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The Prion-like Protein Doppel Enhances Ovine Spermatozoa Fertilizing Ability

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Contents

The function of prion-like protein Doppel was suggested to be related to male fertility. In this study, the importance of ovine Doppel polypeptide on spermatozoa capacitation and fertilization was evaluated. After refolding, recombinant Doppel (rDpl) was supplemented with different concentrations (40, 80 or 190 ng/ml) to ovine spermatozoa during the capacitation process. In experiment 1, post-thawed ovine spermatozoa were incubated with different concentrations of rDpl during 1 h for swim-up, and changes in sperm motility, concentration, vigour, viability and capacitation were monitored (10 replicates). In experiment 2, the fertilization ability of post-swim-up spermatozoa incubated as above was tested through heterologous fertilization of bovine *in vitro* matured oocytes (n = 423, three replicates). Regardless of dosage, rDpl improved ($p \leq 0.03$) spermatozoa viability. Sperm individual motility and vigour were the highest ($p \leq 0.04$) for the group receiving 190 ng/ml rDpl. Sperm supplemented with the highest doses of rDpl achieved higher ($p = 0.02$) fertilization rates (56.0 ± 3.0%) than control (39.1 ± 2.2%) and 40 ng/ml rDpl (39.8 ± 2.7%). Preliminary data suggest that Doppel protein may enhance *in vitro* spermatozoa fertilizing ability.

Introduction

Prions are infectious pathogens that cause a group of invariably fatal neurodegenerative diseases in both humans and animals. A hallmark of prion diseases is the conversion of the cellular prion protein (PrP^C), expressed by the prion protein gene (PRNP), into an abnormally folded isoform, designated as PrP^{Sc} (prion protein associated with scrapie) in ovine, which is the major component of infectious prions (Prusiner et al. 1998). Spontaneous cerebellar neurodegeneration and ataxia syndromes in certain strains of $PRNP^{0/0}$ mice led to the discovery of a novel gene (PRND – prion-like protein Doppel gene), which encodes a prion-like protein designated as Doppel (Moore et al. 1999), also named Dpl. This ataxic phenotype is linked to the overexpression of PRND and is corrected after introducing a wild-type transgene in $PRNP^{0/0}$ mice (Moore et al. 2001), suggesting a direct or indirect interaction between PRND and PRNP. However, PRND does not seem to be required for prion disease progression or for the generation of the PrP^{Sc} isoform (Behrens et al. 2001; Eghiaian et al. 2004).

Under physiological conditions, PRND is poorly expressed in the brain. However, it is highly expressed in the male reproductive tract (Moore et al. 1999; Silverman et al. 2000; Peoc'h et al. 2002). Like PrP^C, Doppel is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein (structured by three α-helices and two β-sheets), although it has only a 25% amino acid similarity to PrP^C and lacks the distinctive PrP^C repeats and the hydrophobic domain (Silverman et al. 2000). In ovine testis, Doppel protein appears to carry two N-glycans but apparently lacks O-glycans (Espenes et al. 2006). After being synthesized in the endoplasmic reticulum, the Doppel polypeptide is processed at its C- and N- terminus and is then exposed to the cell membrane (Uelhoff et al. 2005). However, observations in cells and tissues suggest that Doppel may also exist as an intracellular form devoid of the GPI anchor (Peoc'h et al. 2002; Cordier-Dirikoc et al. 2008). The existence of different Doppel forms either associated with the cell membrane via a GPI anchor or in the intracellular or extracellular spaces was proposed by Peoc'h and Laplanche (2006). Thus, a soluble form of Doppel protein was identified in human seminal fluid while it also presented a transient location within the acrosomal granule/matrix of round spermatids (Peoc'h et al. 2002). In addition, under certain conditions of cell trafficking, PrP^C retains an uncleaved signal peptide, which may also hold for the Doppel polypeptide. This N-terminal α-helical structured region found in unprocessed Doppel proteins could promote the formation of oligomeric aggregates and establish a transmembrane pore (Papadopoulos et al. 2006). Regarding human Doppel protein, a hydrophobic cleft, surrounded by charged residues and evolutionarily conserved, was recognized and might represent a binding site for an unidentified but functionally important factor (Lührs et al. 2003).

Serres et al. (2006) hypothesized that Doppel could be acquired during the passage of maturing spermatozoa through the epididymis, as it has been described for other GPI proteins anchored to spermatozoa, thus suggesting a possible epididymal origin of Doppel. In sheep, Espenes et al. (2006) detected the transient presence of Doppel in the final stages of spermiogenesis which points to an important role of this protein in the final remodelling of spermatids prior to their release into the seminiferous lumen. However, theses authors were unable to identify the Doppel protein on ovine spermatozoa from ejaculated sperm, which may suggest an expression level smaller than the detection limit of the methods and antibodies used. A possible association between PRND gene polymorphisms and ram semen traits/freezability and fertility was recently suggested by Baptista et al. (2008) and Pereira et al. (2009). Nonetheless, the precise role of Doppel in male fertility remains unclear. Sperm from $PRND^{0/0}$ mice appear to be unable to undergo the normal acrosome reaction necessary to penetrate the *zona pellucida* of the oocyte.

Therefore, $PRND^{0/0}$ mice were sterile, although presenting normal sexual behaviour with reduced or normal sperm concentrations, while $PRND^{0/0}$ females were viable and fertile (Behrens et al. 2002; Paisley et al. 2004). The aim of this work was to identify Doppel function on spermatozoa capacitation and on the consequent fertilization process. Thus, recombinant Doppel protein expressed in *Escherichia coli* was used to supplement ovine spermatozoa during the capacitation process allowing studying spermatozoa fertilizing ability.

Material and Methods

Cloning, expression and purification of ovine Doppel protein

The cDNA fragment encoding the entire mature ovine Doppel protein (Genebank™: AF394223) was synthesized (Geneart, http://www.geneart.com) with a codon utilization optimized for *Escherichia coli* expression and cloned into pGA18 vector using *Kpn*I and *Sac*I restriction sites and named pGA18-Dpl.

cDNA corresponding to the mature Dpl protein (amino acids 27–153) was amplified from the pGA18-Dpl vector, using forward and reverse primers containing a *Nhe*I and a *Xho*I restriction sites, respectively, which were used to clone the nucleic acid into the prokaryotic expression vector pET-21a (Novagen, Darmstadt, Germany), generating pET21aDplA. Recombinant Doppel protein contained a C-terminal His tag and was termed rDpl. Aiming to improve the solubility of the rDpl, the DNA fragment encoding the T7 promoter fused with the 'thioredoxin' (trx) coding sequence, that had been previously amplified by our group from the pET32a expression vector (Novagen) using forward and reverse primers containing a *Bam*HI and a *Nhe*I restriction sites respectively, and sub-cloned into the pET28a vector (Novagen), was isolated from pET28a, in *Bam*HI and *Nhe*I restriction sites, and cloned into the pET21aDplA vector *Bgl*II and *Nhe*I restriction sites, respectively, to generate pET21aDplB. *Bam*HI and *Bgl*II generate different-but-compatible ends with the same GATC tetranucleotide sequence. With pET21aDplB plasmid, Dpl protein is expressed as a linear chimera with the trx tag (named TRX-rDPL) following induction with isopropyl-β-D-thiogalactopyranoside (IPTG).

The plasmids employed in this work were used to transform *E. coli* Origami (DE3) strains (Stratagene, La Jolla, CA, USA), which were cultured in *Luria Bertani* medium supplemented with 100 μg/ml of ampicillin. The bacterial cells were grown at 37°C to mid-exponential phase (A600 0.6), and expression of the recombinant genes was induced by the addition of IPTG to a final concentration of 1 mM. Cultures were further incubated for 4 h at 37°C. Recombinant Doppel was expressed by the bacterial cells in the form of inclusion bodies. To purify and refold rDpl, cells were harvested by centrifugation ($8000 \times g$, 4°C for 10 min), and pellets were resuspended in sodium HEPES buffer, pH 8.0, containing 50 mM HEPES, 1 M NaCl, 5 mM $CaCl_2$, 1 mM ß-mercaptoethanol and 10 mM imidazole (Buffer A). Cells were disrupted by sonication (Bandelin Sonoplus HD2070; GmbH e Co. KG, Buchen, Germany), and the inclusion bodies were collected by centrifugation and resuspended in Buffer A containing 2 M urea. After sonication and centrifugation, the debris pellet was resuspended in Buffer A containing 6 M urea, sonicated and stirred overnight at 4°C. The mixture was pelleted by centrifugation ($13\,000 \times g$, 4°C for 30 min), and the supernatant was filtered through a 0.45-μm filter before loading into a 5 ml HiTrap Chelating HP column (GE Healthcare, Pittsburgh, PA, USA) equilibrated in buffer A, containing 6 M urea. After washing with Buffer A containing 6 M urea, rDpl was refolded by gradually removing the urea through the application of a linear gradient into Buffer A. Recombinant Doppel was eluted in Buffer A, containing 300 mM imidazole. Protein purity was assessed by SDS-PAGE. Recombinant Doppel was buffer-exchanged to phosphate-buffered saline (Gibco, Invitrogen, Carlsbad, USA), by gel filtration using PD-10 (GE Healthcare) columns.

Semen collection and cryopreservation

Semen collection was conducted at the experimental farm of INRB in compliance with the requirements of the European Union for farm animals welfare and the Portuguese authority guidelines for animal experimentation.

Semen was collected in autumn from a native Portuguese Merino ram already identified by its good *in vivo* and *in vitro* fertility results, using an artificial vagina. Each ejaculate was immediately evaluated for volume, motility and concentration. Good quality ejaculates (mass motility >4; individual motility $>60\%$; concentration $>2.5 \times 10^9$ spz/ml) were diluted using an extender containing a solution of 45.0 g/l TRIS, 24.4 g/l citric acid, 5.6 g/l glucose, 15% egg yolk (v/v), 6.6% glycerol (v/v) and antibiotics. The diluted semen was loaded into 0.25-ml mini-straws (300×10^6 spz) and refrigerated to 4°C. Afterwards semen was placed in liquid nitrogen (LN2) vapours during 25 min and then submersed and kept in a LN2 container (Valente et al. 2010).

Preparation and evaluation of thawed semen

After thawing, sperm motility was immediately examined by the same experienced observer. Thawed semen was then incubated at 38.5°C in a 5% CO_2 atmosphere in capacitation medium (modified Bracket's medium containing 20% ovine serum) in the absence and presence of different concentrations of purified rDpl (40, 80 or 190 ng/ml) for swim-up. Volumes of 40 μl of the thawed semen were layered under 1 ml of capacitation medium in glass tubes (two for each treatment), and the motile spermatozoa were allowed to swim-up into the medium during an incubation period of 1 h. The upper regions of the overlays were harvested and pooled for each medium used. After centrifugation at $225 \times g$ for 5 min, the supernatant was rejected and the remaining pellet of spermatozoa was evaluated (Pereira et al. 2009).

Spermatozoa motility, morphological parameters and capacitation status evaluation

Post-swim-up sperm individual motility (percentage of progressively motile spermatozoa) and vigour (scale 0–5) were subjectively determined by visual estimation using a phase contrast light microscope (Olympus BX41, Tokyo, Japan). In addition, spermatozoa viability (nigrosin–eosin staining) and concentration, calculated in duplicate using a Neubauer's chamber, were also measured. The capacitation status of post-swim-up spermatozoa was assessed by measuring the binding pattern of chlortetracycline (CTC) staining in spermatozoa membranes using a modified technique described by Perez et al. (1996). Briefly, aliquots (5 μl) of motile spermatozoa were mixed with 5 μl of CTC solution (0.4 g/l CTC-HCl, 0.9 g/l cysteine in 5 ml of 2.4 g/l Tris and 7.6 g/l NaCl solution), 1 μl of 12.5% glutaraldehyde solution and 1 μl of DABCO (Merck, Darmstadt, Germany) on microscope slides before a coverslip was placed over each sample. Slides were observed under fluorescence within 12 h. At least 100 spermatozoa were scored for each slide, and three fluorescent staining patterns were identified according to the acrosomal status of spermatozoa: uncapacitated acrosome intact cells or 'F' pattern, bright post-acrosomal or entire head fluorescence; capacitated acrosome intact cells or 'B' pattern, bright acrosome region fluorescence; and acrosome-reacted cells or 'AR' pattern, no head fluorescence or fluorescence in the equatorial region only (Perez et al. 1996).

Heterologous *in vitro* fertilization

Bovine ovaries were collected and processed as described previously (Pereira et al. 2007). Briefly, selected cumulus-oocyte complexes were matured in M199 with Earle's salts, L-glutamine and 25 mM HEPES (Gibco). This medium was supplemented with 10% superovulated oestrous cow serum, 10 μg/ml FSH (Sigma, St. Louis, MO, USA), 100 UI/ml penicillin and 100 μg/ml streptomycin (Sigma). Maturation was accomplished in an incubator at 38.5°C with humidified atmosphere in air containing 5% CO_2 for 22–24 h. Bovine matured oocytes were denuded and inseminated with swim-up ovine spermatozoa as described by Valente et al. (2010). Twenty hours after insemination, presumptive zygotes were washed in a 1% sodium citrate solution and fixed in ethanol : acetic acid (3 : 1) prior to staining with 1% aceto-lacmoid. Oocytes were considered fertilized if a decondensed sperm head, two pronuclei or sincariosis were observed under a light microscope. Polyspermy was defined by the presence of more than two swollen sperm heads, or more than two pronuclei within a single oocyte.

Experimental design

These experiments were designed to understand the importance of the Doppel protein during the capacitation of ovine spermatozoa and the consequent fertilization processes. As both rDpl (from pET21aDplA) and TRX-rDpl (from pET21aDplB) were expressed in the form of insoluble inclusion bodies, implying the latter an additional step to remove thioredoxin from the fusion partner, only refolded and purified rDpl was supplemented with different concentrations to ovine spermatozoa during the capacitation process in two experiments. In experiment 1, post-thawed ovine spermatozoa were incubated in the absence and presence of different concentrations of purified rDpl (40, 80 or 190 ng/ml) during 1 h for swim-up, and changes in sperm motility, concentration, vigour, viability and capacitation were monitored. Different ejaculates collected and frozen during the breeding season from the same Merino ram were used in a total of 10 replicates. In each session, a pool of semen from two straws collected and frozen in the same day was used. In experiment 2, ovine spermatozoa were incubated in the absence and presence of different concentrations of rDpl (40, 80 or 190 ng/ml) for swim-up as described earlier, and their fertilization ability was tested. Heterologous fertilization was performed using bovine matured oocytes (n = 423) and frozen-thawed semen from different ejaculates in three replicates (105–180 oocytes/ejaculate). The percentage of fertilized oocytes [(decondensed sperm head, two pronuclei, sincariosis and polyspermic oocytes)/total matured oocytes] and of each fertilization stage (decondensed sperm head, two pronuclei, sincariosis or polyspermic oocytes)/fertilized oocytes) were determined.

Statistical analysis

All results were expressed as mean value ± standard error of mean. Data representing 10 replicates of spermatozoa motility, morphological parameters and capacitation status as well as three replicates for heterologous fertilization rates analysis were performed by analysis of variance using a MIXED procedure. The mixed linear model included rDpl doses treatment as fixed effect and replicates as random effect. When significant effects were identified, group means were compared by the least significant difference. Pearson's coefficient of correlation and regression equations were calculated to determine the relationship between variables and rDpl increment. Mann–Whitney *U* test was used to compare heterologous fertilization stages among groups (Statsoft Inc, 2000). Differences were considered significant when $p \leq 0.05$.

Results

Production of ovine Doppel protein

Recombinant Doppel protein, here termed rDpl, was purified and refolded from inclusion bodies, after expression in *E. coli* Origami (DE3) transformed with pET21aDplA and pET21aDplB. The molecular mass of the recombinant proteins was in accordance with the expected molecular weight deduced from the protein primary sequences, and the proteins were considered to be more than 95% pure (Fig. 1). The fusion of the Doppel protein to the thioredoxin sequence had no impact on the overall solubility of the protein *in vivo,* which led us to select the recombinant Doppel protein

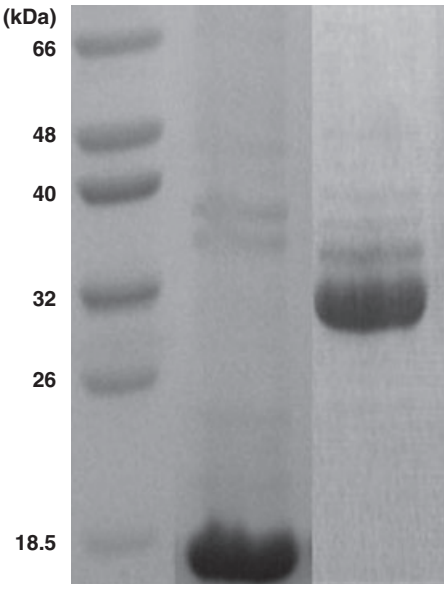

Fig. 1. SDS-PAGE (14%) Doppel (rDpl) protein expressed individually (first lane) or in fusion with the thioredoxin protein coding sequence (second lane). Both proteins were obtained by purification from inclusion bodies. The molecular masses (kDa) of protein standards (NZYTech Ltd., Lisbon, Portugal) are indicated

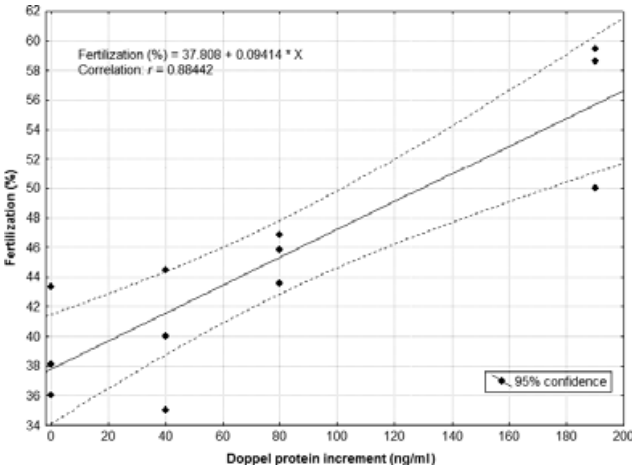

Fig. 2. Linear regression between purified mature ovine Doppel protein (rDpl) increment during spermatozoa capacitation process and fertilization rate

derived from the expression vector pET21aDplA for the experiments described below.

Doppel effect on spermatozoa motility, morphological parameters, capacitation and fertilizing ability

In experiment 1, sperm individual motility and vigour were higher after swim-up in the presence of 190 ng/ml rDpl than with 80 ng/ml ($p = 0.002$ and $p = 0.04$, respectively), 40 ng/ml ($p = 0.0009$ and $p = 0.04$, respectively) or control ($p < 0.0001$ and $p = 0.009$, respectively) (Table 1). Spermatozoa viability was improved ($p \leq 0.03$) by Doppel protein regardless of dosage while the concentration and the capacitation status were not significantly ($p > 0.05$) changed (Table 1). In experiment 2, a total of 423 bovine matured oocytes were used to assess the fertilizing ability of ovine spermatozoa incubated in the presence and absence of different rDpl concentrations. Fertilization rate was higher after spermatozoa incubation with 190 ng/ml rDpl during the swim-up than with 40 ng/ml rDpl ($p = 0.02$) or control ($p = 0.02$) (Table 2). Moreover, a positive correlation ($r = 0.884$, $p < 0.001$) between rDpl increments during capacitation and fertilization rates was identified (Fig. 2). Nevertheless, no differences ($p > 0.05$) in the stages of heterologous fertilization were found among groups.

Table 1. Effect of different concentrations of ovine Doppel protein on spermatozoa morphological parameters and capacitation status (10 replicates)

	Morphological parameters				Capacitation status		
Groups	Motility (%)	Vigour (1–5)	Viability (%)	Conc. (10^6/ml)	NCap (%)	Cap (%)	AR (%)
Control	50.0 ± 3.3[a]	4.5 ± 0.1[a]	34.1 ± 1.4[a]	37.3 ± 8.3	32.0 ± 3.3	47.5 ± 2.4	20.5 ± 2.9
40 rDpl	53.5 ± 3.5[a]	4.5 ± 0.1[a]	42.1 ± 2.9[b]	25.9 ± 3.8	32.2 ± 4.5	45.5 ± 3.2	23.2 ± 3.3
80 rDpl	54.0 ± 2.1[a]	4.6 ± 0.1[a]	40.7 ± 2.2[b]	23.4 ± 3.6	31.0 ± 3.7	50.8 ± 2.8	18.2 ± 2.1
190 rDpl	62.0 ± 2.6[b]	4.9 ± 0.1[b]	44.6 ± 2.5[b]	33.1 ± 7.1	27.8 ± 2.6	53.5 ± 2.2	18.7 ± 2.9

Motility, individual spermatozoa motility; conc., spermatozoa concentration; NCap, non-capacitated spermatozoa; Cap, capacitated spermatozoa with intact acrosome; AR, acrosome-reacted spermatozoa.
Data within columns with different superscripts are statistically different ($p \leq 0.05$); 40 rDpl, 80 rDpl and 190 rDpl = 40, 80 and 190 ng/ml of mature ovine Doppel protein (rDpl) in the capacitation medium, respectively.

Table 2. Effect of different concentrations of ovine Doppel protein on the spermatozoa fertilizing ability (n = 423, three replicates)

Groups	Fertilization (%)	Decondensed spz (%)	Pronucleus (%)	Sincariosis (%)	Polyspermy (%)
Control	39.1 ± 2.2[a]	43.2 ± 13.6	34.1 ± 16.6	22.8 ± 8.1	0.0 ± 0.0
40 rDpl	39.8 ± 2.7[a]	32.2 ± 9.7	44.8 ± 2.6	20.2 ± 7.6	2.8 ± 2.8
80 rDpl	45.4 ± 1.0[ab]	51.1 ± 5.7	20.8 ± 2.9	20.8 ± 2.9	7.2 ± 1.0
190 rDpl	56.0 ± 3.0[b]	33.9 ± 5.4	31.2 ± 12.2	31.5 ± 12.0	3.5 ± 1.8

Data within columns with different superscripts are statistically different ($p \leq 0.05$); 40 rDpl, 80 rDpl and 190 rDpl = 40, 80 and 190 ng/ml of mature ovine Doppel protein (rDpl) in the capacitation medium, respectively.

Discussion

To our knowledge, this is the first time that the role of ovine Doppel protein in improving the fertilizing ability of ram spermatozoa is reported. Data presented here suggest that sperm supplementation with 190 ng/ml of rDpl during *in vitro* capacitation significantly improves spermatozoa motility, vigour, viability and fertilization rate. This observation suggests an important function for Doppel during ovine sperm capacitation and also in the consequent fertilization process. Furthermore, during the capacitation process, ovine spermatozoa were supplemented with different concentrations (40, 80 and 190 ng/ml) of Doppel protein, and the enhanced spermatozoa viability was achieved regardless of dosage.

Doppel function in male fertility is not clearly defined. In mice, Behrens et al. (2002) found that in the absence of Doppel, spermatids were malformed and showed a defect in acrosome function which resulted in the inability to achieve oocyte fertilization. Moreover, Paisley et al. (2004) found that $PRND^{0/0}$ mice spermatozoa, although presenting normal morphological characteristics, only rarely fertilized oocytes *in vivo* because of an inability to perform the acrosome reaction. In addition, $PRND^{0/0}$ and also $PRNP^{0/0}/PRND^{0/0}$ sperm are capable of fertilizing oocytes *in vitro*, albeit at reduced rates, but unable to support the development of the generated embryos beyond the morula stage. Elevated levels of oxidative spermatozoa DNA damage were found in both mutants. Mammalian sperm capacitation is a maturation step that enables sperm to achieve an acrosome reaction and penetrate the oocyte. During capacitation, several Ca^{2+}-induced membrane modifications occur, resulting in an increased motility and hyperactivation (Parrish and First 1993). Reports linking Doppel protein to the control of Ca^{2+} movements in local cell domains, such as the endoplasmic reticulum and the mitochondrial matrix or beneath the plasma membrane, have emerged (Brini et al. 2005; Cordier-Dirikoc et al. 2008). After exposure to rDpl, enhanced spermatozoa viability (all doses, $p \leq 0.03$) and motility ($p < 0.0001$), vigour ($p = 0.001$) as well as fertilization rate at the highest dose ($p \leq 0.02$) were observed. Perhaps the positive effect of Doppel protein on ovine sperm is through its ability to influence Ca^{2+} transients. Nonetheless, the ability of spermatozoa to undergo the calcium ion-induced capacitation or acrosome reaction, as identified through CTC staining, was not improved in this study.

Human Doppel protein was found on mature ejaculated spermatozoa in particular on the flagella (Peoc'h et al. 2002). These authors suggest the involvement of Doppel in the motility of spermatozoa which comes in agreement with the results presented here regarding sperm individual motility. On the other hand, the Doppel polypeptide being described as a GPI-linked membrane protein is present in unusual locations such as the acrosomal matrix (Serres et al. 2006) and seems to bind to partner(s) and induce the activation of intercellular Ca^{2+} signalling pathway in B cells. This may argue in favour of putative physiological functions of soluble forms of Doppel (Cordier-Dirikoc et al. 2008), like the one used in this study. For instance, human PrP^{C} was reported as N-terminally truncated isoforms in the human brain (Peoc'h et al. 2002), as well as C-terminally truncated isoform in ejaculated spermatozoa (Shaked et al. 1999). The N-terminal part of Doppel (that embraces the signal peptide) not present in the rDpl may, as previously mentioned, adopt a transmembrane location, suggesting a possible channel formation mechanism (Papadopoulos et al. 2006). The possibility that these transmembrane pores could help in cell–cell interactions, between Sertoli and germ cells, throughout spermatogenesis, along with other reported cell adhesion molecules should be further investigated.

Another possible explanation for the Doppel effect on ovine spermatozoa motility, vigour and viability as well as on the consequent fertilization process could be related to its action on the modulation of nitric oxide synthase enzyme (Cui et al. 2003). Hence, the synthesis of nitric oxide in spermatozoa has been associated with the enhancement of tyrosine phosphorylation of sperm proteins, an essential component of the cascade of biochemical changes leading to sperm capacitation (Aitken et al. 1995), and also in the different events of fertilization (Reyes et al. 2004).

In sheep, cervical insemination with frozen-thawed semen results in low pregnancy rates which is the primary reason for the limited use of artificial insemination in this species (Valente et al. 2010). Following either transcervical or oviductal insemination of ewes, conception rates and percentage of fertilized ova with frozen-thawed ram semen were approximately 20% less than with fresh semen (Maxwell et al. 1993). Cryopreservation initiates 'cryo-capacitation', producing a sperm subpopulation with a shortened life span *in vivo*, effectively reducing the fertilization efficiency of the population as a whole (Bailey et al. 2000). The data presented here reveal that during the capacitation process, Doppel protein, although not significantly affecting spermatozoa capacitation status as identified by CTC staining, improves ram semen post-thawed characteristics namely spermatozoa motility, vigour and viability, particularly in the highest dose. These better sperm post-thawed characteristics resulted in an increased fertilizing capacity. For a preliminary approach, heterologous fertilization was chosen to test the fertilizing ability of ram semen. Several authors reported heterologous fertilization as a useful tool for predicting the fertility of frozen-thawed ram semen either *in vivo* or *in vitro* because a high relationship was showed between heterologous IVF and *in vivo* male fertility as well as embryo production rates (Choudhry et al. 1995; Garcia-Alvarez et al. 2009; Valente et al. 2010). The enhanced fertilizing ability of frozen-thawed ram semen after exposure to rDpl suggests the possibility of using this protein to improve conception rates. Behrens et al. (2002) and Paisley et al. (2004) proposed that Doppel is involved in sperm protection from oxidative stress known to be increased during the frozen-thawed process. A possible association between PRND gene polymorphisms and ram semen traits/freezability and fertility was identified. Pereira et al. (2009) used specific functional *in vitro* assays to disclose the ability of frozen-thawed spermatozoa from PRND polymorphic rams to undergo complicated processes such as capacitation,

acrosome reaction, fertilization of oocytes and embryo development *in vitro*. In addition, preliminary data indicated that sperm from Churra Galega Mirandesa rams heterozygous for 78G > A polymorphism in codon 26 of the PRND gene is characterized by a higher viability and lower occurrence of abnormalities (Baptista et al. 2008).

In conclusion, the data presented in this report indicate that mature ovine Doppel protein may enhance *in vitro* spermatozoa fertilizing ability. Whether Doppel binds to spermatozoa or is involved in direct membrane interactions remains unclear. The clarification of the biological mechanisms involving the Doppel protein during capacitation and fertilization could be of fundamental importance to elucidate possible causes of male infertility and on the other hand to develop improved methods of sperm cryopreservation for commercial application.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

J. Pimenta, J.A.M. Prates, C.A. Fontes and R.M. Pereira were responsible for the experimental design. They also actively participated on the experimental procedures, analysis and interpretation of data, and on the preparation and revision of the manuscript. F. M.V. Dias, C.C. Marques, M.C. Baptista, M.I. Vasques, A.E.M Horta, J.P. Barbas, R. Soares, P. Mesquita and E. Cabrita participated on the experimental procedures, analysis and interpretation of data and on the critical review of the manuscript.

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