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Identification of bovine doppel protein in testis, ovary and ejaculated spermatozoa

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Abstract

Doppel (Dpl) protein is a recently identified prion-like protein. Although Dpl might be expressed in the brain after prion gene deletion, in both human and mice Dpl is normally expressed only in testis and spermatozoa, where it appears to be involved in male fertility. Little information is available so far about the expression pattern of Dpl in bovines, thus, hampering possible research on the role of this protein in bovine infertility. We have thus, designed, produced and validated through Western blotting a polyclonal antibody against bovine Dpl. With this antibody we then screened bovine tissues for Dpl expression by immunohistochemistry. Ejaculated spermatozoa were screened by flow cytometry and immunocytochemistry.

Bovine Dpl was expressed in all the developing stages of germinal cells, from spermatogones to ejaculated spermatozoa, in Sertoli cells and in ovarian follicles (granulosa cells and follicular fluid). Dpl immunoreactivity was also found on other tissues, where endothelial cells, peripheral nerves and scattered lymphocytes stained positive. This distribution pattern suggests that Dpl might be involved in sperm maturation/capacitation in bovines, like it might be in mice. This hypothesis needs to be verified by widespread application of the flow cytometric protocol established in this paper on spermatozoa from animals with reduced fertility.
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Keywords: Doppel protein; Fertility; Western blotting; Immunohistochemistry; Flow cytometry

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1. Introduction

Doppel (Dpl) is an acronym for downstream prion protein-like [1]. *Prnd* is the gene encoding for Dpl protein and is located 16–20 kb (depending on the species) downstream from *Prnp*, the gene encoding for prion proteins (PrP) [2,3]. *Prnd* contributes, together with *Prnp* and with the recently discovered *Prnt*, to the so called ''prion gene complex'', *Prn*. Dpl and PrP present a 25% sequence homology [4,5]. Nevertheless, some structural differences and the lack of Dpl overexpression in patients with PrP-induced diseases [6] suggests that Dpl is not involved in prion pathology. Dpl expression induces neurological signs only in PrP knockout mice [1,5,7,8] and except for transient expression in the brain of neonatal mice [9] *Prnd* mRNA and/or Dpl protein are not expressed in the central nervous system (CNS) of healthy animals. On the other hand, intense expression has been recorded in both testis and heart [1,7,10]. Furthermore, the localization of human Dpl on Sertoli cells and spermatozoa [10] and the lack of expression observed in cases of male sterility in mice [11] suggests that the main function of this protein is male fertility regulation. The demonstration of a similar role in cattle could assist in the study, diagnosis and prevention of bovine infertility. Studies concerning Dpl function, however, require a precise knowledge of cellular types expressing the protein. Unfortunately, Dpl distribution in bovine tissues was studied only at the mRNA level [12]: *Prnd* mRNA was mainly detected in testis, ovary and spleen. The tissue distribution of the protein and its localization within each tissue have not yet been investigated in cattle.

Aims of the present study were: to produce a specific polyclonal antibody for bovine Dpl and to validate it's specificity by Western blotting against recombinant bovine Dpl; to characterize bovine Dpl in tissues by a panel of Western blotting approaches; to establish an immunohistochemical protocol and to verify whether Dpl distribution in bovine tissues is consistent with that recorded in other species; to identify bovine Dpl in ejaculated spermatozoa by immunocytochemistry and flow cytometry techniques.

2. Materials and methods

2.1. Production and validation of a polyclonal anti-bovine Dpl antibody

Polyclonal antibody anti-bovine Dpl (boDpl67–81) was raised in rabbits following standard immunization procedures and using the synthetic peptide DIDFGVEGN-RYYEAN, corresponding to the residues 67–81 of bovine Dpl.

Recombinant bovine Dpl (rBoDpl) was produced using Dpl cDNA kindly provided by Prof. L. Ferretti (University of Pavia, Italy). After amplification, cDNA gene was cloned into the plasmid pQE30 (Qiagen SpA, Milano, Italy) via the BamH1 and KPN1 restriction enzyme sites and the plasmid was then overexpressed in *Escherichia coli* cultures. Recombinant BoDpl was then purified by chromatography, using MC-POROS column, 100 mm $\times$ 4.6 mm i.d. (Applied Biosystems, Milano, Italy), charged with Cu^{2+} ions according to manufacturer's instructions, and equilibrated in 10 mM Tris pH 7.5, 6 M Urea and 0.5 mM imidazole. rBoDpl was then eluted with 100 mM imidazole in equilibrating buffer. Urea was removed by dialysis.

Purified rBoDpl was used to test the reactivity of the polyclonal antibody boDpl67–81 by Western blotting: 500 ng of the protein were analysed onto 12% SDS–PAGE and Western blotted onto nitrocellulose membrane. Nitrocellulose blots were blocked for 1 h with 2% (w/v) milk powder in PBS at room temperature. Polyclonal antibody boDpl67–81 was used as primary antibody at a concentration of 1:1000, 1:2000, 1:5000 and 1:10,000. An alkaline phosphatase-conjugated anti-rabbit secondary antibody was used, and the blots were developed using the Amplified AP Immuno-Blot Kit (Bio-Rad Laboratories Srl, Milano, Italy).

In order to assess the specificity of the antibody, the same Western blotting procedure was performed against bovine PrP^c purified as described by Pergami et al. [13]

2.2. Sample collection

The distribution of bovine Dpl was investigated in both tissues and ejaculated spermatozoa.

Tissues samples were collected from bovine foetuses, from newborn calves and from 18 month-old cows. Foetuses were aborted at different stages of gestation (4, 6 and 8 months of gestation) due to traumatic events. Newborn calves died during parturition due to maternal dystocia. Cows were sampled during slaughtering. Gross findings recorded during necropsies (foetuses and newborn calves) or slaughtering (adults) excluded the presence of both infectious diseases and congenital malformations.

The following tissues were sampled from each animal: intestine, liver, spleen, mesenteric lymph nodes, kidney, testis or ovarium, heart, lung, diaphragm, and central nervous system (five sites were sampled: cerebellum, encephalic cortex and three sequential parts of the brain stem). Three specimens per tissue were collected: two were immediately frozen in liquid nitrogen and stored at −80 °C to be homogenised for Western blotting and to obtain 6 μm-thick cryostatic sections. The third specimen was fixed in buffered iso-osmotic 10% formalin, routinely processed, and embedded in paraffin wax to prepare 3 μm-thick microthomic sections. Cryostatic and microthomic sections were stained with haematoxilin and eosin and with immunohistochemistry procedures (see below).

Frozen bovine semen was thawed and diluted 1:1 (w/v) with Nutrient mixture F-10 Ham (HF-10, Sigma Chemical Co., St. Louis, USA) medium supplemented with 2 mM L-glutamine l-1 (Sigma). Following washes, the pellet was diluted 1:1 with HF-10 medium and sperm suspension samples were evaluated for spermatozoa motility and morphology. Samples with at least 70% progressive motility and 80% normal morphology were chosen. Samples were then diluted with HF-10 to reach a final concentration of 5×10^6/ml and cytocentrifuged in a multiwell cytocentrifuge (Neuroprobe, inc Cabin John, MD, USA).

2.3. Western blotting

Tissue Dpl expression was assessed with four different Western blotting protocols:

(A) Conventional Western blotting: 500 mg of frozen tissue were homogenized in 5 ml 20 mM Tris pH 8.0, NaCl 150 mM, 0.5% (w/v) Triton X-100, sodium deoxycholate 0.5% (w/v), one complete EDTA free mini-protease inhibitor tablet (Roche Diag-

nostic, Monza, Italy). Samples were incubated for 30 min at 4 °C, centrifuged (13,000 × g, 4 °C, 10 min) and the supernatants were made to 10 mg/ml protein concentration. Five ml were loaded onto SDS–PAGE.

(B) Western blotting after purification of microsomal membranes: One gram of each tissue was homogenized at 4 °C in 5 ml homogenisation buffer (0.32 M Sucrose, with mini complete tablets proteinases inhibitors, EDTA free), using a Tissumizer. The pellet was rehomogenised using the same procedure, and the supernatants were ultra-centrifuged (100,000 × g, 60 min, 4 °C) on a 0.85 M Sucrose cushion. The pellets containing the microsomal membranes were resuspended in Tris 10 mM, pH 7.5, 1% (w/v) Zwitterion 3–12, with mini EDTA-free complete tablet protease inhibitors, and proteins were released by gently stirring at room temperature for 30 min. The last step was a final high-speed centrifugation (100,000 × g, 60 min, 10 °C), then the supernatants were made to 10 mg/ml protein concentration, and 5 ml were loaded onto SDS–PAGE.

(C) Immunoprecipitation: the immunoprecipitation of Dpl was carried out as proposed by Shaked [14] using boDpl67–81 as primary antibody and starting from 500 mg of tissues. The bound material was eluted off the antibody beads by incubating them for 10 min at 100 °C with sample buffer.

(D) Endoglycosidase digestion of tissue homogenates: 50 μg aliquots of tissue homogenate were digested with peptide N-glycosidase F (PNGaseF) as described by Peoc'h et al. [10]. All samples were incubated at 37 °C for 2 h.

Fifty microgram of each sample were directly analysed onto 12% SDS–PAGE and Western blotted onto nitrocellulose membrane. Nitrocellulose blots were blocked for 1 h with 2% (w/v) ECL Blocking Reagent (Amersham Biosciences) in PBS at room temperature. Polyclonal antibody boDpl67–81 was used as primary antibody at a concentration of 1:5000 in 2% (w/v) ECL blocking reagent (Amersham Bioscences) at room temperature for 1 h. Blots were visualized using Advanced ECL (Amersham Biosciences).

2.4. *Immunohistochemistry*

We firstly assessed boDpl67–81 reactivity on cryostatic sections in order to avoid possible antigen masking due to formalin fixation. Immunohistochemistry was performed using the Avidin–Biotin-Complex (ABC) technique with a commercially available kit (Vectastain Elite, Vector Laboratories Inc. CA, USA). Endogenous peroxidase activity was inhibited placing 30 min the slides in Tris buffer, pH 7:6 added with H_2O_2 (0.3%) and 0.1% sodium azide. Blocking solution (Tris-buffered solution, added with 2% (v/v) normal goat serum and 2%, w/v milk powder in) was then applied (30 min at room temperature) on the slides. The blocking solution was also used to prepare serial dilutions (1:20; 1:50; 1:100; 1: 200; 1:500; 1:800; 1:1000; 1:1500) of primary antibody boDpl67–81 (incubation timing: 1 h at 37 °C in humid box). After three washes in 100 mM Tris, biotinylated anti-rabbit antibody was applied on the slides (30 min at room temperature), then, after three washes in Tris, the sections were covered with the ABC complex (30 min at room temperature). Diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories Inc) or 3-amino-9-ethyl-carbazole (AEC, Vector Laboratories Inc.) served alternatively as chromogen for the

reaction. Reaction was blocked by washing in running tap water and the slides were counterstained with Mayer's haematoxylin. Coverslip was applied on DAB-stained slides, after dehydratation, using non-aqueous mounting medium (Eukitt, Kindler, Freiburg, Germany): Kaiser's glycerine (Sigma) was used to coverslip slides stained with AEC. Histological sections of each sample were used as negative controls, substituting primary antibody with non-immune rabbit IgG serum (DAKO A/S, Glostrup, Denmark). In order to verify the specificity of boDpl67–81 antibody, some sections were also stained by using as a primary antibody, boDpl67–81 pre-incubated with recombinant boDpl and with the immunogenic peptide Dpl 67–81.

Once the results on cryostatic sections furnished preliminary data on the distribution of Dpl positive cells, the ABC technique previously described was also applied on formalin-fixed and paraffin embedded sections, using boDpl67–81 at the final dilution of 1:800, after deparaffination, inhibition of endogenous peroxidases (H_2O_2, 0.3% in methanol), rehydration. The following antigen unmasking protocols were applied:

- no unmasking.
- microwave pre-treatment (two cycles of 5 min in citrate-buffer solution, 10 mM, pH 6) [15].
- pepsin digestion with a commercially available kit (Digest All 3, Zymed Laboratories Inc., San Francisco, CA, USA): the solution was applied on the slides for 6 min at 37 °C.
- SDS pre-treatment, slightly modifying the protocol suggested by Brown et al. [16]: 0.1% (w/v) SDS was applied on rehydrated sections for 3 min at room temperature.

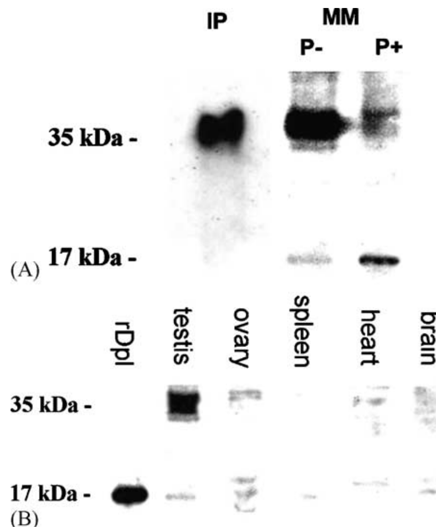
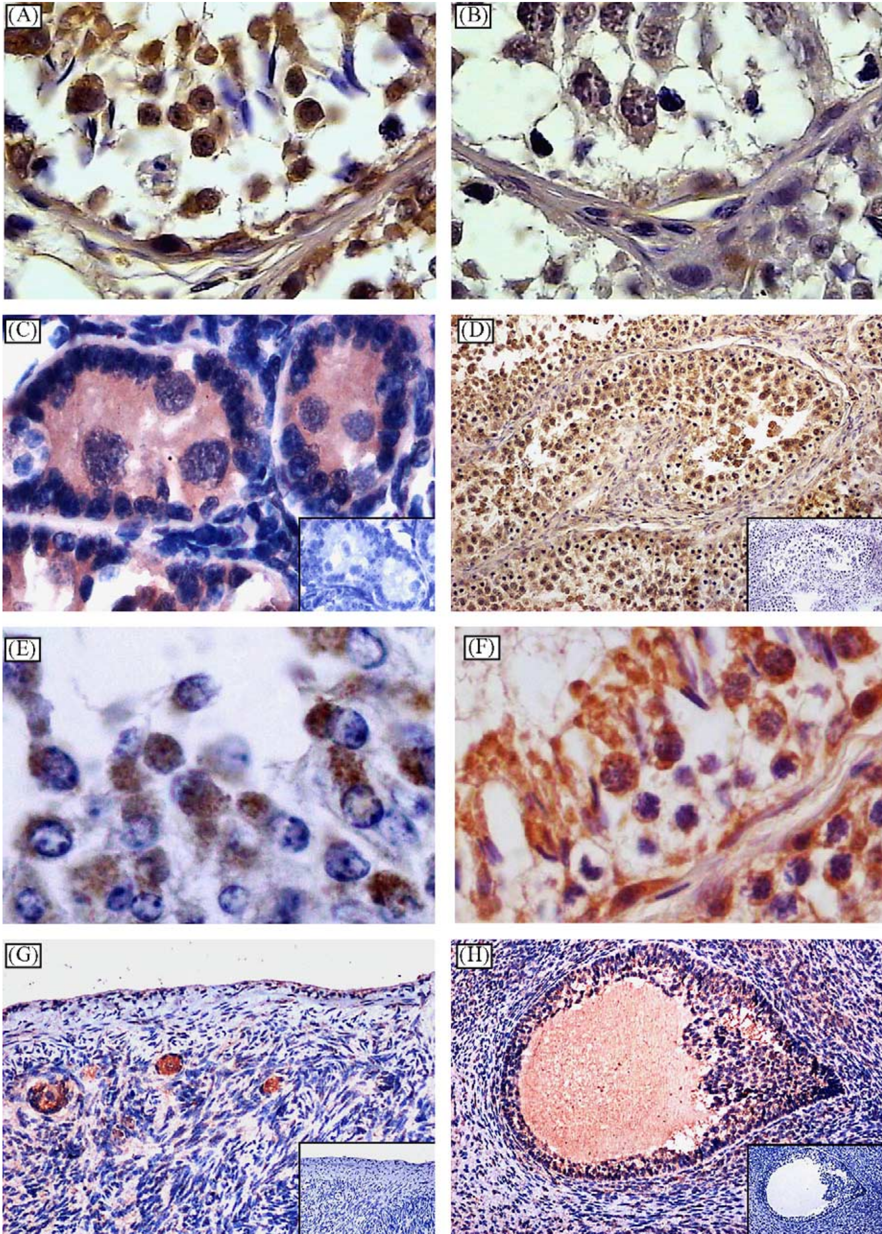


Fig. 1. Dpl protein expression in tissue homogeneates by Western blotting. A: immunoprecipitation (lane 1, IP) of Dpl protein using polyclonal boDpl67–81: an heterodisperse band with an apparent MW ranging from 34 to 38 kDa is detectable. A similar band was detected by Western blotting on untreated microsomal membranes (lane 2, P−); after deglycosylation with PNGaseF (lane 3, P+), the positive signal corresponds to a protein with a MW similar to that of recombinant bovine Dpl (about 17 kDa). B: an heterodisperse band similar to that evidenced by immunoprecipitation expreiments was detectable only in testis homogeneates (rDpl: recombinant boDpl).

2.5. Flow cytometry

Spermatozoa were respectively incubated with the boDpl67–81 or with a non-immune rabbit IgG (DAKO), as a control, for 20 min at 4 °C. Then they were washed twice in RPMI



+ FCS + sodium azide, resuspended and incubated with the secondary antibody (phycoerythrin conjugated anti-rabbit IgG; Serotec Ltd., Oxford, UK) for 20 min at 4 °C.

Fluorescence was measured in a minimum of 10,000 cells using a FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analysed using CELLQuest software (Becton Dickinson).

3. Results

3.1. Characterization of the boDpl67–81 antibody by Western blotting

The antibody produced specifically recognised bovine rBoDpl, but not purified bovine PrP^c in Western blotting tests (data not shown).

3.2. Characterization of bovine Dpl by Western blotting

Bovine Dpl was detected by Western blotting only in testis (Fig. 1A). Specifically, the positive signal was clearly detectable as an heterodisperse band with an apparent MW ranging from 34 to 38 kDa only after previous enrichment for microsomal membranes and immunoprecipitation (Fig. 1B).

It has been reported that digestion with the endoglycosidase PNGaseF [10,14] reduces the original heterodisperse signal of Dpl to a single band that runs at 17 kDa. This also occurred in our case (Fig. 1). The prolonged incubation with PNGaseF did not reduce bovine Dpl beyond 17 kDa, thus indicating that two *N*-linked glycosylation sites are present.

3.3. Immunohistochemical localization of Dpl in bovine tissues

Immunohistochemical protocol was set up on cryostatic sections of testis. Bovine Dpl was then labeled in formalin-fixed, paraffin embedded sections only after potent unmasking techniques, such as pepsin digestion and SDS treatment. As a consequence, the background staining intensity increased. In order to assess whether the background was due to the unmasking protocol or to a poor specificity of the antibody, we pre-incubated boDpl67–81 with the rBoDpl and with the immunogenic peptide. The positive signal disappeared in sections stained with the pre-incubated antibodies (Fig. 2A and 2B) confirming the specificity of boDpl67–81.

Fig. 2. boDpl distribution in gonads by immunohistochemistry using the polyclonal antibody boDpl67–81. Bovine Dpl staining was often associated to an intense background (A) but sections stained with boDpl67–81 pre-incubated with recombinant bovine Dpl stained negative (B); thus, confirming the specificity of boDpl67–81 antibody. Positive cells were mainly detected in fetal spermatogones; (C) and in the large majority of the cells within testicular tubuli from adults (D): specifically, spermatocytes (E); spermatides and Sertoli cells; (F) stained positive for bovine Dpl. In the ovary, granulosa cells stained positive in both young (G) and mature (H) follicles. Follicular fluid was also diffusely and weakly positive. Serial sections stained with isotype matched irrelevant antibody (see boxes in C, D, G and H) did not show any positive signal. Immunohistochemistry, ABC technique, pepsin digestion. Chromogens: DAB (A, B, D, E, F) or AEC (C, G, H); Haematoxylin counterstained, 100× (D, G, H), 400× (A, B, E, F) or 1000× (C). Boxes in C, D, G, H represent the negative controls (isotype matched irrelevant antibody).

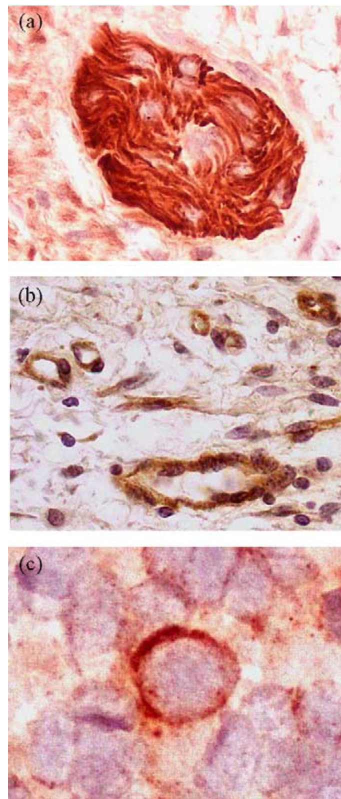


Fig. 3. boDpl distribution in tissues other than gonads. Bovine Dpl was detected on peripheral nerves (a), endothelial cells (b) and scattered lymphocytes (c). Immunohistochemistry, ABC technique with pepsin digestion. Chromogens: DAB (b) or AEC (a, c); haematoxylin counterstained, 1000×.

Positive cells were abundant in sections from testis (Fig. 2C–2F). Specifically, in foetuses, spermatogones had a diffuse positivity (Fig. 2C). In newborn animals and in adult bovines, all the cells within the tubules stained positive (Fig. 2D): spermatocytes showed granular intracytoplasmic positivity (Fig. 2E). Spermatide tails were intensely positive (Fig. 2F). A granular to diffuse positivity was occasionally detectable in Sertoli cells (Fig. 2F). In the ovary, Granulosa cells stained intensely both in young follicles (Fig. 2G) and in mature follicles, where a diffuse intrafollicular positivity was also detectable (Fig. 2H). Other than the gonads, peripheral nerves (Fig. 3a) and endothelial cells (Fig. 3b) were also intensely positive. Moreover, occasional membrane staining was detected in some follicular lymphocytes within lymphoid tissue (Fig. 3c). The distribution of Dpl protein was similar in foetuses and in adult animals.

3.4. Detection of Dpl on ejaculated spermatozoa

Bovine Dpl was detected on ejaculated bovine spermatozoa. Intense positive staining extending from the neck to the middle piece was detectable by immunocytochemistry

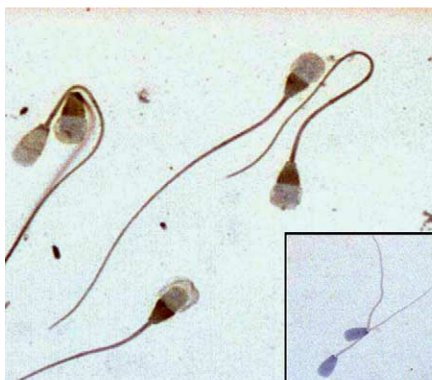


Fig. 4. boDpl detection in ejaculated spermatozoa: intense staining extending from the neck to the middle piece was detected in samples on which the anti boDpl67–81 antibody was applied. No Dpl positivity was detected in samples on which an isotype matched irrelevant antibody was applied (negative control). Immunocytochemistry, ABC technique. Chromogen: DAB; Haematoxylin counterstained, 1000×. The box represents the negative control (isotype matched irrelevant antibody).

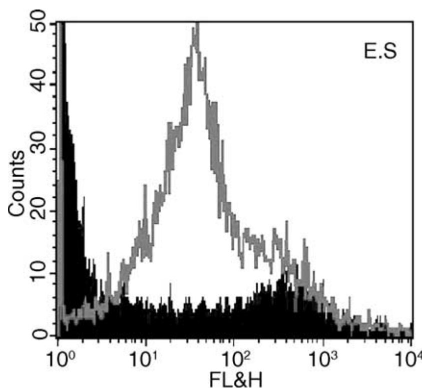


Fig. 5. Flow cytometry: Dpl expression in ejaculated spermatozoa (E.S.). A strong positive signal is detected on cells labelled with the boDpl67–81 antibody (gray line), compared to cells stained with a non-immune rabbit IgG (black area).

(Fig. 4), and an increased fluorescence signal compared to controls was detected by flow cytometry (Fig. 5).

4. Discussion

Dpl protein plays a role in regulating male fertility in several mammalian species [11]. If a similar function could be demonstrated in bovines, the identification of any possible

changes in Dpl expression on bovine semen would have a strong practical implication. The expression pattern of bovine *Prnd* mRNA has already been investigated [12] but, to our knowledge, the distribution of the bovine protein has never been explored. In this paper a vast screening of bovine tissues for Dpl expression was performed. Dpl was detected by Western blotting in testis as a diglycosylated protein, while human Dpl has two N-glycosylation sites and one O-glycosylation site [10]. We have not investigated the possible presence of O-glycosylation, so we cannot exclude that BoDpl is also O-glycosylated. Neverthelss, it should be underlined that species-specific differences in the glycosylation pattern of Dpl have already been reported: murine Dpl, as an example, seems to have only N-glycosylation sites [10].

Western blotting results are consistent with the results obtained by immunohisto/cytochemistry and by flow cytometry, which showed bovine Dpl in all the maturative stages of germinal cells, including spermatozoa. We did not analyse freshly collected and/or capacitated spermatozoa, but sperm cells that underwent a process of cryopreservation. Nevertheless, our results are in agreement with the detection of Dpl in human and murine spermatozoa after capacitation [10,11]. On the contrary, immature human germinal cells do not express Dpl [10]. This might suggest a different biological behaviour of bovine Dpl or a possible role of bovine Dpl in germinal cell ontogeny. Moreover, Dpl was expressed in fetal spermatogones while, in adult animals, the immunoreactivity was mainly detectable in spermatocytes and/or in spermatides. In other species, Dpl has been reported to be a membrane bound protein [17]. Although we have not used experimental approaches that are able to localise the positive signal (e.g. confocal microscopy or electron microscopy analysis), the staining pattern of immature cells was suggestive of an intracytoplasmic localisation of Dpl, which may further support a possible role of this protein in regulating germinal cell maturation. Moreover, Dpl was detected in Sertoli cells, in agreement with that reported in humans [10].

Although our study did not investigate Dpl function, we might hypothesize that Dpl contributes to fertility by means of its rich glycan moiety that may help to prevent sperm self-aggregation and/or unspecific binding to the genital tract [18] or to prevent loss of the acrosome content [19]. The granulosa cells and the follicular fluid produced by them, stained positive by immunohistochemistry, while Western blotting carried out on homogenised ovaries, from which the fluid was removed during sample processing, was negative. This finding may suggest a possible role of Dpl in regulating fertility, since follicular fluid has been shown to influence sperm motility and fertility [20].

Dpl was also distributed throughout the body in endothelium, in peripheral nerves, and in scattered lymphocytes within lymphoid tissues as detected by immunohistochemistry (Western blot is probably not sensitive enough to detect the low amount of Dpl expressed on these few cells). Further, investigations would clarify the role of bovine Dpl in these tissues.

Literature is progressively ruling out that Dpl is connected with spontaneous prion diseases, but our detection of bovine Dpl in scattered tissue cells in different organs merits to be further investigated. Nevertheless, our data confirm that also in bovine, Dpl is mainly expressed in gonads and its presence in germinal cells and in both Sertoli cells and follicular fluid suggests that this protein might be involved in sperm maturation. It is our

opinion that Dpl expression and/or its structural modification in spermatozoa may be a new parameter to consider during bovine infertility studies. This hypothesis needs to be verified by extensively applying the flow cytometric protocol established in this paper on spermatozoa from animals with reduced fertility.

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