isoform in culture).

BOEC expressed sFn mRNA and the expression levels were significantly higher than those measured in explants (Supplementary Figure S5b). The incubation of BOEC with spermatozoa did not modify the Fn mRNA levels (data not shown).

α5 expression in cryopreserved bovine spermatozoa

Immunocytochemistry results indicated that bovine spermatozoa expressed α5 in both the head and the tail of the cells (Figure 4). However, four patterns were distinguished in the head: acrosomal (A), post-acrosomal (P), acrosomal+postacrosomal (A+P) and without label (WL) (Figure 4a). The quantification of the patterns at basal conditions (t=0) indicated that α5 is mainly localized in the post-acrosomal region of bovine spermatozoa (Figure 4b). When spermatozoa were incubated in the presence of Fn for 45 min, we observed an increase in the A+P pattern to the detriment of the P label alone (data not shown). No changes were found in the percentage of spermatozoa with the A or WL patterns at the head. We also analyzed the localization of α5 in spermatozoa bound to BOEC. In this case, α5 was mainly localized in the acrosomal region (Figure 4c).

We also performed determinations by flow cytometry to support immunocytochemical studies. Spermatozoa were incubated with Fn for 0, 15 and 45 min and the percentage of sperm expressing α5 was quantified using a FITC-conjugated α5 antibody. Results indicated that approximately 70 % of the sperm population expressed α5 on the surface (Figure 5a), both when incubated in the presence of Fn and when incubated in the presence of sp-TALP alone (control). However, the analysis of fluorescence intensity indicated that, after 45 min incubation with Fn, spermatozoa showed a significant increase in fluorescence intensity related to control incubations (Figure 5b).

Participation of Fn from the epithelial oviduct and α5β1 in sperm-oviduct binding

Taking into account that BOEC expressed Fn and that bovine spermatozoa presented α5β1, we next studied the possible participation of oviductal Fn in sperm-oviduct binding. Cultures of BOEC or spermatozoa were pre-incubated with an anti-α5β1 or an anti-α5 antibody. BOEC pre-incubated with the antibody or sp-TALP (control) alone were incubated with non-treated spermatozoa and the sperm bound to BOEC were counted after 60 min incubation. The number of spermatozoa bound to BOEC was similar in both treated BOEC and controls, indicating that the blocking of oviductal integrins by the antibody did not inhibit the binding of sperm to BOEC (Figure 6a). However, when spermatozoa were pre-incubated with an anti-α5 antibody and later incubated with non-treated BOEC, the number of bound spermatozoa was significantly lower than that in control samples (spermatozoa pre-incubated with sp-TALP alone) (Figure 6b). No effect was observed when spermatozoa were incubated with the control antibody isotype, indicating that the anti-α5 inhibition was specific to sperm integrin (Figure 6c). Similar results were obtained when we used an anti-α5β1 antibody (data not shown).

Fn participation in the sperm release from the bovine oviduct

In another set of experiments, we evaluated the participation of Fn in the sperm release from oviductal cells. These experiments were performed with co-cultures of BOEC and spermatozoa in the presence of sp-TALP or with increasing concentrations of Fn (1-100 µg/ml).

The number of spermatozoa bound to BOEC was significantly lower in the presence of Fn (100 µg/ml) than in control ones, suggesting that Fn might be stimulating the release of spermatozoa from oviductal cells (Figure 7a). In agreement with the results obtained with monolayers, the incubation of co-cultures of sperm-oviduct explants with Fn (100 µg/ml) for 15 min also induced sperm release from epithelial cells (Supplementary Figure S3).

To evaluate the specificity of the effect of Fn, co-cultures were incubated with the anti-α5 antibody, which inhibits Fn binding to its receptor α5β1. The presence of the antibody inhibited the effect of Fn, indicating that its action was mediated through α5β1 (Figure 7b). We obtained similar results when we used an anti-α5β1 antibody (data not shown).

Characterization of the sperm population released by Fn

In our model, Fn induced sperm release from oviductal cells and seems to modulate the capacitation of bovine sperm adhered to the oviduct epithelium. Thus, we next studied whether Fn acts as a capacitating agent, evaluating functional parameters of the released spermatozoa, such as their viability, the capacitation status and progressive motility. The number of spermatozoa released by Fn was significantly higher than that in control samples (Figure 8a). The sperm population released by Fn showed a higher percentage of spermatozoa with progressive motility and capacitation status (pattern B of the CTC assay)

than those spontaneously released with sp-TALP alone (Figure 8c and 8d). In addition, Fn had no effect on sperm viability (Figure 8b).

These results indicate that the sperm population released from oviductal cells by Fn was enriched in motile and capacitated spermatozoa, functional sperm parameters that increase when spermatozoa are incubated in the presence of a capacitating agent.

Discussion

In mammals, most spermatozoa that reach the oviductal isthmus bind to epithelial cells to form the sperm reservoir. This reservoir represents more than simple binding of spermatozoa to epithelial cells because this interaction directly affects the sperm function (Miller, 2015).

Several molecules participate in both the binding and release of spermatozoa from the oviduct (Miller, 2015; Suarez, 2015). However, the participation of proteins from the extracellular matrix in these processes has not been studied in depth. In the present work, we showed, for the first time, the participation of Fn and its interaction with α5β1 integrin in the regulation of the sperm-oviduct interaction in cattle.

The oviduct is a dynamic organ where different modifications both in the epithelium and oviductal fluid take place during the estrous cycle (Coy et al., 2012; Gervasi et al., 2013; Hunter, 2012; Nah et al., 2015). In the human oviduct, the expression of different components of the extracellular matrix varies through the estrous cycle. For example, Fn mRNA levels are higher in the peri-ovulatory phase than in the luteal one (Diaz et al., 2012). However, the levels of transcription of the different Fn variants had not been described in the mammalian oviduct. In this work, we characterized the expression of Fn mRNA isoforms in the bovine oviduct during the estrous cycle. Our results indicate that the expression of both

the soluble and fibrillar isoforms of Fn was finely regulated at different phases of the estrous cycle in the bovine oviduct. In addition, the expression of EDA-, EDB- and V+ was higher than that of the other isoforms in the bovine oviduct, coinciding with the expression profile of sFn.

In this sense, the differential expression of the Fn isoforms present in the oviductal epithelium might be related to the participation of this glycoprotein in the regulation of the oviductal function, mainly in events associated with epithelial changes such as sperm binding to or release from the oviduct.

Several molecules of the oviductal epithelium and oviductal fluid regulate the sperm-oviduct interaction, modulating sperm function. Here, we described the localization of Fn in the epithelium of the bovine oviduct. Fibronectin was localized in the oviductal ampulla and isthmus, specifically in muscle layers, basal laminae and the apical region of epithelial cells. This location is in agreement with that previously described for Fn in the human oviduct (Makrigiannakis et al., 2009).

Physiologically, the isthmus is the region where sperm attach to oviductal cells to form the sperm reservoir (Miller, 2015). Several annexins have been described to be present in the luminal side of the epithelium and to regulate the sperm-oviduct interaction in cattle and swine (Suarez, 2015; Talevi and Gualtieri, 2010). Thus, the expression of Fn in the apical zone of the isthmus epithelium observed in this work suggests that Fn may be involved in the binding of spermatozoa to the oviduct in cattle.

On the other hand, the composition of the oviductal fluid varies through the estrous cycle. It has been proposed that some molecules from the oviductal fluid increase their concentration in the peri-ovulatory period and might participate in the sperm release from the oviductal reservoir to fertilize the oocyte (Coy et al., 2012; Gervasi et al., 2013; Grippo et al., 1994;

Hunter, 2012). In this way, Fn from ampulla may be involved in acrosome reaction since it has been shown that Fn induces the acrosome reaction in human sperm (Diaz et al., 2007) and spermatozoa that reach the ampulla at ovulation time are acrosome-reacted (La Spina et al., 2016).

In the present work, we determined, for the first time, the presence and levels of Fn in the bovine oviductal fluid during the estrous cycle. The concentration of Fn fluctuated from 15 to 30 ng/ml during the cycle period, and the levels were higher in the luteal and pre-ovulatory periods than in the post-ovulatory one. Regarding the increase in protein levels in the luteal phase, Fn is sensitive to degradation by metalloproteases, such as MMP-2 and MMP-9 (Theocharis et al., 2016), described in the bovine oviductal fluid (Gabler et al., 2001). Furthermore, the proteolytic activity of MMP-2 decreases during the luteal phase (days 6-12) and increases during the post-ovulatory period (Gabler et al., 2001). These facts might explain the difference observed in this work between the protein levels and mRNA expression of sFn in this cycle phase. Moreover, spermatozoa should not be present in the oviduct during the luteal phase because estrus is the only stage of the estrous cycle when a cow is receptive for mating. Then, the regulation of metalloproteases and the increase in Fn concentration from oviductal fluid during the luteal phase might be related to its involving in the extracellular matrix remodeling, that takes place during the estrous cycle, but not in sperm reservoir formation.

The increase in Fn levels in the pre-ovulatory period correlates with the increase in the levels of mRNA of the soluble form. In addition, Fn content from follicular fluid might contribute, after ovulation, to the total Fn amount of the oviductal fluid in the ampulla.

These observations suggest a possible involvement of Fn in the modulation of the sperm-oviduct interaction. In our laboratory, we have developed an *in vitro* model to study the sperm-oviduct interaction in cattle (Gervasi et al., 2015, 2009). To evaluate the participation

of Fn in sperm binding to oviductal epithelial cells (BOEC), we first characterized the expression of Fn and its receptor, integrin α5β1, in our system. BOEC expressed the mRNA of sFn and presented Fn in their extracellular matrix. In addition, the sFn mRNA levels detected in BOEC in culture were significantly higher than those detected in BOEC *ex vivo*. This may be because cells are sensitive to the microenvironment, i.e. to the density of the extracellular matrix, which modifies cell behavior and/or differences in the components of the extracellular matrix (Marinkovic et al., 2016).

On the other hand, in other mammals, α5β1 has been described mainly in the acrosomal and equatorial region of spermatozoa (Rohwedder et al., 1996; Thys et al., 2009; Al-Dossary et al., 2015). Here, we found that 70 % of bovine cryopreserved spermatozoa expressed α5β1 at basal conditions and that α5β1 was localized in the tail and head of these cells. Our results also indicate that the incubation with Fn increased the fluorescence intensity for α5β1, suggesting exposure of the receptors on the surface of sperm. In addition, the presence of Fn increased the population with label in the acrosomal and post-acrosomal regions (data not shown), suggesting that Fn might activate the receptor to play a role in the capacitation process or in the acrosome reaction. Supporting these results, Diaz et al. showed that, in humans, Fn is involved in sperm capacitation (Martínez-León et al., 2015) and in acrosome reaction (Diaz et al., 2007). Previously, Thys and coworkers (2009) reported the localization of α5β1 in the equatorial region in bovine reacted spermatozoa but did not detect α5β1 in cryopreserved cells in basal conditions (Thys et al., 2009). In this work, we detected α5β1 using two different techniques, thus the discrepancy with the results of Thys et al. may be due to the antibodies used.

Taking into account that BOEC in culture and spermatozoa express Fn and α5β1 respectively and that the bovine oviduct expresses Fn in the apical region of epithelial cells, we analyzed the participation of Fn/α5β1 in sperm binding to oviductal cells. We found that the

attachment of spermatozoa to BOEC was α5β1-dependent. In accordance with our observations, Reeve et al. reported an inhibition of sperm binding to oviductal cells using spermatozoa preincubated with RGD peptide, suggesting an involvement of integrins in this attachment (Reeve et al., 2003). However, these authors proposed that oviductal integrins are the mediators of this interaction through their binding to Fn or laminin from the sperm surface.

The presence of α5 in the mammalian oviduct has not been yet described. Therefore, no effect should be observed on sperm binding in experiments in which BOEC were pre-incubated with anti-α5β1. Although α5β1 is the classical receptor for Fn, Fn also can act through other integrins such as α4β1, αIIbβ3, αvβ3 and αvβ6 (Lodish et al., 2008). The expression of several subunits of integrins like αv, β3 and β1 is increased during the peri-ovulatory period in the bovine oviduct, suggesting a possible participation of these receptors in the signaling pathway of Fn during the estrous cycle (Gabler et al., 2003). We cannot discard the participation of other oviductal integrins in our model.

So far, there is little information about the molecular pathways involved in the mechanism of sperm release from the oviduct. However, this mechanism is regulated by the estrous cycle, especially at the peri-ovulatory period (Suarez, 2015). It is possible that Fn expression is hormonally regulated and that this regulation is related to time for sperm capacitation. Taking into account that higher levels of Fn were detected in the oviductal fluid in the pre-ovulatory period, we investigated whether it may affect sperm release in cattle. Our results also indicate that Fn induced sperm release from BOEC and that Fn action was via α5β1. The sperm population released from oviductal cells presented a higher percentage of capacitated spermatozoa with progressive motility than those released spontaneously in the presence of sp-TALP alone. These results suggest that Fn might favor sperm capacitation, allowing

sperm release from the oviductal cells. In accordance with our results, Martinez-León et al. described the capacitating effect of Fn in human spermatozoa (Martínez-León et al., 2015).

Spermatozoa bind to the oviductal epithelium and form the oviductal reservoir. This interaction keeps the fertilizing capacity of sperm until ovulation-associated signals induce sperm release from the oviductal epithelium, allowing the transit of spermatozoa to the fertilization site. From our results, the presence of Fn in the apical region of epithelial cells suggests a possible participation of Fn in the sperm binding during the formation of the reservoir. In addition, the increase in sFn levels during the pre-ovulatory stage indicates a possible involvement of this glycoprotein in the regulation of the sperm release from the oviductal epithelium.

Soluble Fn exhibits selective binding to cell surface receptors and interacts with α5β1 but not with other RGD-dependent integrins (Huveneers et al., 2008). In this context, sFn from oviductal fluid might compete with fibrillar Fn deposited in the apical region of the epithelial cells of the isthmus, allowing the release of sperm attached through their α5β1 integrins. In our model, sFn induces sperm release from the oviduct, probably by inducing sperm capacitation (one of the causes of sperm release). Our results indicate that the sperm population released from oviductal cells by Fn is enriched in motile and capacitated spermatozoa. All these events are related to the acquisition of fertilizing ability in spermatozoa.

Sperm are sequentially exposed to different reproductive fluids such as oviductal and follicular fluid and secretions of the granulosa cells surrounding ovulated eggs all along the female tract up to the site of fertilization in the oviduct (Yanagimachi, 1994). The presence of Fn in these fluids and the detection of its receptor α5β1 in bovine spermatozoa suggest that the signaling triggered by this ligand/receptor interaction may contribute to sperm oviduct

interaction. In addition, our observations suggest that oviductal Fn levels may be hormonally controlled during the bovine estrous cycle and are important to further understand the role of the extracellular matrix in the regulation of sperm and oviductal functions in cattle.

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Figure Legends

Figure 1: **mRNA expression levels of Fn isoforms in the bovine oviduct during the estrous cycle.** The oviducts were classified into one of four different stages of the estrous cycle. The relative composition of each splice isoform was determined for the three alternatively spliced regions (EDA exon, EDB exon, V exon). The expression of the different Fn isoforms and GAPDH was determined by real-time PCR. The results were analyzed using the ΔΔCt method. Results are shown as means ± SEM. a≠b; vs.1-5 $p<0.01$, (n=6).

Figure 2: **Localization of Fn in the isthmus and ampulla of the bovine oviduct**. The images are representative of at least four oviducts. Scale bars and magnification are indicated in each photograph. H&E: Hematoxylin and Eosin.

Figure 3: **Fn levels in the bovine oviduct fluid during the estrous cycle**. The oviducts were classified into one of four different stages of the estrous cycle. The fibronectin levels were determined by ELISA. Results are shown as means ± SEM. a≠b, vs 1-5 $p<0.05$; (n=6).

Figure 4: a) **α5 integrin localization in bovine spermatozoa**. Spermatozoa were fixed and the integrin α5 subunit was immunocytochemically detected. The localization of the integrin α5 subunit in non-capacitated bovine sperm was evaluated by fluorescence microscopy. Arrows indicate the different patterns in α5 integrin localization in bovine sperm head (left panel: acrosomal and without label; right panel: post-acrosomal and post-acrosomal + acrosomal). The images are representative of at least five sperm samples and taken at 1000x. b) **α5 integrin**

localization in bovine sperm head. Spermatozoa were fixed and α5 was immunocytochemically detected. The localization of the integrin was evaluated by fluorescence microscopy. The population was quantified according to α5 localization in sperm head: acrosomal (A), post-acrosomal (P), acrosomal and post-acrosomal (A+P) and without label (WL). Bars indicate the percentage of classified sperm of total population. Results are shown as means ± SEM. a≠b, $p<0.05$; (n=5). c) **α5 integrin localization in sperm bound to BOEC**. Co-cultures were fixed and incubated with anti- α5 conjugated to FICT (α5) or the specific isotype control (C). The localization of the integrin was evaluated by fluorescence microscopy. The photographs are representative of at least three experiments.

Figure 5: a) **α5 integrin expression in bovine sperm.** Spermatozoa were incubated for 0, 15 and 45 min in sp-TALP alone (control) or supplemented with Fn 100 µg/ml. Then, cells were fixed and incubated with an anti-α5 antibody conjugated to FITC or with the corresponding isotype control. Bars indicate the percentage of sperm expressing α5 on its surface by flow cytometry. Results are shown as means ± SEM; (n=5). SPZ = spermatozoa. b) **Evaluation of α5-FITC mean fluorescence in bovine sperm**. Spermatozoa were incubated for 0, 15 and 45 min in sp-TALP alone (control) or supplemented with Fn 100 µg/ml. Cells were fixed and incubated with an anti-α5 antibody conjugated to FITC or the corresponding isotype control. The mean fluorescence intensity emitted by the sperm population that expressed α5 was evaluated using a flow cytometer. Data obtained for 45 min by flow cytometry are represented. The bars indicate the percentage change of mean fluorescence values relative to the control (= 100%). Data are expressed as the mean ± SEM. a ≠ b, $p<0.05$; (n= 5).

Figure 6: a) **Participation of oviductal α5β1 in the sperm-oviduct interaction.** BOEC monolayers were pre-incubated with either sp-TALP alone or an anti-α5β1 antibody. After that, BOEC were co-cultured with sperm cells for 15 min. Bars indicate the number of spermatozoa that remained attached to the monolayers and represent the mean of bound spermatozoa/0.11 mm2 monolayer. Results are shown as means ± SEM. (n = 5). b) **Participation of sperm α5β1 in the sperm-oviduct interaction.** Sperm suspension was pre-incubated with sp-TALP alone, an anti-α5β1 antibody or its isotype (control). After that, spermatozoa were co-cultured with BOEC monolayers for 15 min. Bars indicate the number of spermatozoa that remained attached to the monolayers and represent the mean of bound spermatozoa/0.11 mm2 monolayer. Results are shown as means ± SEM. a≠b; $p<0.05$; (n=5).

Figure 7: **Effect of Fn on sperm release from bovine oviductal monolayers (BOEC).** a) Sperm cells and BOEC were co-cultured and then incubated for 15 min with BSA-free sp-TALP alone (0) or increasing concentrations of Fn. Bars indicate the number of spermatozoa that remained attached to the monolayers and represent the mean ± SEM of bound spermatozoa/0.11 mm2 monolayer. a≠b; $p<0.05$; (n=5). b) Sperm cells and BOEC were co-cultured and then incubated for 15 min with BSA-free sp-TALP alone (0) or Fn (100 µg/ml) in the presence or absence of an anti-α5β1 antibody. Bars indicate the number of spermatozoa that remained attached to the monolayers and represent the mean ± SEM of bound spermatozoa/0.11 mm2 monolayer. a≠b; $p<0.05$; (n=5).

Figure 8. **Effect of Fn on the released sperm population.** Measurement of different sperm parameters in the released spermatozoa population after incubation of the sperm-BOEC co-cultures with sp-TALP (control) or Fn (100 µg/ml). We analyzed a) number of released sperm

b) viability c) progressive motility and, d) capacitation status (pattern B of the CTC assay). Results are shown as means ± SEM. a≠b; $p<0.05$; (n=5).

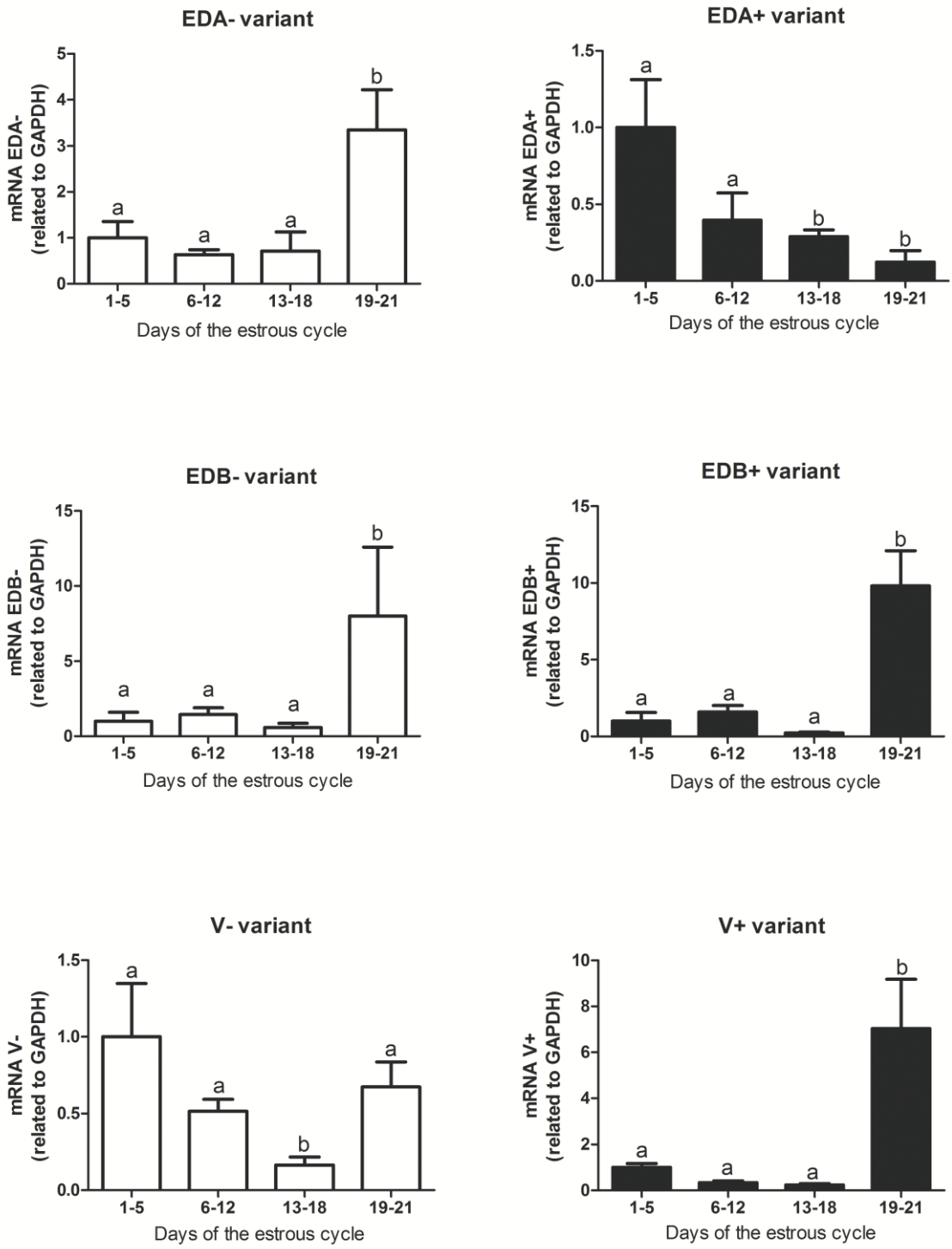
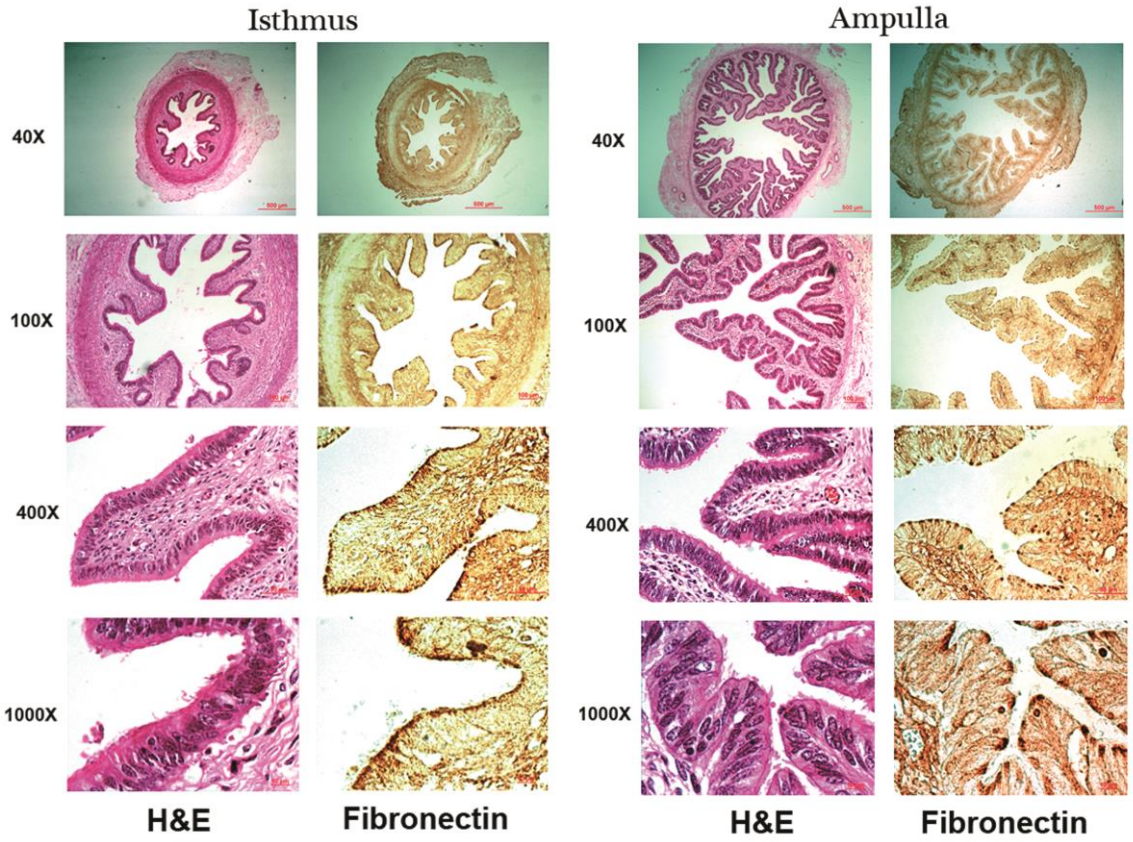


Figure 1



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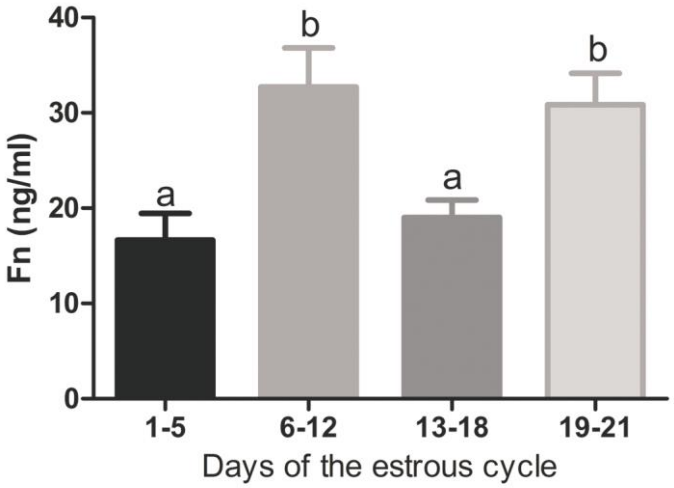

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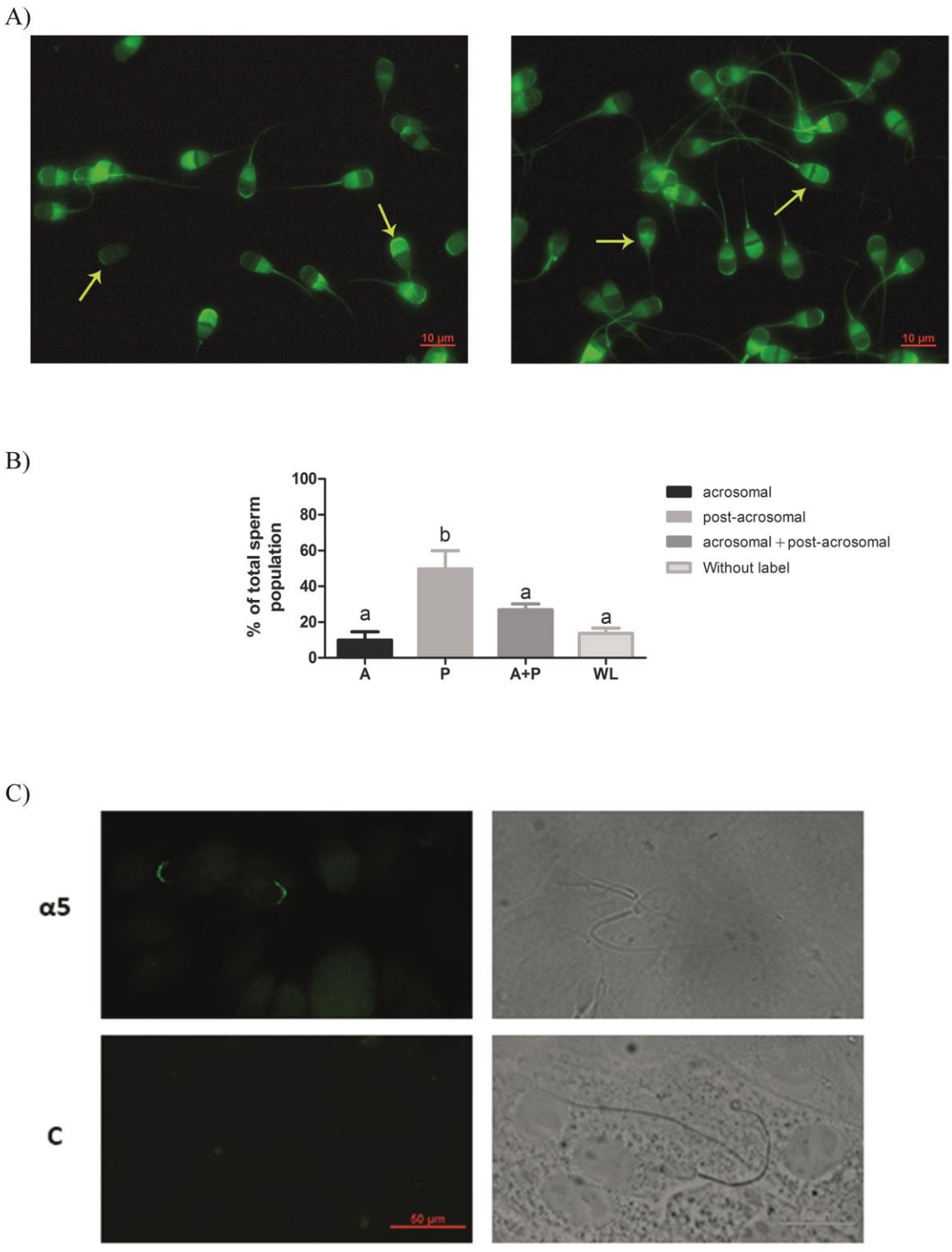

Figure 4

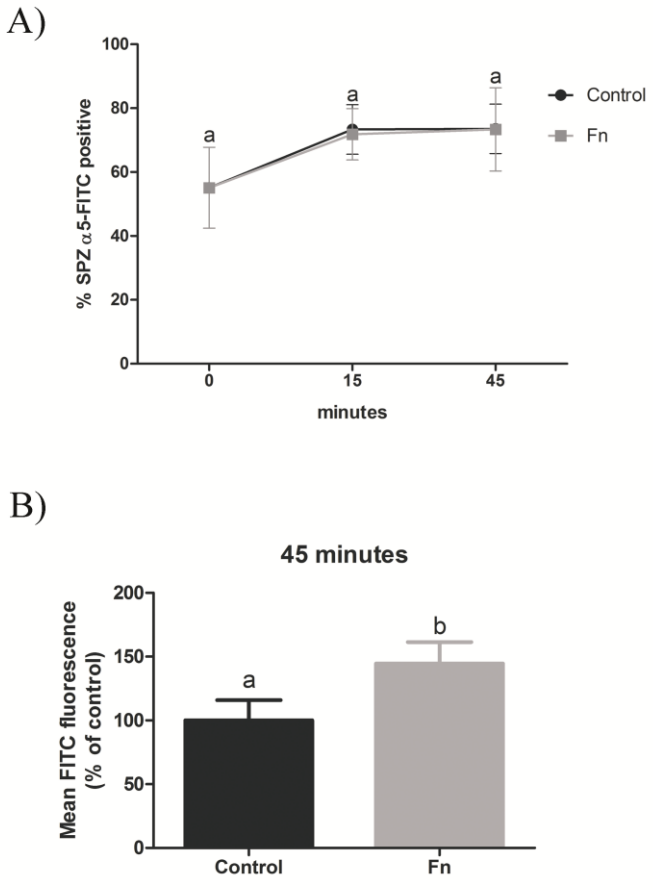

Figure 5

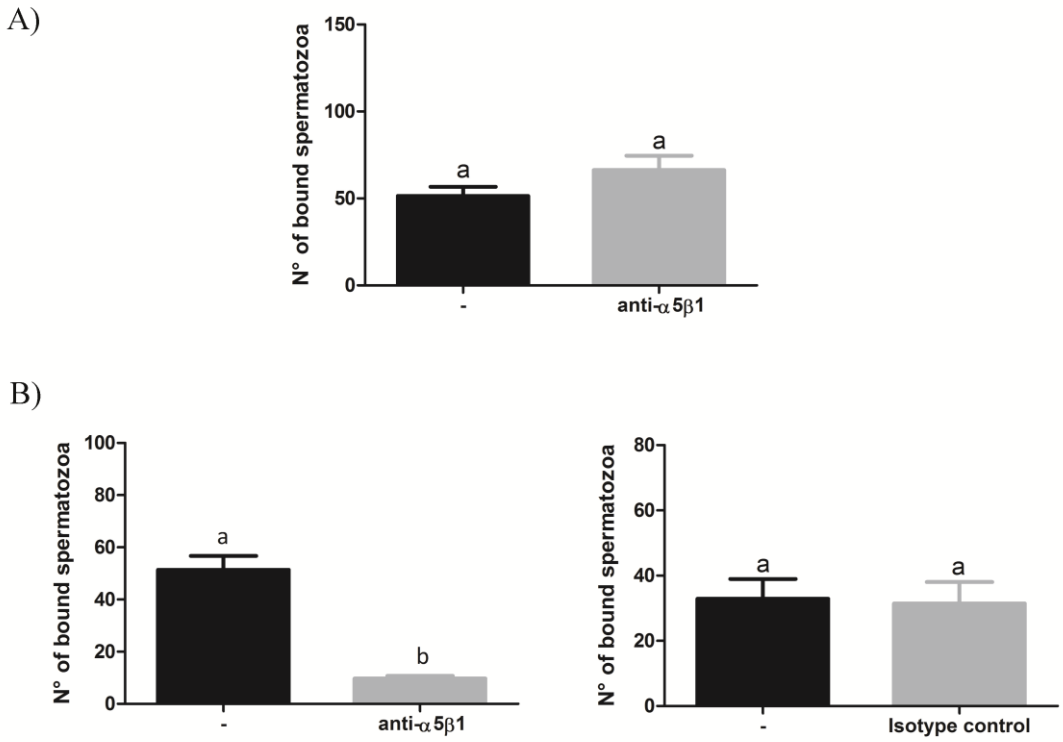

Figure 6

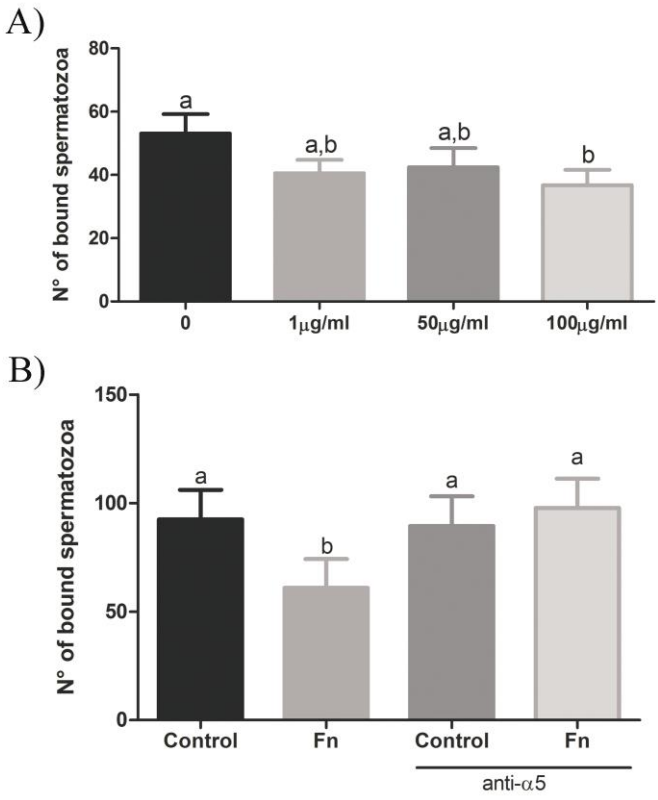

Figure 7

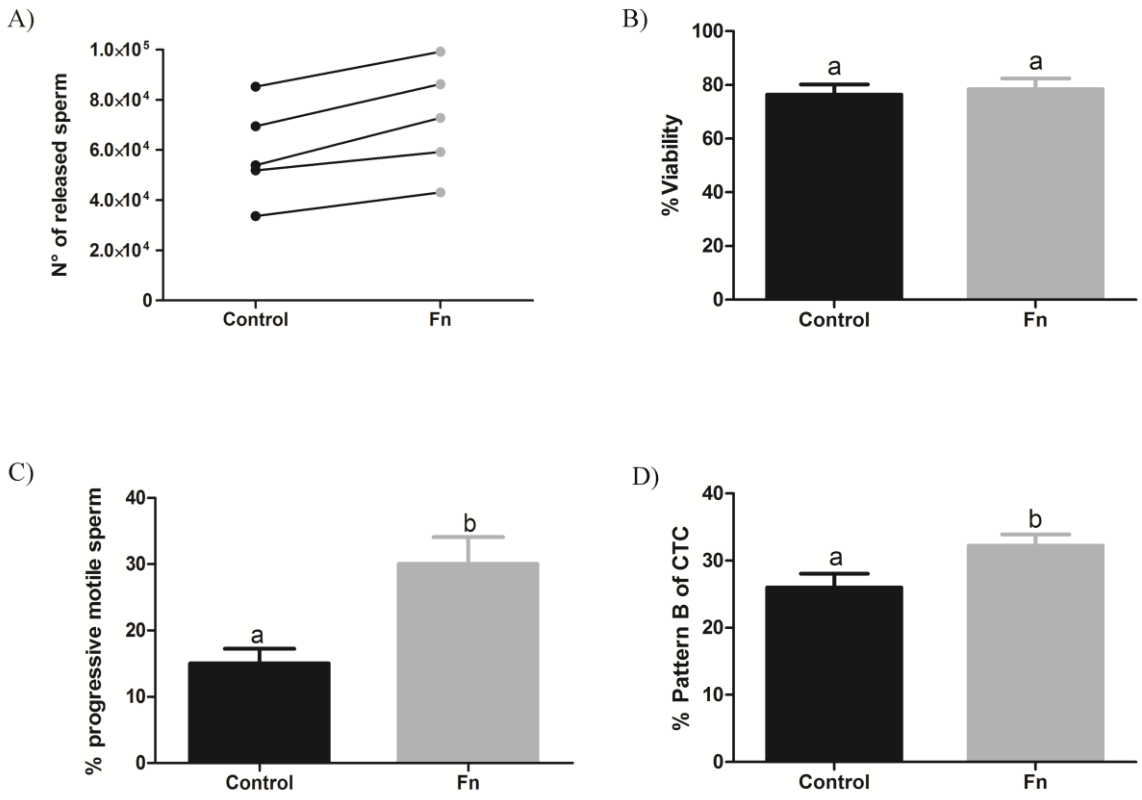

Figure 8