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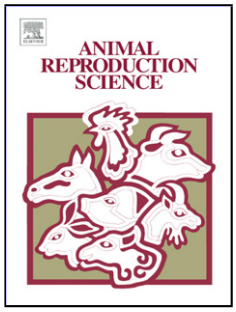

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Recombinant peptide reverses cryo-capacitation in ram sperm and improves *in vitro* fertilization

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Abstract

Semen cryopreservation is a very important technique for assisted reproduction; however, the cryopreservation process is harmful because it results in a reduction in sperm motility and viability, and leads to premature signals of capacitation, resulting in lesser than desirable fertility rates after artificial insemination. A fraction of seminal plasma, enriched in proteins that contain type II fibronectin domains (FNII) can reverse molecular indicators of cryo-capacitation. The beneficial effects of these proteins, however, depend on the relative abundance in seminal plasma. To create a safe additive for improving frozen sperm functionality, in the present study there was cloning and expression of a recombinant peptide containing four FNII domains (named TrxA-FNIIx4-His_6) and evaluation of its effect after addition to frozen/thawed ram sperm. The cDNA for this protein was expressed in *E. coli* and after denaturation and re-naturalization of the protein, toxicity and binding capacity were assessed. By fluorescent labelling assessment, there was binding of the protein to the thawed sperm. At the two doses used (0.15 and 0.3 µM), TrxA-FNIIx4-His_6 had the capacity to reverse the molecular indicators of cryo-capacitation as indicated by the reduction on phosphorylated substrates of PKA. Furthermore, the supplementation with this protein resulted in a normal capacitation process as evidenced by the increase in the *in vitro* fertilization rate when the greatest concentration of the protein was evaluated (73.25 ± 2.95; 40.13 ± 11.82 for 0.3 µM and control, respectively). There was no effect of protein supplementation on sperm objective motility compared to untreated sperm. In conclusion, the use of TrxA-FNIIx4-His_6 is a promising biotechnological approach for cryopreserving ram sperm and maintaining sperm viability.

Keywords: Recombinant peptide; Fibronectin (FNII) domains; Cryopreservation; Ram; Seminal plasma

1. Introduction

The cryopreservation process, involving cooling, freezing and thawing, induces serious detrimental changes in sperm function (Watson 1981). Sperm sensitivity to this process is species-specific due to its shape and size and the biophysical-biochemical properties of plasmatic membranes that differ between species and males (Curry, 2007). Ram sperm are more sensitive to structural damage than sperm from bulls, rabbits or men (White, 1993). Cryopreservation reduces viability, motility, and mitochondrial activity, as well as imposing the process induces chromatin damage, increases reactive oxygen species, activates apoptosis mechanisms (Said et al., 2010) and perhaps most importantly, is the premature induction of a capacitation-like process known as cryo-capacitation (Bailey et al., 2003; Reddy et al., 2010). Cryo-capacitation is characterized by a membrane reorganization with loss of polyunsaturated fatty acids and cholesterol (Maldjian et al., 2005; Chakrabarty et al., 2007) with further development of molecular hallmarks of capacitation such as an intracellular calcium increase (Hagiwara et al., 2009; Oldenhof et al., 2010) and protein phosphorylation (Kumar and Atreja, 2012; Singh et al., 2012). All these alterations might reduce longevity of the cryopreserved spermatozoa within the female reproductive tract, decreasing the likelihood of typical oviductal-sperm interactions or affecting the fertilization process.

Incubation of frozen/thawed ram sperm with seminal plasma (SP) resulted in improvements in sperm variables including motility, viability (Ollero et al., 1997; Domínguez-Rebolledo et al., 2007; Domínguez et al., 2008) and resulting fertilization rate when SP was added to the incubation media (Maxwell et al., 2007). The beneficial effect of SP on sperm has been attributed to its protein components, because seminal plasma proteins (SPP) function to increase sperm resistance against cold-shock (Barrios et al., 2000; Pérez-Pé et al., 2001; Colás et al., 2009). The SPP support survival of ram spermatozoa functioning not only at the plasma membrane but also by inhibiting cryo-

capacitation (Desnoyers and Manjunath, 1992; Barrios et al., 2005) and apoptosis-like process changes (Mendoza et al., 2013). Bernardini et al. (2011) reported that a fraction of SPP with affinitive binding properties for the sperm membrane, termed interacting SP proteins (iSPP), had the capacity to reverse ultrastructural cell damage and improve motility of frozen/thawed ram sperm. Furthermore, the addition of iSPP to frozen/thawed sperm reversed the development of molecular indicators of cryo-capacitation such as tyrosine protein phosphorylation (Ledesma et al., 2016). The iSPP fraction is enriched in two proteins, RSVP14 and RSVP20, that belong to the BSP protein family (Binder of Sperm Proteins), for which the primary characteristic is the presence of two fibronectin type II (FNII) tandem domains. The FNII domains interact with the choline-phospholipids of the plasma membrane of sperm (Plante et al., 2016) and it has been proposed that the phospholipid-domain interaction would prevent the free movement of phospholipids in the membrane, stabilizing the membrane structure (non-capacitated stage) (Manjunath and Therien, 2002) until a signal of physiological induction occurs. Because RSVP14 and RSVP20 concentration in SP varies according to the reproductive season (Pérez-Pé et al., 2001; Domínguez et al., 2008), there has been a focus in many studies on the development of protocols to produce these proteins *in vitro*. Given the binding properties reported for the FNII domain (Therien et al., 2005), it was hypothesized that a recombinant peptide with FNII tandem repeats would mimic the beneficial effects of SP on frozen-thawed ram sperm.

The aim of the present study, therefore, was cloning and over-expression of a recombinant fragment of the epididymal sperm binding protein 1 (ELSPBP1) gene from *Bos taurus* cattle with the resulting protein being composed of four fibronectin type II domains in the heterologous system of *Escherichia coli (E. coli).* Furthermore, to gain insight into the physiological effect of this recombinant protein, motility, capacitation status and fertilizing potential were evaluated in frozen-thawed ram sperm.

2. Materials and methods

2.1. Bovine epididymis library construction

Epididymis RNA was obtained from Zyagen (BR-402; San Diego, US). Total RNA (100 µg) was examined on an Agilent Bioanalyzer 2100 system using the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA; Figure 1A). Subsequently, mRNA was purified from those samples using an Oligotex kit (QIAGEN, USA) and the concentration and quality were determined by RNA 6000 Nano chips were processed using an Agilent Bioanalyzer 2100 system (Agilent Technologies) resulting in 6 µg mRNA (figure 1B). A cDNA Library was constructed in the pDONR222 vector (Gateway compatible) using the CloneMiner kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's non-radiolabeling protocol with some modifications. After attB1 adapter ligation, the cDNA content was assessed and then fractionated using column chromatography. Each fraction (1 µL) was evaluated using capillary electrophoresis (Bioanalyzer 2100) with which the DNA 7500 chip kit was compared with the internal standard (figure 1C). Fractions were selected and pooled based on the criteria suggested by the manufacturer, the pooled samples were ethanol precipitated, suspended in TE buffer and concentration of cDNA was calculated on a DNA 7500 chip. All subsequent methods of the CloneMiner cDNA library construction kit protocol were performed according to the manufacturer´s instructions.

Serial dilutions of the library were plated on LB plates containing 50 ug/mL kanamycin (LBkan) and incubated overnight at 37 °C to determine the libraries titer.

A minimum of 24 kanamycin-resistant clones were randomly selected and cultured using LBkan. Plasmid from harvested colonies were isolated using the QIAprep Spin Miniprep Kit (QIAGEN) and digested with *BsrGI* to determine the average size of the inserts and percentage of recombinants (Figure 1D). The amplification of an aliquot of the primary cDNA libraries was performed a semi-solid amplification protocol provided by Invitrogen. The resulting library had a primary titer of 6.5 x 10^6 cfu/mL, a secondary 9.75 x 10^{14} cfu/mL and average insert size of 1.21 Kbp with 88% recombinants.

2.2. Recombinant protein in E. coli and purification

The EPIDIDYMAL SPERM BINDING PROTEIN 1 (ELSPBP1) contains four characteristic fibronectin type II domains (FNII) in tandem. The sequences encoding these FNII domains (FNII x4 cDNA) (ELSPBP1) were amplified from a bovine epididymal cDNA library. The restriction sites KpnI and Xho were included in the forward (Fw: 5'-131CGGGGTACCTCATGCATCTTTC-3') and reverse (Rv: 5' CCGCTCGAGGCAGTACAC-3') primers, respectively. The PCR reactions were prepared in a reaction mixture (20 µL) containing: Taq 1x buffer, 200 µM dNTPs, 1.5 mM Cl_2Mg, 1 µM primers Fw and Rv, 0.5U Taq DNA polymerase (0.5 U/µL) and 5 µL of template (bovine epididymal library dilution containing 9.75 x 10^{10} cfu/mL). The PCR cycling conditions consisted of 2 min at 94 °C, 35 cycles of 1 min at 94 °C, a step of hybridization at 53 °C for 1 min, an elongation step at 72 °C for 1 min and at the end 72 °C for 7 min. A negative control cycle without inclusion of the template was also performed. A positive control was included consisting in the amplification of the β-actin cDNA by 1 µM primers: β ACT-Fw 1405'-TTCCGATGCCCTGAGGCTCTTTTC-3'and β ACT-Rev 5'-CTTGCTGATCCACATCTGCTGGAA-3'. The amplified fragments (597 bp) were visualized in 1% (wt/vol) agarose gel, excised and purified using the Puro I Clean

Up kit (Biological Products).

The cDNA encoding the four FNII domains of ELSPBP1 (FNII x4 cDNA) was sub-cloned in the expression vector pET-32 Ek/LIC (Novagen, 5.917 bp). This vector was selected because it generates thioredoxin fusion proteins, which was expected to contribute increasing protein solubility in *E. coli.* Futhermore, this vector contains two histidine tags that allowed purifying the protein later by nickel affinity chromatography. For subcloning, 400 ng cDNA and 500 ng pET-32 Ek/LIC vector were subsequently digested with 10 IU of the restriction enzyme XhoI (Fermentas) and then with 10 IU of KpnI (Promega) according to the manufacturer's instructions. Digested fragments were purified with the DNA PuriPrep-GP Highway kit and Pure Link kit (Invitrogen) respectively. Ligation was carried out in a 1:5 relation with T4 DNA Ligase enzyme (3 IU/μL, Promega) and 100 mM ATP and incubating ON at 18 °C. DH5α competent cells were transformed with the putative FNIIx4-pET-32 Ek construction. The colonies were analyzed using Colony PCR with the first specific Fw primer of the ELBSP1 cDNA and as the RV Terminator T7 primer from the vector. The putative positive clones (product amplification size of 729 bp) were then analyzed using a DNA sequencing service (Macrogen, Korea) to discard mutations. Sequences were analyzed using the BioEdit Sequence Alignment program.

2.3. Protein cDNA over expression and purification

Rosetta™ *E. coli* competent cells (Novagen) were transformed using thermal shock with 50 ng of the plasmid containing the FNIIx4-pET-32 Ek construction. From an isolated colony, cultures were grown in liquid LB medium supplemented with 100 μg/mL ampicillin and 20 μg/mL chloramphenicol and incubated at 37 °C utilizing agitation for 16 h. A 25 mL of LB medium with the corresponding antibiotics was inoculated (1/100) and

incubated at 37 °C while stirring the medium until the OD = 0.4. Subsequently, the incubation continued at 18 °C until there was an OD = 0.6. The cultures were induced with 0.1 mM isopropyl-b-D-thiogalactoside (IPTG) and incubated 16 h at 18 °C until there was an OD = 2. Cells were harvested by centrifugation at 1700 x *g* for 15 min and re-suspended in lysis buffer (phosphate-buffered saline, pH 7.4 containing 1 mg/mL lysozyme, 2.5% v/v triton and 0.5 mM PMSF) and subsequently lysed using sonication (ten cycles of 60 Hz power of 30 s). The cell lysate was centrifuged (13,000 x *g*, 30 min) to separate supernatant from pellet. Both, soluble and insoluble fractions were separated using 12% w/v SDS-PAGE to verify the expression of the cDNA for the protein of interest.

To recover TrxA-FNIIx4-His_6 from the inclusion bodies, the insoluble fraction was washed twice in washing buffer I (20 mM Tris-HCl, pH 8, 20,000 x *g*, 30 min at 4 °C) and was subsequently placed in washing buffer II to denature and solubilize the proteins (50 mM Tris, 50 mM ClNa, 1.5 mM β-mercaptoethanol, 2 M urea, 2 % v/v triton, pH 8, 20,000 x *g*, 30 min at 4 °C). Subsequently, the cell pellet was re-suspended in binding buffer (50 mM Tris, 500 mM ClNa, 10 mM β-mercaptoethanol, 8 M urea, 20 mM imidazole), stored for 1 h while stirring the medium at 4 ºC and centrifuged at 40,000 x *g* to separate the supernatant.

The TrxA-FNIIx4-His_6 purification was conducted using a HiTrap nickel affinity column (GE Healthcare) equilibrated with binding buffer. Refolding of the bound protein was induced using an on-column decreasing linear gradient of 14 mM urea/min from binding buffer to a buffer without urea and dihydrotreithol (50 mM Tris, 500 mM NaCl, 20 mM imidazole) with a total volume of 300 mL for 12 h. The refolded protein was eluted with a gradient elution buffer containing increasing concentrations of imidazole (50 mM Tris-HCl, 500 mM NaCl with100 mM, 200 mM or 400 mM imidazole, respectively). Eluted fractions were pooled, dialyzed against 50mM phosphate buffer pH 7.5 and analyzed

using 12% (w/v) polyacrylamide gel. Protein concentration was quantified using the by Bradford method (Bradford, 1976).

2.4. Sperm collection and freezing-thawing procedure

All animals used in this study were managed in strict accordance with good animal practice and the conditions approved by the Animal Ethics Committee at INTA, Argentina. Semen collections were made from five fertile mature (5 years old) Texel rams, with a mean body condition score of 2.9 ± 0.8 (scale 1–5) housed at the Experimental Station of Balcarce (Instituto Nacional de Tecnología Agropecuaria, INTA Argentina; 37°450 south, 58°180 west), during the natural breeding season (March-June; autumn). Ejaculates were obtained using an artificial vagina. At least three samples with a sperm mass motility ≥ 4 (scale of 1 to 5) and concentration of ≥ 3 x 10^9 cells/mL were included in a semen pool for each week and there were 3 weeks of collections conducted. Each semen pool was composed of one ejaculate from at least three of the five rams used in the study. The composition of pools was different each week, as rams were selected randomly. Pooled ejaculates were diluted at 35 °C in one step with a TRIS-glucose-citric acid extender supplemented with egg yolk (10 % v/v) and glycerol (7 % v/v) and cooled to 5 °C for 2 h. Samples were subsequently stored at 5 °C for 2 additional hours before freezing and packaging in 0.25 mL plastic straws. Semen samples were packaged at a final concentration of 100 x 10^6 spermatozoa/mL, corresponding to 25 x 10^6 spermatozoa per straw. Straws were sealed with polyvinyl alcohol and frozen in liquid nitrogen vapors, 5 cm above the liquid nitrogen surface for 10 min, before being plunged into the liquid nitrogen for storage.

2.5. Experimental design

Different sperm variables were evaluated to study the effect of the recombinant protein over post-thawed sperm. Two different protein concentrations were assayed and compared to a control group without protein. To define the concentrations of the protein to be evaluated, a dose response assay for sperm viability was conducted using a subjective motility analysis as follows: 5 µl of semen was placed on a pre-warmed glass slide with a cover slip being applied and there was visual assessment using a phase-contrast microscope (4009; Nikon Diaphot, Japan). The initial dose of TrxA-FNIIx4His_6 that was evaluated was similar to that reported by Barrios et al. (2005) for RSVP14 (13 µM). The following doses were assessed: 13, 6.5, 3, 1.5, 0.3 and 0.15 µM. Because of the complete loss of sperm viability (evaluated as subjective total motility) after an incubation of 30 min at 37 °C with the greater concentrations, the decision was made to use 0.15 µM and 0.3 µM in the assays for the present study.

Regarding the assessment of the effects of recombinant protein when considering the various sperm variables, three straws were randomly thawed by immersion in a water bath (37 °C, 20 s), layered over 1 mL Androcoll-OTM colloid and centrifuged to remove dead cells and SP (800 x *g* by 5 min and then 1200 x *g* for 10 min), and subsequently sperm cells were washed with PBS (800 x *g*, 5 min) and incubated at 37 ºC in PBS. At the initiation of the incubation period, there was addition of the protein immediately after the selection process. At the indicated times for each assay, an aliquot was collected. The evaluation of sperm motility was performed at 30, 60, 90 and 120 min of the incubation period, phosphorylation status was assessed after 60 min incubation as previously described (Ledesma et al., 2016) and in vitro fertilization was evaluated after 40 h of incubation. There were three experimental replicates for each assay. Within each replicate, straws from the same pool of semen were used to evaluate the different sperm variables.

2.6. Fluorescent labeling and binding of TrxA-FNIIx4-His6x to spermatozoa

The TrxA-FNIIx4-His_6 (1.32 µM) was incubated with 130 mM FITC (in DMSO) in 0.1M buffer Sodium bicarbonate (pH 9.2, 2 h, room temperature). Non-conjugated dye was subsequently removed by dialysis against 50 mM buffer phosphate pH 7.5 containing 0.15 M of NaCl. The protein labeling was confirmed by evaluating the absorption spectrum (280-500 nm). To evaluate the interaction of FITC-TrxA-FNIIx4-His_6 with ram the sperm cells, 2 x 10^8 frozen/thawed sperm were incubated with 0.37 µM FITC-TrxA-FNIIx4-His_6 solution in PBS during 15 min at room temperature as previously described for recombinant SPINK3 (Zalazar et al., 2016). There was also inclusion of a group where sperm were incubated with unlabeled protein as a control of autofluorescence. Furthermore, there was incubation of sperm with FITC and without protein as a specificity control. Samples were then fixed in 4 % (v/v) formaldehyde and washed in PBS (600 x g, 5 min). Binding was confirmed using fluorescence visualization under confocal microscope (600 x) (Nikon C1siR). Images were analyzed using the ImageJ free software (https://imagej.nih.gov/ij/).

2.7. Sperm analyses

2.7.1. Capacitation status by phosphorylation of PKA substrates

Thawed sperm (5 x 10^6) were treated with 0.15 or 0.3 µM of recombinant protein for 60 min at 37 °C and then centrifuged at 7500 x *g* 5 min at 4 °C. Sperm pellets were mixed with 5X Laemmli sample buffer under reducing conditions (100 mM DTT) and boiled for 5 min. Solubilized proteins were separated by electrophoresis on 10 % (w/v) acrylamide SDS–PAGE (Laemmli et al., 1970) and transferred to Immuno-Blot 251PVDF membranes (BIO-RAD). Non-specific binding sites on membranes were blocked with 5 % w/v BSA in TBS-T (10 mM Tris–HCl, pH 8, 120 mM NaCl, 0.05 % Tween) for 60 min h at room temperature. There was immuno-detection of

phosphorylated proteins in PKA substrates by incubating with the primary antibody (AntiPKA monoclonal mouse, Santa Cruz, 1:500 in blocking solution), overnight at 4 °C with constant agitation of medium occurring. After three washes in TBS-T, the membranes were incubated for 60 min at room temperature with the secondary antibody (anti-mouse IgG conjugated with HRP, BioRad, 1:10000 in blocking solution). The membranes were developed using the C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). Blots were subsequently stripped for 20 min at 60 °C using a stripping buffer (62.5 mM Tris-HCl, 2 % w/v SDS, 100 mM β-mercaptoethanol, pH 6.5), re-blocked and sequentially incubated overnight with a mouse monoclonal anti-tubulin antibody (1:5000) (Sigma, T6094). Secondary antibody incubation and development were performed as previously described in this manuscript. Chemiluminescence was developed in a C-Digit blot Scanner (Li-Cor, US) and images were analyzed using Image Studio Lite 5.0. Bands intensities per area unit were normalized to the corresponding tubulin densities.

2.7.2. Sperm motility variables

Objective motility was evaluated every 30 min up to 120 min of incubation by using a CASA computerized analysis system developed by Buchelly Imbachi et al. (2018). Aliquots of 1 x 10^9 spermatozoa were treated with 0.15 µM, 0.3 µM of TrxA-FNIIx4-His_6 or without protein at 37 °C in PBS. A Cell-Vu chamber (20 µm, Millennium Sciences) warmed to 37 °C, was filled with 7 µL of sample and examined using a 10 X objective (negative phase contrast field) on a Nikon Eclipse E200 microscope. Images were captured with a Coolpix S10, Nikon digital camera at 30 frames per second (fps). Four fields containing among 700 and 2000 cells were analyzed for each treatment, time and replicate. The kinematic variables were analyzed for each spermatozoon were: total motility (TM; %), progressive motility (PM; %), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), velocity path average (VAP; µm/s), linearity (LIN; %),

straightness (STR; %), wobble (WOB; %), amplitude of the lateral movement of the head (ALH; μm) and beat-cross frequency (BCF; Hz).

2.7.3. Fertilization potential

To evaluate sperm fertilizing potential, the heterologous *in vitro* fertilization described by García-Alvarez et al. (2009) with modifications was used. Bovine ovaries were collected from a local slaughterhouse and transported within 2 h in a thermic container at room temperature. Cumulus–oocyte complexes (COCs) with homogeneous ooplasm and more than four complete layers of cumulus cells, corresponding to grade 1 and 2 according to De Loos et al. (1989), were selected using a stereomicroscope and washed three times in M199 supplemented with 0.5 % HEPES (w/v). COCs were cultured in TCM199 supplemented with 50 lg/mL gentamycin, 5.5 mM Ca lactate, 2.3 mM Na pyruvate, 36 mM $NaHCO_3$, 5 mM Hepes, 0.01 UI/mL rhFSH (Gonal F-75, Serono, UK). Maturation was induced in four-well plates (Nunc, Roskilde, Denmark) in groups of 50 to 60 COCs with 400 μl of maturation media for 24 h at 38.5 °C under 5 % CO_2 in air with maximum humidity. Matured COCs were transferred to 400 μL of fertilization medium (SOF supplemented with 10% of estrous sheep serum and 40 μg/ml gentamycin). Thawed ram sperm were selected on a Percoll discontinuous density gradient (45/90) and capacitated in the fertilization medium for 10 min. Sperm was co-incubated with oocytes at a final concentration of 2.5 x 10^5 cells/mL per 40-50 COCs at 38.5 °C in 5 % CO_2 for 40 h, in the presence or absence of 0.15 μM or 0.3 μM TrxA-FNIIx4-His_6. To discount any parthenogenic effect of the recombinant protein, a control was performed supplementing un-inseminated oocytes with the same concentrations of TrxA-FNIIx4-His_6. After co-incubation, oocytes were fixed in 400 μL of glutaraldehyde and stained with Hoechst 33342. Fertilization rate was assessing after 40 h using an inverted microscope by assessing the presence of

cleaved oocytes (two to eight cells) or the presence of two or more nuclei.

2.8. Statistical analysis

Data were analyzed using a generalized linear mixed effect model for continuous response variables of the statistical package SAS (Statistical Analysis System version 8.2 (SAS). The statistical model included the effect of the addition of TrxA-FNIIx4-His_6 in two concentrations 0.15 µM and 0.3 µM and the effect of the time (discrete variable). To test normality and homoscedasticity, a histogram and plot of residuals for each model was performed. When model assumptions were not fitted, data were transformed by square root function. Results were expressed as least square means (LSM) ± standard error of means (SEM). Differences were considered to exist $P \leq 0.05$.

3. Results

3.1. Construction of the cDNA and expression of recombinant TrxA-FNIIx4-His_6

The cDNA encoding four tandem FNII repeats from ELSPBP1 was amplified from a bovine epididymal cDNA library (figure 1) with specific primers obtaining a product of 597 bp and sub-cloned in the expression vector pET-32 Ek/LIC (Figure 2). The resulting recombinant TrxA-FNIIx4-His_6 cDNA was expressed in competent E. *coli* Rosetta. As the protein was detected primarily within inclusion bodies, there was a denaturation and on-column refolding-purification protocol applied using nickel affinity chromatography (Figure 2).

3.2. Analysis of the TrxA-FNIIx4-His_6 binding to spermatozoa

The sperm binding capacity of the purified TrxA-FNIIx4-His_6 was assessed

by incubating thawed ram spermatozoa (2 x 10^8 cells/mL) with 2.8 µM FITC-conjugated-TrxA-FNIIx4-His_6. With use of fluorescence confocal microscopy analysis, there were differences detected in the intensity and distribution patterns of fluorescence for sperm incubated with and without TrxA-FNIIx4-His_6 having a greater fluorescence intensity when TrxA-FNIIx4-His_6 was attached to sperm (Figure 3). Fluorescence was observed mainly in the acrosomal region and middle piece, which is consistent with what occurs with the native protein ELSBP1 and other proteins with FNII domains (Ekhlasi-Hundrieser et al., 2007; Plante et al., 2014).

3.3. Analysis of TrxA-FNIIx4-His_6 capacity to repair sperm cryodamage

3.3.1. Capacitation status

To evaluate the possible effect of the recombinant protein on the reversion or minimization of cryo-capacitation molecular signals, the status of substrates phosphorylated by the cAMP-dependent protein kinase (PKA) were analyzed. Proteins extracted from post-thawed sperm previously incubated for 60 min with or without 0.15 µM or 0.3 µM of TrxA-FNIIx4-His_6 were analyzed using western blot procedures. A pattern with four defined and quantifiable bands was detected in each treatment (Figure 4). Densitometry assessments of each band indicated that there were differences in the intensities of all bands (approximately 102, 76, 52 and 31 kDa) detected for substrates phosphorylated as a result of PKA (P <0.05) between treatments and control. With use of the two concentrations of TrxA-FNIIx4-His_6 evaluated, there was a reduction of the phosphorylation signal. Conversely, there was no statistical differences when the two concentrations of TrxA-FNIIx4-His_6 were evaluated (Figure 4).

3.3.2. Sperm motility

Considering sperm motility is the most frequently assessed variable for determination of cell quality, different kinetic variables were evaluated using CASA every 30 min during the 120 min of the incubation period (37 °C) in thawed spermatozoa supplemented with TrxA-FNIIx4-His_6. Results were contrasted with those of untreated sperm (control). There was no significant beneficial effect of TrxA-FNIIx4-His_6 on sperm motility percentages or values for kinetic variables at any of the evaluation periods (Figure 5, Table 1).

3.3.3. Fertilizing potential

Considering that TrxA-FNIIx4-His_6 is a decapacitating protein, it was important to evaluate the fertilization capacity of the treated sperm. For this, a heterologous *in vitro* fertilization assessment was conducted (García-Alvarez et al., 2009). Addition of 0.3 µM of TrxA-FNIIx4-His_6 induced an increase in fertilization rate compared to control samples (P = 0.024) because there were a greater number of cells where cleavage had occurred and/or there was the presence of two or more nuclei. There, however, were no differences in cleavage rates between sperm treated with 0.15 µM of TrxA-FNIIx4-His_6 and control samples (Figure 6).

4. Discussion

The results from the present study indicate there can be cloning, expression of an encoding cDNA and purification of a recombinant peptide composed of four tandem FNII domains as a first approach to create a media with defined composition that can protect/reverse the damage caused by cryopreservation. The cDNA fragment was obtained from a bovine epididymis library constructed and the resulting recombinant peptide had the capacity to bind to frozen/thawed ram sperm

at the acrosomal region and the midpiece, reversed cryo-capacitation indicators and improved *in vitro* fertilization when added at 0.3 µM to fertilization media. Sperm motility, however, was not affected by the treatment at any of the concentrations assayed.

For several years, additives for freezing/thawing media have been studied to prevent/reverse the undesirable effects of cryopreservation on sperm cells (Kumar and Atreja 2012; Mukherjee et al., 2016; Zalazar et al., 2016; Del valle et al., 2017; İnanç et al., 2018). Cryo-capacitation, a premature capacitation-like status, reduces the functional sperm lifespan decreasing the fertilization potential when these sperm are used for artificial insemination. Preventing and even reversing this cryo-damage allowing the normal capacitation (physiological) to occur when the sperm are located in the oviduct with the oocyte is the desired outcome with the use of these treatments. Because SP is known to contain decapacitating proteins, the addition of SP has been used prior to freezing/after thawing to prevent/reverse molecular changes as a result of cryo-capacitation such as protein phosphorylation in several species such as rams (Pérez-Pé et al., 2001), boars (Vadnais and Althouse, 2011) and horses (De Andrade et al., 2012). The effect of SP, however, depends on its variable composition, making it difficult to obtain consistent results when using these approaches. The capacity of SP to protect and reverse the damage caused by freezing/thawing has been attributed to a group of SP proteins that interact with membrane components of the sperm. Most of these proteins belong to BSP (Binder of Sperm Proteins) family characterized to contain two or four fibronectin type II domains, which mediate the interaction with spermatozoa through choline phospholipids of the sperm membrane (Plante et al., 2016). Serrano et al. (2013) cloned and expressed two proteins from this family, RSVP14 and RSVP20 (Serrano et al., 2015). The effect of inclusion of these proteins in the freezing/thawing media, however, has not yet been reported and the mechanism of action of these

recombinant proteins remains unknown.

Most recombinant proteins for biotechnological purposes are produced in *E. coli* (Ferrer-Miralles et al., 2009) due to relatively lesser cost as compared with other procedures and protein production efficiencies when using this approach. This protein production approach, however, jeopardizes the occurrence of post-translational modifications such as formation of di-sulfide bridges (Wilkinson and Harrison, 1991). In the present study, despite the addition of a thioredoxin tag (La Vallie et al., 1993), the recombinant peptide was expressed in the insoluble fraction using all the conditions utilized (data not shown). Solubilized and refolded TrxA-FNIIx4-His_6 bound to the head and midpiece, as reported for other proteins with FNII domains in bull, mice and human sperm (Plante et al., 2012). As reported by Barrios et al. (2005) for RSVP14, in the present study there was evaluation of the addition of 13 µM of TrxA-FNIIx4His_6 in the thawing media, however, there was loss of sperm motility completely after 30 min of incubation at 37 °C. The doses used in the present study were similar to those used by Serrano et al. (2013) where there was expression of recombinant RSVP14.

In regard to results from the sperm motility CASA assessments in the present study, there was no difference between control and sperm supplemented with TrxA-FNIIx4His_6 neither for population nor values for kinetic variables at any of the incubation periods and concentrations evaluated. This finding is similar to those from a previous study (Ledesma et al., 2016), in which the addition of a fraction of seminal plasma proteins that interact with sperm surface that is known to be rich in FNII containing proteins (Bernardini et al., 2011) did not modify mitochondrial potential and sperm motility. This result indicates that the lack of effect for these variables was not due to the presence of other components of SP present in enriched fraction, but instead, it may explain that this domain did not function at the mitochondria, or there may be actions of this protein only at a subpopulation of sperm in the samples.

In the present study, the addition of 0.15 and 0.3 µM of TrxA-FNIIx4His$_6$ reversed the phosphorylation of some PKA substrates. Protein kinase A (PKA) is known to be responsible for the phosphorylation of proteins at Ser and Thr residues (Visconti et al., 2011) as part of the signal transduction pathway when the normal capacitation processes occur in the oviduct. Although there are some similarities between *in vitro* capacitation and cryo-capacitation such as plasma membrane reorganization, increase in $[Ca2+]_i$ and occurrence of protein phosphorylation, there are striking difference in pattern of phosphorylation and it should not be considered as the same capacitation process that occurs for sperm in the oviduct after insemination of the female (Green and Watson, 2001; Kumar and Atreja, 2011; Pommer and Meyers, 2002). The effect of reducing phosphorylation that occurred in the present study are consistent with those reported previously after supplementation of ram sperm with seminal plasma proteins that interact with the sperm surface (Ledesma et al., 2016). The advantages of reversing cryo-capacitation in ram sperm was previously reported by other groups. Fang et al. (2018) reported that addition of boar SP inhibited cryo-capacitation of frozen-thawed ram sperm while improving the normal physiological capacitation process at *in vitro* capacitating conditions, increased sperm penetration and pregnancy rate when there was intra-cervical AI conducted. The use of a recombinant peptide, however, has the advantages related with the production practices and the capacity to have a more precisely defined composition. There has been identification as a result of a recent proteomics study of a tyrosine-protein kinase isoform X2 and a phospholipid scramblase isoform X1 with use of LC-MS/MS and SWATH and there were different quantities in lysates of ram spermatozoa after cryopreservation (Pini et al., 2018) with both alterations being related to cryo-capacitation. The FNII rich proteins might function to reverse these variations as a result of the affinity of these proteins for sperm membrane phospholipids.

The promising results of the present study regarding the decapacitating effect of

TrxA-FNIIx4His$_6$ were associated with improvements in the *in vitro* fertilization rate when the treated sperm were used for this purpose. Addition of 0.3 µM of TrxA-FNIIx4His$_6$ to the spermatozoa improved the fertilization rate compared with control. To the best of our knowledge, this is the first study where it has been reported that the addition of a recombinant peptide resulted in improvements in the fertilizing potential of frozen/thawed sperm. Decapacitating proteins are known to be removed from the sperm surface on the female reproductive tract environment and/or in the oviduct where the ovum is located prior to the time of fertilization (Mannowetz et al., 2011; Young et al., 2009). As a result, the TrxA-FNIIx4-His$_6$ protein supposedly has a de-capacitating activity in sperm media; however, it is supposed to detach from the sperm during the FIV co-culture. Obviously, video imaging is needed to confirm that there is detaching of TrxA-FNIIx4His$_6$ from the fertilizing sperm. Because capacitation is a known pre-requisite for fertilization, the results of the present study not only confirm that the protein did not prevent physiological capacitation but also improved the percentage of sperm with the capacity for fertilization as evidenced by the IVF results. It has to be noted that although both TrxA-FNIIx4His$_6$ doses reduced protein phosphorylation, only at the larger doses were there the more desirable results with the IVF assay. It is not surprising that there were no indications with the Western blot analysis of a dose-response effect when due to background phosphorylation induced by freezing because the treatment cannot affect the whole sperm.

In view of the results of the present study, there was cloning of a cDNA and expression resulting in the production of a peptide containing four domains of type II fibronectin fused to a thioredoxin and a tag of six terminal histidines, named TrxA-FNIIx4-His$_6$. As a promising biotechnique, further analyzes of post thawing sperm variables using flow cytometry (plasma membrane permeability, mitochondrial activity, membrane fluidity, production of intracellular reactive oxygen species) needs to occur

and furthermore, incorporation of the recombinant peptide into the freezing extender is planned for future research.

Declaration of interest

The authors have not declared any conflicts of interest.

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References

Bailey, J., Morrier, A., Cormier, N., 2003. Semen cryopreservation: successes and persistent problems in farm species. Can J. Anim. Sci. 83, 393-401.

Barrios, B., Pérez-Pé, R., Gallego, M., Tato, A., Osada, J., Muiño-Blanco, T., Cebrián Pérez, J., 2000. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol. Reprod. 63, 1531-1537.

Barrios, B., Fernández-Juan, M., Muiño-Blanco, T., Cebrián-Pérez, J., 2005. Immunocytochemical localization and biochemical characterization of two seminal plasma proteins that protect ram sperm against cold shock. J. Androl. 26, 539-549.

Bernardini, A., Hozbor, F., Sánchez, E., Fornes, M., Alberio, R., Cesari, A., 2011. Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. Theriogenology 76, 436-447.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Buchelly Imbachí, F., Zalazar, L., Pastore, J., Greco, M., Iniesta-Cuerda, M., Garde, J. Soler, A., Ballarin, V., Cesari, A., 2018. Objective evaluation of ram and buck sperm motility by using novel sperm tracker software. Reproduction 156, 11-21.

Chakrabarty, J., Banerjee, D., Pal, D., Majumbder., 2007. Shedding off specific lipid constituents from sperm cell membrane during cryopreservation. Cryobiology 64, 27-35.

Colás, C., Junquera, C., Pérez-Pé, R., Pérez, J., Muiño-Blanco, T., 2009. Ultrastructural study of the ability of seminal plasma proteins to protect ram spermatozoa against cold-shock. Microsc. Res. Techniq. 72, 566-572.

Curry, M. R., 2007. Cryopreservation of mammalian semen. In: Cryopreservation and Freeze-Drying Protocols (pp. 303-311). Humana Press.

De Andrade, A., Zaffalon, F., Celeghini, E., Nascimento, J., Bressan, F., Martins, S., De Arruda, R., 2012. Post-thaw addition of seminal plasma reduces tyrosine phosphorylation on the surface of cryopreserved equine sperm, but does not reduce lipid peroxidation. Theriogenology 77, 1866-1872.

de Loos, F., van Vliet, C., van Maurik, P., Kruip, T. A. 1989. Morphology of immature bovine oocytes. Gamete Res. 24, 197-204.

Del Valle, I., Casao, A., Pérez-Pé, R., Holt, W., Cebrián-Pérez, J., Muiño-Blanco, T., 2017. Seminal Plasma Proteins Prevent Detrimental Effects of Ram Sperm

Cryopreservation and Enhance the Protective Effect of Lecithin. Biochem. Anal. Biochem. 6, 2161-1009.

Desnoyers, L., Manjunath, P., 1992. Major proteins of bovine seminal plasma exhibit novel interactions with phospholipid. J. Biol. Chem. 267, 10149-10155.

Domínguez-Rebolledo, A., Navarrete Sierra, L., Cruz Tamayo, A., Aguiar Lorio, A., Erosa Denis, S., Bolio Oses, R., González Parra, E., Paredes Monsreal, L., Ramón Ugalde, J., 2007. Fertilidad en ovejas de pelo inseminadas con semen congelado rediluido con plasma seminal. Revista Científica. 17, 73-76.

Domínguez, M., Falcinelli, A., Hozbor, F., Sanchez, E., Cesari, A., Alberio, R., 2008. Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. Theriogenology 69, 564-573.

Ekhlasi-Hundrieser, M., Schafer, B., Philipp, U., Kuiper, H., Leeb, T., Mehta, M., Kirchhoff, C., Topfer-Petersen, E., 2007. Sperm-binding fibronectin type II-module proteins are genetically linked and functionally related. Gene 392, 253-265.

Fang, Y., Zhong, R., Zhang, X., Zhang, J., Zhou, D., 2018. Boar seminal plasma inhibits cryo-capacitation of frozen-thawed ram sperm and improves fertility following intracervical insemination. Theriogenology 105, 84-89

Ferrer-Miralles, N., Domingo-Espin, J., Corchero, J., Vázquez, E., Villaverde, A., 2009. Microbial factories for recombinant pharmaceuticals. Microb. Cell. Fact. 8, 17-25.

García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Fernández-Santos, M., Esteso, M., Pérez-Guzmán, M., Soler, A., 2009. Heterologous in vitro

fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. Theriogenology 71, 643-650.

Green, C., Watson, P, 2001. Comparison of the capacitation-like state of cooled boar spermatozoa with true capacitation, Reproduction 122, 889-898;

Hagiwara, M., Choi, J., Devireddy, R., Roberts, K., Wolkers, W., Makhlouf, A., Bischof, J., 2009. Cellular biophysics during freezing of rat and mouse sperm predicts post-thaw motility. Biol. Reprod. 81, 700-706.

İnanç, M., Uysal, O., Ata, A., 2018. Cryopreservation and evaluation of Akkaraman ram semen with 7-dehydrocholesterol. Ankara Üniversitesi Veteriner Fakültesi Dergisi, 65, 187-192.

Kumar, R.; Atreja, S., 2012. Effect of Incorporation of Additives in tris-based egg yolk extender on Buffalo (Bubalus bubalis) sperm tyrosine phosphorylation during cryopreservation. Reprod. Dom. Anim. 47, 485-490.

Laemmli, U., Beguin, F., Gujer-Kellenberg, G., 1970. A factor preventing the major head protein of bacteriophage T4 from random aggregation. J. Mol. Biol. 47, 69-85.

La Vallie, E., Di Blasio, E., Kovacic, S., Grant, K., Schendel, P., Mc Coy, J., 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. Nat. Biotechnol. 11, 187-193.

Ledesma, A., Fernández-Alegre, E., Cano, A., Hozbor, F., Cesari, A., Martínez-Pastor, F., 2016. Seminal plasma proteins interacting with sperm surface revert capacitation indicators in frozen-thawed ram sperm. Anim. Reprod. Sci. 173, 35-41.

Maldjian, A., Pizzi, F., Gliozzi, T., Cerolini, S., Penny, P., Noble, R., 2005. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology 63, 411-421.

Manjunath, P., Therien, I., 2002. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J. Reprod. Immunol. 53, 109-119.

Mannowetz, N., Wandernoth, P., Hornung, J., Ruffing U., Raubuch, M., Wennemuth, G., 2011. Early activation of sperm by HCO3- is regulated hormonally in the murine uterus. Int. J. Androl. 34, 153–64.

Maxwell, W., De Graaf, S., Ghaoui, R., Evans, G., 2007. Seminal plasma effects on sperm handling and female fertility. Soc. Reprod. Fert. 64, 13.

Mendoza, N., Casao, A., Pérez-Pé, R., Cebrián-Pérez, J., Muiño-Blanco, T., 2013. New insights into the mechanisms of ram sperm protection by seminal plasma proteins. Biol. Reprod. 88, 1-15.

Mukherjee, K., Basu, S., Sahoo, A., Datta, U., Ray, K., Chattaraj, S., 2016. Cryoprotective effect of EDTA, lactose, ascorbic acid and L-cysteine as additives on garole ram (Ovis aries) semen. Int. J. Adv. Res. Biol. Sci, 3, 92-98.

Oldenhof, H., Friedel, K., Sieme, H., 2010. Membrane permeability parameters for freezing of stallion sperm as determined by Fourier transform infrared spectroscopy. Cryobiology 61, 115-122.

Ollero, M., Cebrián-Pérez, J., Muiño-Blanco, T., 1997. Improvement of cryopreserved ram sperm heterogeneity and viability by addition of seminal plasma. J. Androl. 18, 732-739.

Pérez-Pé, R., Cebrián-Pérez, J., Muiño-Blanco, T., 2001. Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenology 56, 425-434.

Pini, T., Rickard, P., Leahy, T., Crossett, B., Druart, X., de Graaf, S., 2018. Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa. J. Proteom. 181, 73-82.

Plante, G., Thérien, I., Manjunath, P., 2012. Characterization of recombinant murine binder of sperm protein homolog 1 and its role in capacitation. Biol. Reprod. 87, 1-11.

Plante, G., Fan, J., Manjunath, P., 2014. Murine Binder of Sperm Homolog 2 (BSPH2): The Black Sheep of the BSP Superfamily. Biol. Reprod. 90, 20-21.

Plante, G., Prud'homme, B., Fan, J., Lafleur, M., Manjunath, P., 2016. Evolution and function of mammalian binder of sperm proteins. Cell Tissue Res. 363, 105-127. A.

Pommer, A., Meyers, S., 2002. Tyrosine phosphorylation is an indicator of capacitation status in fresh and cryopreserved stallion spermatozoa, Theriogenology 58, 351-355.

Reddy, N., Mohanarao, G., Atreja, S., 2010. Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (Bubalus bubalis) sperm quality following cryopreservation. Anim. Reprod. Sci. 119, 183-190.

Said, T., Gaglani, A., Agarwal, A., 2010. Implication of apoptosis in sperm cryoinjury. Reprod. BioMed. 21, 456-462.

Serrano, E., Martínez, A., Arruga, D., Pérez-Pé, R., Sánchez-Ferrer, A., Muiño-Blanco, T., Pérez-Pé, J., 2015. New insights into the phylogeny and gene context analysis of binder of sperm proteins (BSPs). PLoS ONE. 10: e0137008. https://doi.org/10.1371/journal.pone.0137008.

Serrano, E., Pérez-Pé, R., 2013. Characterization of the cDNA and in vitro expression of the ram seminal plasma protein RSVP14. Gene 519, 271-278.

Singh, S., Atreja, S., Kumar, R., Chillar, S., Singh, A., 2012. Assessment of intracellular Ca2+, AMPc and 1,2 diacylglycerol in cryopreserved buffalo (Bubalus bubalis) spermatozoa on supplementation of taurine and trehalose in the extender. Reprod. Dom. Anim. 47, 584-590.

Therien, I., Bergeron, A., Bousquet, D., Manjunath, P., 2005. Isolation and

characterization of glycosaminoglycans from bovine follicular fluid and their effect on sperm capacitation. Mol. Reprod. Dev. 71, 97-106.

Vadnais, M., Althouse, G., 2011. Characterization of capacitation, cryoinjury, and the role of seminal plasma in porcine sperm. Theriogenology 76, 1508-1516.

Visconti, P., Krapf, D., De La Vega-Eltán, J., Acevedo, J., Darzon, A., 2011. Ion channels, phosphorylation and mammalian sperm capacitation. Asian J. Androl. 13, 395-405.

Watson, PF. 1981. The effects of cold shock on sperm cell membranes. G.J. Morris, A. Clarke (Eds.), Effects of Low Temperatures on Biological Membranes, Academic Press, New York, NY (1981), pp. 189-218

White, I., 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. Reprod. Fert. Dev. 5, 639-658.

Wilkinson, D., Harrison, R., 1991. Predicting the solubility of recombinant proteins in Escherichia coli. Nat. Biotechnol. 9, 443-448.

Young, C., Grasa, P., Coward, K., Davis, L., Parrington, J., 2009. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. Fertil. Steril. 91, 2230–42.

Zalazar, L., Ledesma, A., Hozbor, F., Cesari, A., 2016. Heterologous recombinant protein with decapacitating activity prevents and reverts cryodamage in ram sperm: an emerging biotechnological tool for cryobiology. Anim. Reprod Sci. 164, 31-39.

Figure legends

Fig. 1**.** Bovine Epididymis cDNA library construction; Bioanalyzer electropherograms of total RNA, mRNA and cDNA fractions (A, B and C respectively); Panel D. depicts the range of inserts in 24 random clones from library after excision from the pDONR plasmid using the BsrG I restriction enzyme; Samples were processed using a 1.5% agarose gel compared to 1Kb ladder (Invitrogen)

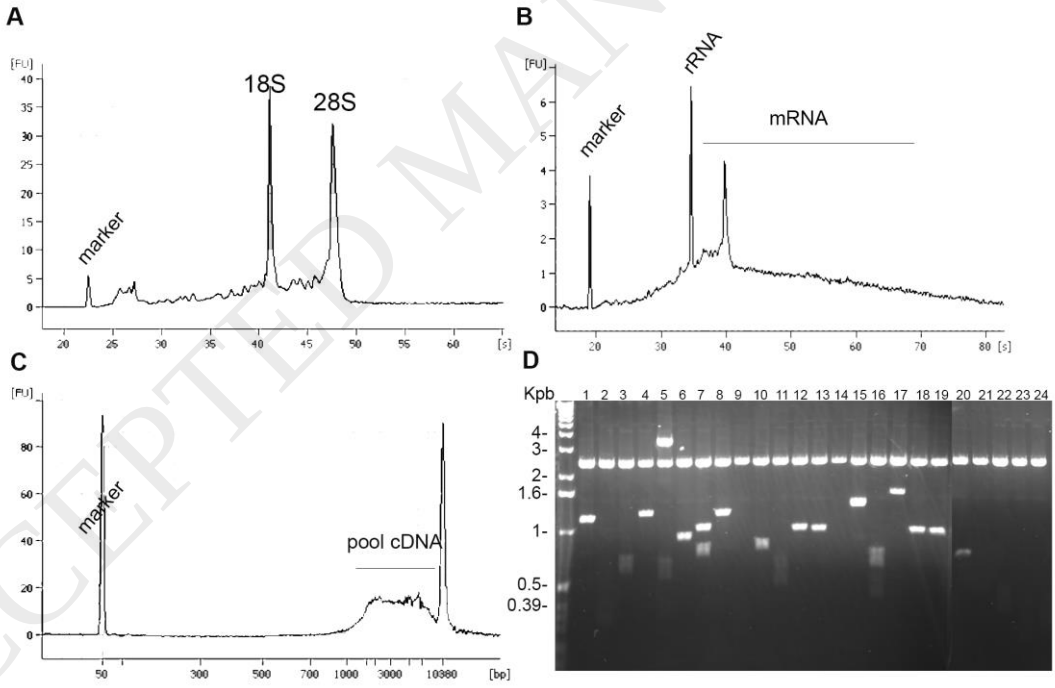


Fig. 2**.** Cloning, gene expression and purification of TrxA-FNIIx4-His_6; A. cDNA encoding four tandem FNII repeats from Epididymal Sperm Binding Protein 1 precursor (ELSPBP1) was amplified from a bovine epididymal cDNA library and

cloned into the expression vector pET-32 Ek/LIC (Novagen, 5.917 bp); Construction included a thioredoxin tag (TrxA) at the N-terminal and a 6 Histidine tag (Hisx6) at the C-terminal; B. E. coli Rosetta cells were transformed with the construction FNIIx4-pET-32 Ek; Electrophoretic profile using SDS–PAGE indicates the soluble (S) and insoluble (I) fractions of E. coli cells after 16 h induction (+IPTG T16) with 0.1 mM IPTG at 18 °C; A soluble fraction of cells without induction is depicted (control); TrxA-FNIIx4-His_6 protein solubilized from inclusion bodies was refolded and purified using a HiTrap nickel affinity column (Purification); Molecular weight marker is depicted at left; Arrow indicates the expected size for the protein (38 kDa)

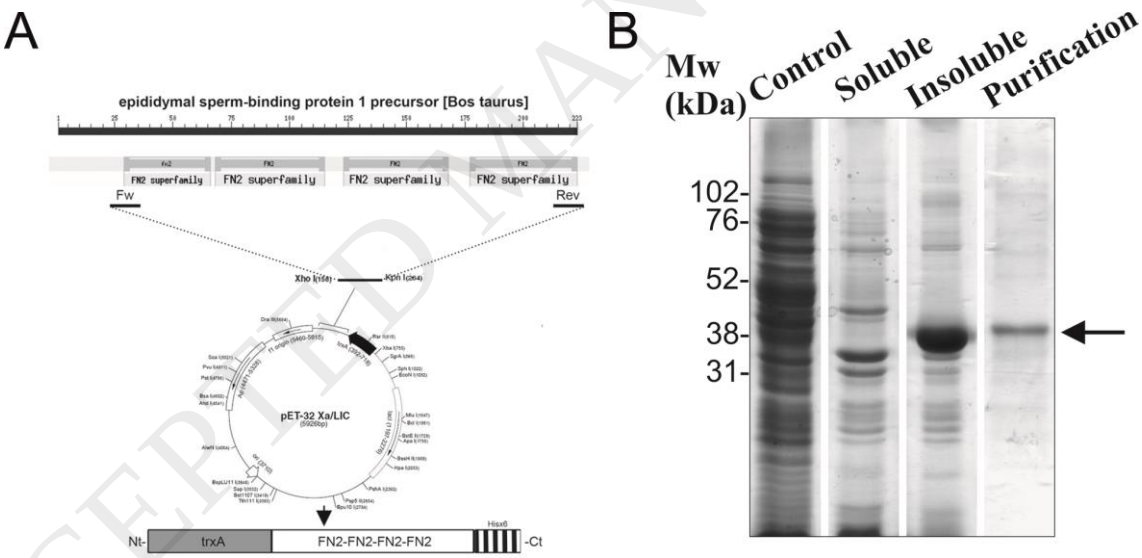


Fig. 3. Interaction between FITC-TrxA-FNIIx4-His_6 and thawed ram sperm using fluorescence confocal microscopy; Thawed ram sperm (20 x 10^6 cells/mL) were incubated with 25 µg 0.37µM FITC-TrxA-FNIIx4-His_6 during 60 min at room temperature and fixed; Left panel: Spermatozoa incubated with FITC-TrxA-FNIIx4-His_6 (TrxA-FNIIx4-His_6) or FITC without protein (control) were visualized using a microscope (60x); Right panel: The graph depicts the distribution fluorescence intensity

from the apical region to the flagella of each sperm; Upper line corresponds to spermatozoa incubated with FITC-TrxA-FNIIx4-His_6 and bottom line corresponds to control

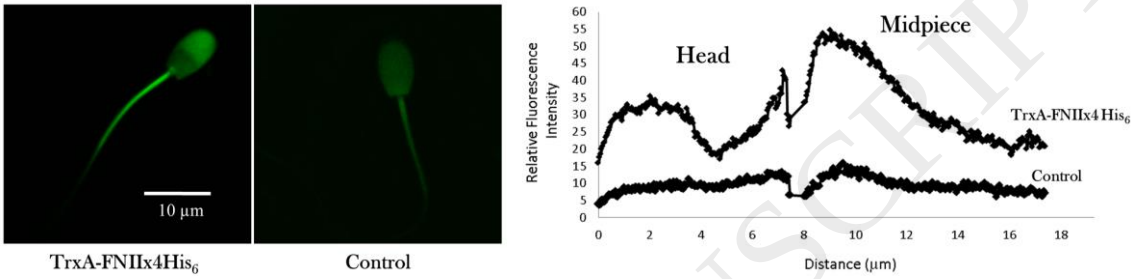
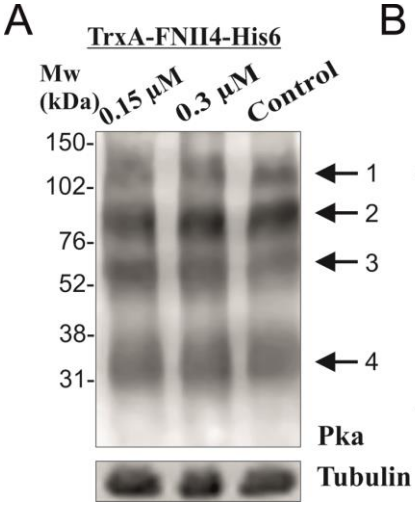


Fig. 4. Phosphorylated PKA substrates pattern in frozen/thawed ram sperm; A. Phosphorylation was detected by western blot using an antibody anti-PKA on total proteins obtained after 60 min incubation of frozen/thawed ram sperm with 0.15 µM (Lane 1), 0.3 µM TrxA-FNIIx4-His_6 (Lane 2) and contrasted with control without protein (Lane 3); Experiment was performed three times and a representative western blot is included; B. Quantification of signal intensity detected for each protein band (1-4) was normalized to the corresponding tubulin densities; Mean values ± SEM of each phosphorylation signal band (area x intensity); For each protein band, different superscripts letters indicate differences between treatments

A



B

Protein band	TrxA-FNIIx4-His6xtreatment		Control
	0.15 µM	0.3 µM	
1	0.173 ± 0006[a]	0.176 ±0.006[a]	0.209 ± 0007[b]
2	0.227 ± 0008[a]	0.240 ± 0008[a]	0.279 ± 0010[b]
3	0.236 ± 0010[a]	0.241 ± 0010[a]	0.287 ± 0012[b]
4	0.162 ± 0006[a]	0.159 ±0006[a]	0.200 ± 0007[b]

Fig. 5. Motility analysis on frozen/thawed ram sperm treated with TrxA-FNIIx4-His_6; Thawed spermatozoa were incubated with or without 0.15 µM, 0.3 µM of TrxA-FNIIx4-His_6 at 37 °C in PBS for 30, 60, 90 and 120 min; Values for kinetic variables VSL (A), VCL (B), VAP (C), ALH (D), BCF (E), LIN (F) and STR (G) were determined using a CASA system; *Value differences compared to control samples (P< 0.05); VAP: Average Path Velocity, VSL: Straight-Line Velocity, VCL: Curvilinear Velocity, ALH: Amplitude of Lateral Head Displacement, BCF: Beat Cross Frequency, LIN: linearity coefficient, STR: straightness coefficient; Four fields containing among 700 and 2000 cells were analyzed for each treatment, time and replicate (n = 3)

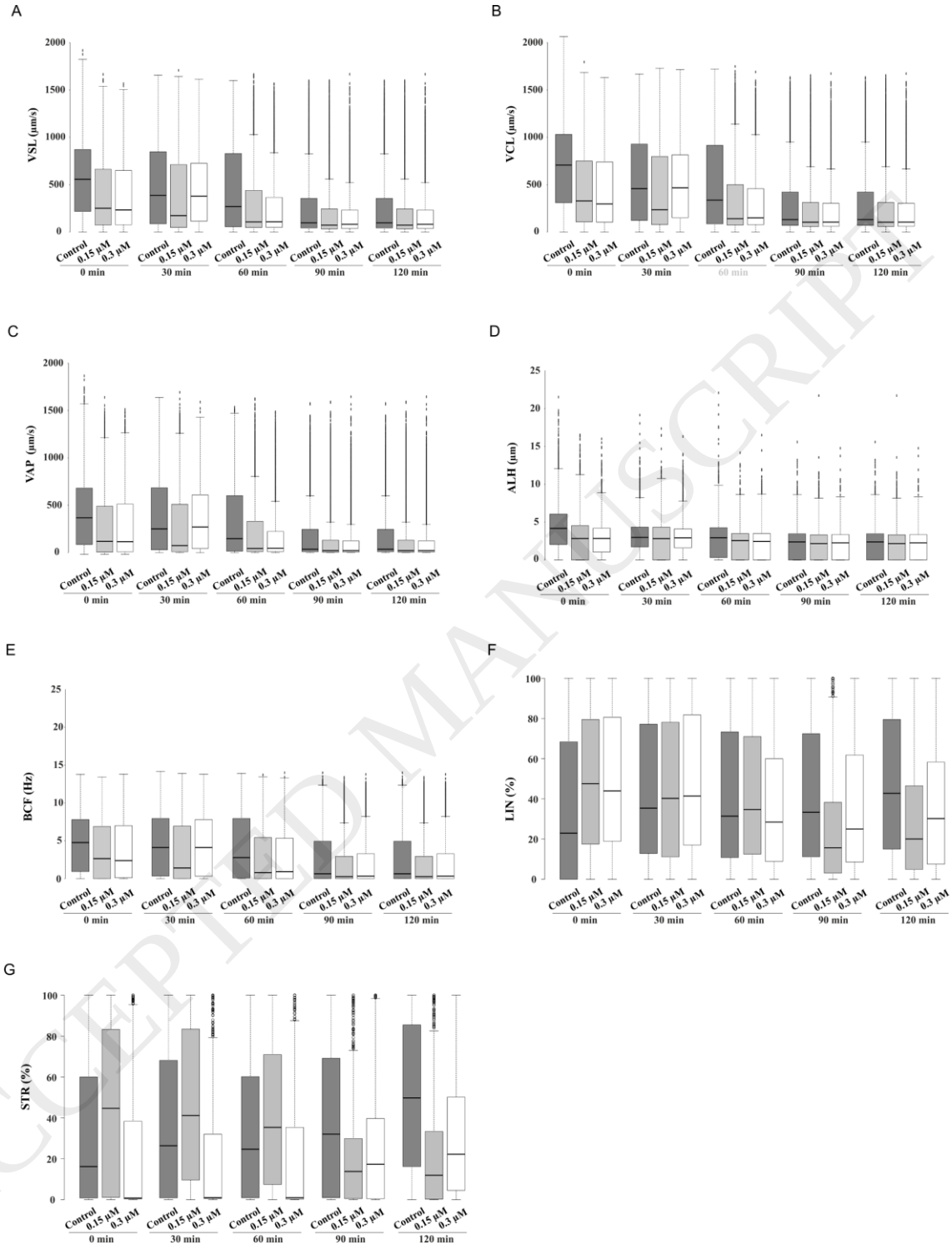


Fig. 6. Heterologous *in vitro* fertilization rate of frozen/thawed ram sperm treated with 0.15 μM or 0.3 μM TrxA-FNIIx4-His$_6$; Fertilization rate was assessed by the presence

of cleaved oocytes (two to eight cells) and/or the presence of two or more nuclei after 40 h insemination; Mean values ± SEM; Different letters indicate differences between treatments (*n* = 3)

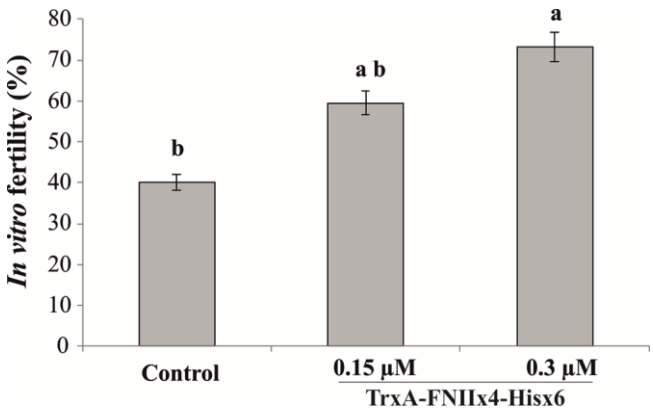


Table 1

Effect of TrxA-FNIIx4-His_6 (0.15 µM or 0.3 µM) on total and progressive motility of thawed ram sperm after 30, 60, 90 and 120 min of incubation at 37 ºC

Treatment	Incubation time (min)	Total motility (%)	Progressive motility (%)
0.15 µM	0	95.9 ± 0.1	36.7 ± 2.4
	30	94.7 ± 1.0	30.0 ± 4.8
	60	94.6 ± 1.2	26.2 ± 3.0
	90	92.1 ± 4.3	18.6 ± 7.8
	120	91.7 ± 0.9	16.0 ± 0.5
0.3 µM	0	95.8 ± 1.0	36.6 ± 3.9
	30	96.4 ± 1.9	38.0 ± 5.1
	60	94.1 ± 1.3	24.8 ± 1.6
	90	93.1 ± 1.5	21.2 ± 4.5
	120	95.2 ± 1.4	15.0 ± 6.2
Control	0	93.8 ± 4.9	31.3 ± 9.7
	30	95.8 ± 1.3	37.5 ± 8.7
	60	95.1± 2.7	31.8 ± 2.0
	90	92.4 ± 1.3	25.4 ± 3.5
	120	94.2 ± 1.3	26.0 ± 5.9

Data represent means ± SEM: values for variables were determined using a CASA system; *Value different compared to control samples (P< 0.05); Four fields containing 700 and 2000 cells were analyzed for each treatment, time and replicate (n = 3)