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Expression and distribution of zona pellucida proteins 3 and 4 in morphologically abnormal canine oocytes: a confocal microscopic observation-based study[1)]

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Summary

The morphology of mammalian oocytes significantly determines their ability to grow and develop. It has been clearly demonstrated that cumulus-oocyte complexes (COCs) of abnormal quality have low developmental competence, especially in terms of their maturation and fertilization ability. However, few data are available on the expression of proteins responsible for fertilization in morphologically abnormal oocytes as a possible cause of failed fertilization. This study was aimed at analyzing the expression and distribution of ZP3 and ZP4 glycoproteins within canine oocytes.

Canine COCs were morphologically evaluated following their recovery from anestrus mongrel bitches after ovariohysterectomy. Only morphologically abnormal, denuded oocytes with a strongly heterogenous and dark cytoplasm were used in further stages of experiments.

The authors found a decreased expression of ZP3 glycoprotein within the oocyte's cytoplasm, whereas the expression of ZP4 was significantly more pronounced. In almost all oocytes, ZP3 glycoprotein was distributed in the oocytes' cytoplasm. However, a less pronounced expression of ZP4 was detected within the zona, as well. Using the DAPI staining assay, a proper chromatin configuration was found in only approximately 15% of canine oocytes analyzed. All confocal microscopic observations and analyses were performed by the Imaris 7.2 software (BitPlane, Zurich, Switzerland).

Our results suggest that a decreased expression of ZP3 glycoprotein as the primary sperm receptor, as well as its pronounced cytoplasmic location, might explain failed fertilization in this group of canine oocytes. Moreover, the specific staining of ZP4, distributed within the zona, might reflect a significant role of this glycoprotein in fertilization in canines, or this partial distribution may represent remnants of the protein's strong expression during the early stages of oogenesis and zona formation. Several irregularities found in the expression profiles of both ZP3 and ZP4 reflect the differential distribution of these proteins in oocytes during oogenesis.

Keywords: zona pellucida, protein expression, confocal microscopy

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In most cases, developmental competence of mammalian oocytes is determined by their maturation and fertilization ability, as well as by the potential of the embryo to reach the blastocyst stage and successful implantation (22, 33). It is suggested that all of these stages of the oocyte's/embryo's "life" are determined by the morphology of female gametes, which ultimately strongly influence the embryo's viability and ability to grow and develop (20). The morphology of normal mammalian oocytes is characterized by an appropriate number of cumulus cell layers (more than five in most species), granularity and color of cytoplasm, perivitelline space, and first polar body, as well as a fully competent structure of the *zona pellucida* (ZP). It has been shown that oocytes with dark, highly granular cytoplasm have a low ability to mature (to reach the MII stage) and become fertilized (24). Additionally, oocytes with low number of cumulus layers or completely denuded oocytes are described as degenerated female gametes. Although several reports have already been published on ZP glycoprotein expression in oocytes characterized by normal morphology, its expression and cellular distribution in morphologically abnormal gametes have yet to be investigated, especially with the aim of pinpointing the cause of their much lower fertilization potential. Jackowska et al. (10) used porcine oocytes as a model, and found that a differential expression or cellular distribution of ZP3 glycoprotein is highly dependent on the morphology of female gametes. The distribution of ZP3 protein within the oocyte changed between the *zona pellucida* and cytoplasm, which depended on the morphology of the oocyte.

The mammalian *zona pellucida* is formed by a glycoprotein structure that surrounds the oocyte's membrane. The main proteins building the structure of the *zona* include ZP1, ZP2, ZP3, and, in some species, ZP4. It is well recognized that an appropriate expression of these proteins determines to a significant extent the ability of spermatozoa to recognize, interact, and fuse with oocytes in the course of a successful fertilization (4, 8, 11, 13, 18, 32). The ZP3 protein is described as the primary sperm receptor, responsible for interaction between male and female gametes, and, as the sperm-oocyte receptor, activity of the *zona*. Rankin et al. (27) found that ZP1 plays a mostly structural role and is the most important protein for the formation and structural integrity of the *zona* matrix in mice. Furthermore, ZP2 is recognized as a secondary sperm receptor and is involved in sperm-oocyte fusion only after the induction of sperm acrosome reaction. Dean (3) characterized all three forms of ZP glycoproteins, ZP1, ZP2, ZP3, documenting their molecular weights of 90-110 kD, 64-76 kD, and 57-73 kD, respectively.

It has been well established in several studies that oocytes with normal morphology are characterized by an appropriate expression and distribution of all three forms of ZP glycoproteins, as well as by an increased ability to become fertilized. However, few data are available on the expression of these proteins in canine oocytes (16). Therefore, the aim of this study was to show a differential distribution of ZP3 and ZP4 in canine oocytes characterized by defective morphology as a potential cause of failed fertilization in this group of gametes.

Material and methods

Collection of oocytes. Ovaries and reproductive tracts were recovered from 15 normal healthy females, aged 2-5 years, after routine ovariohysterectomies at the clinic of the Department of Veterinary Medicine, Poznań University of Life Sciences, and transported to the laboratory within a few minutes at 39°C. Only samples from bitches at the anestrus stage of the estrus cycle were used. Vaginal cytology swab specimens and venous blood samples were collected from each female after the induction of anesthesia for surgery. The stage of the estrus cycle was assessed on the basis of vaginal cytology data as described by Otoi et al. (25). The blood serum P4 concentration was analyzed by a P4-kit (OvucheckPremate®; Camelot Farms, College Station, TX), and the reproductive tissues were evaluated morphologically. The females were considered to be in anestrus when vaginal cytology revealed parabasal cells on vaginal swabs, and progesterone concentrations were lower than the kit standard for low progesterone. Furthermore, the morphological criteria for the estrus stage in bitches were evaluated using a classification described by Kempisty et al. (16).

The ovaries were placed in physiological saline solution (PSS) at 39°C and then washed three times with PSS. After washing, the ovaries were sliced with a scalpel blade at room temperature to collect COCs.

Confocal microscopic observation of ZP3 and ZP4 distribution in canine oocytes. The oocytes with abnormal morphology (n = 50) were incubated with bovine testicular hyaluronidase (Sigma-Aldrich Co., St. Louis, MO, USA), 300 µg/ml, for 2 min at 38°C to remove cumulus cells. Then, the oocytes were fixed with an aceton/methanol mixture at –20°C for 10 min and washed three times in PBS. They were incubated for 1 hour at room temperature (RT) with goat polyclonal anti-ZP3 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-23717) antibody (Ab) or goat polyclonal anti-ZP4 (I-14) Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-49587) diluted 1: 500 in PBS/3% BSA. After several washes with PBS, the samples were incubated for 1 hour at RT with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG Ab, produced in rabbit, diluted 1: 500 in PBS. Following this wash in PBS, the oocytes were mounted on glass slides in an antifade drop and observed under LSN 510 (Pro Long Gold Antifade Reagent, Life Technologies USA) on the Olympus microscope Fluoview 10i confocal system. FITC was excited by an argon laser at 488 nm, and the emissions were imaged with a 505-530 nm filter. All confocal microscopic images were analyzed by the Imaris 7.2, 40x software (BitPlane, Zurich, Switzerland).

Assessment of nuclear maturation. Cumulus cells were removed from COCs by vortexing. The oocytes were stained with 0.1 µg/ml 4.6-diamino-2-phenylindole (DAPI; Santa

Cruz Biotechnology, Santa Cruz, CA, USA) in mineral oil and mounted on slides. The evaluation of the nuclear status was conducted by confocal microscopy (Fluoview 10i, Olympus, Tokyo, Japan). The oocytes were classified either as MII, i.e. oocytes with a polar body or two shiny chromatin spots, or as aberrations, i.e. oocytes with an abnormal chromosomal organization. In this study canine COCs with modifications were classified morphologically according to data published by Antosik et al. (1). The homogeneity of cytoplasm and the structure of the *zona pellucida* after staining with antibodies were considered as morphological criteria. Additionally, both of these parameters were used for statistical calculations.

Statistical analysis. Student's t-test was used for statistical calculations, carried out by means of the GraphPad Prism version 4.0 software (GraphPadSoftware, San Diego, CA).

Results and discussion

After collection, the canine COCs were morphologically assessed. Only oocytes with strongly heterogeneous, dark cytoplasm and completely absent cumulus oophorus were used in further stages of experiments. ZP3 expression was significantly decreased compared with the cellular distribution of ZP4 protein within the oocytes, as ZP3 was distributed in the oocyte cytoplasm of only 97% of all gametes analyzed. (Figure 1 A, B, C, D, E, F, G, H, I). However, the expression of ZP4 glycoprotein was significantly higher in all oocytes analyzed, as compared to the expression of ZP3 (Figure 2 A, B, C, D, E, F, G, H). Moreover, ZP4 was differentially distributed within the oocytes: approximately 75% of denuded canine oocytes showed cytoplasmic

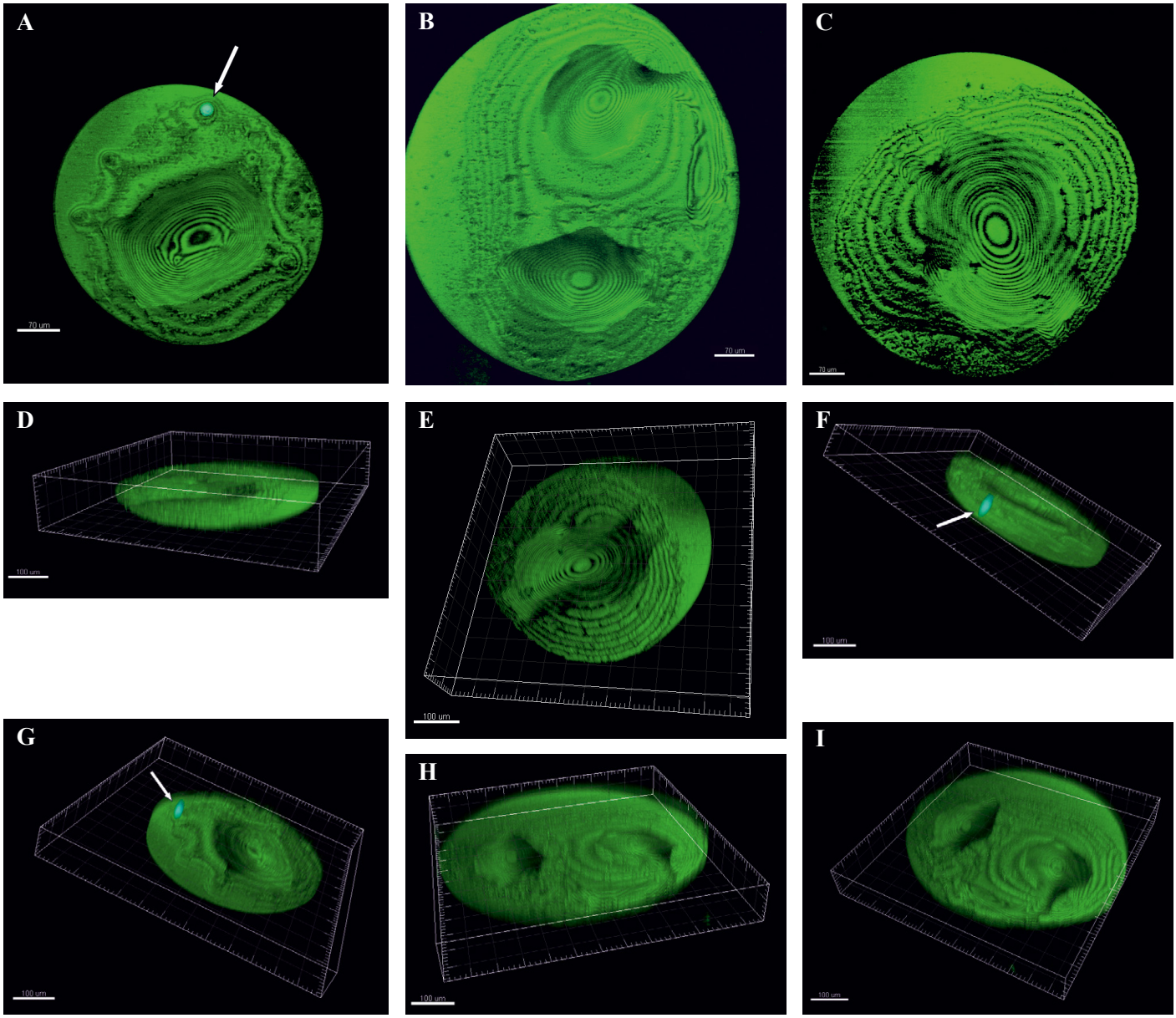

Fig. 1. Confocal microscopic observation of ZP3 glycoprotein expression in canine oocytes
Explanations: Canine oocytes collected from ovariohystorectomized bitches were stained with canine goat polyclonal anti-ZP3 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-23717), (Fig. 1A, B, C). The treated oocytes were labeled for 1h with FITC-conjugated anti-goat IgG Ab at a 1: 500 dilution in PBS. Bars represent 70 µm. Next, the confocal microscopic images were analyzed by the Imaris 7.2 software (BitPlane, Zurich, Switzerland) and presented in 3D emission (Fig. 1 D, E, F, G, H, I). Bars represent 100 µm. The arrows point to a DAPI stained example of chromatin configuration.

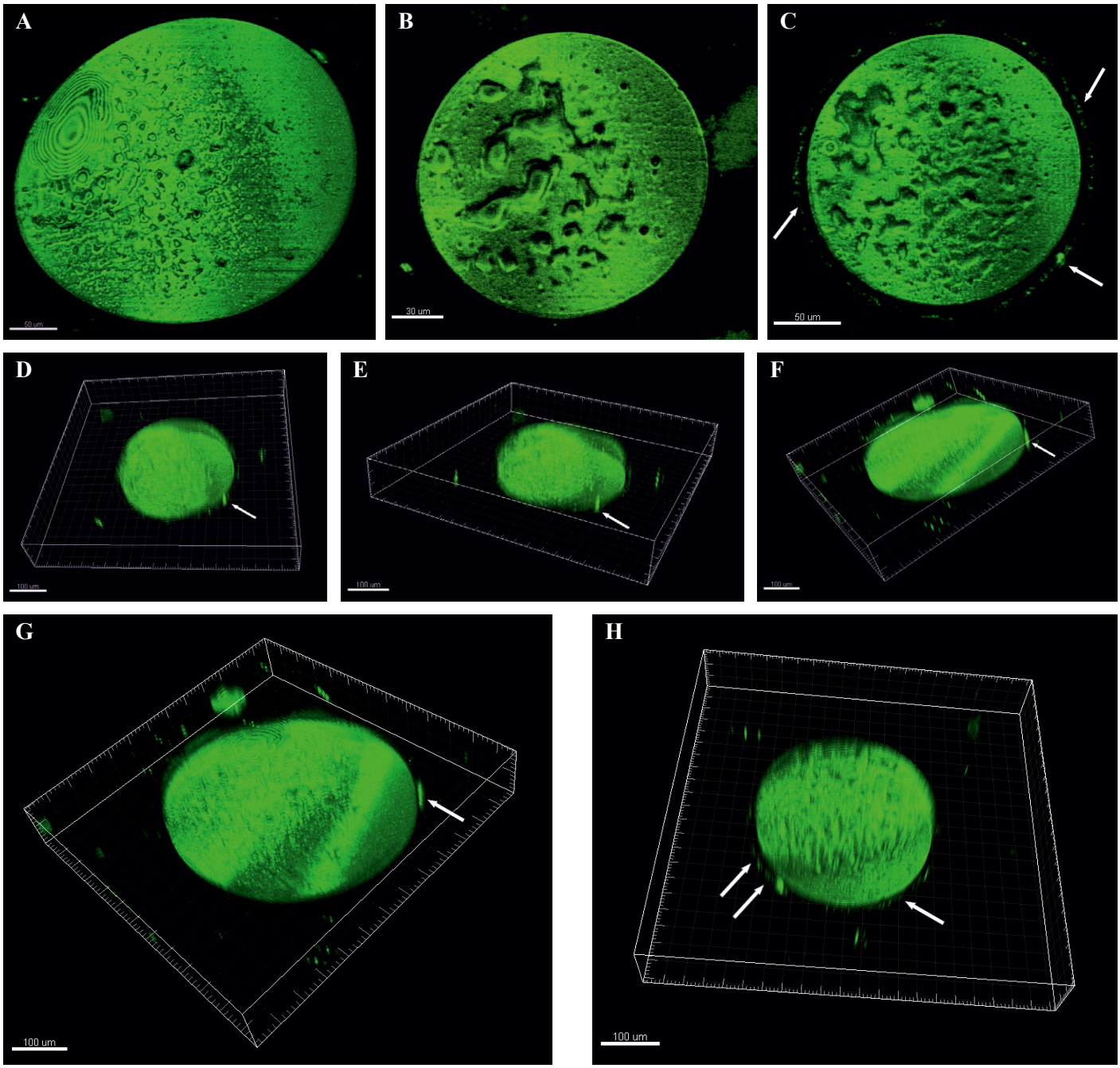

Fig. 2. Confocal microscopic observation of ZP4 glycoprotein expression in canine oocytes
Explanations: Canine oocytes collected from ovariohystorectomized bitches were stained with canine goat polyclonal anti-ZP4 (I-14), (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-49587), (Fig. 1A, B, C). The treated oocytes were labeled for 1 h with FITC-conjugated anti-goat IgG Ab at a 1: 500 dilution in PBS. Bars represent 70 µm. Next, the confocal microscopic images were analyzed by the Imaris 7.2 software (BitPlane, Zurich, Switzerland) and presented in 3D emission (Fig. 1 D, E, F, G, H). The arrows point to the *zona*-specific location of ZP4 glycoprotein expression. Bars represent 100 µm.

location of ZP4, whereas in 25% of images this protein was detected in the *zona pellucida* (Figure 2 C, D, E, F, G, H). After DAPI staining and confocal microscopic observation, chromatin staining was detected in only 3% of oocytes analyzed (Figure 1 A, F, G). In degenerated, denuded canine oocytes, several narrow sites and irregularities in the distribution of both ZP3 and ZP4 were detected within oocyte cytoplasm (Figure 3 A, B, C, D, E). In these incompetent and abnormal canine oocytes, a low number of MII stage oocytes were observed after maturation and DAPI staining (approximately 7%). Moreover, in the group of immature canine oocytes, 20% of gametes showed abnormal morphology.

In several species of mammals, the morphology of female gametes has been found to significantly influence the ability of oocytes to mature and become fertilized *in vitro* (7, 31). In most species of mammals, including pigs and bovines, the *in vitro* maturation and fertilization efficiency is on a high, stable level. However, it has been shown in several studies that in canine species only about 15-20% of recovered

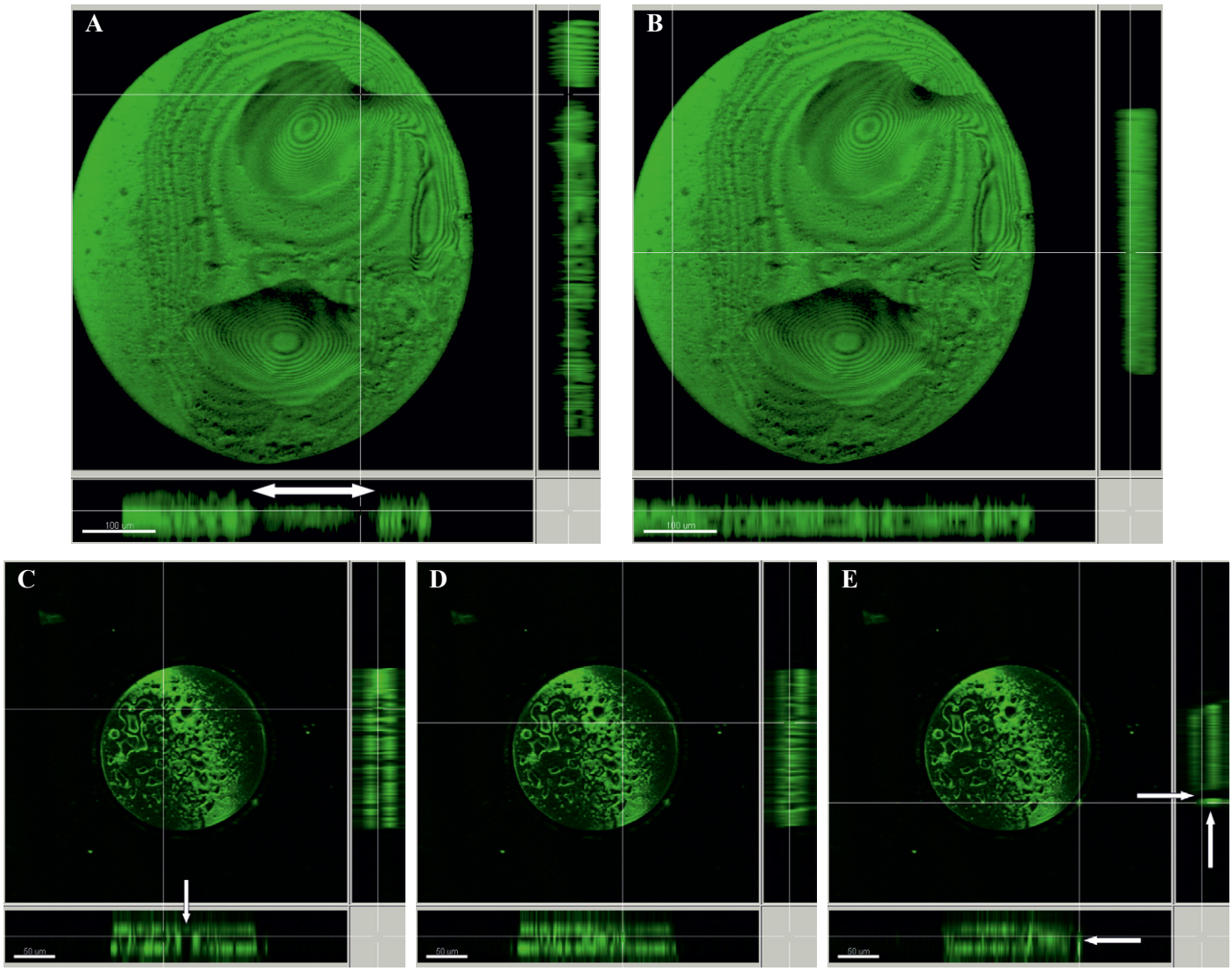

Fig. 3. Confocal microscopic observation of the vertical sections of canine ZP3 and ZP4 glycoprotein expression throughout oocytes

Explanations: The recovered oocytes were stained for ZP3 and ZP4 proteins, and then stained with FITC-conjugated anti-goat IgG Ab, reacting with both primary antibodies. The images show irregularities in the expression pattern of ZP3 and ZP4 glycoproteins throughout the oocytes. The arrows show narrow sites in both ZP3 (Fig. 3 A, B) and ZP4 (Fig. 3 C, D) protein distribution. The arrow in figure 3E points to the perivitelline space and location of ZP4 in the *zona pellucida*. All vertical section images were obtained by the Imaris 7.2 software (BitPlane, Zurich, Switzerland)

oocytes are able to undergo successful maturation *in vitro* (IVM) and eventually reach the MII stage. There are only few published data related to the production of blastocysts and embryo production (IVP) following *in vitro* fertilization (IVF) in canines. Moreover, there has been no offspring in dogs after IVF (28, 29). Thus, the production of healthy offspring after IVF is still an unsolved problem of reproductive biology (6, 19). It is also worth noting that, in contrast to other mammalian species, there are still few published data concerning the use of molecular biology techniques for the identification or recognition of processes important for oocyte/embryo viability in canines. Therefore, this study was aimed at investigating the effect of oocyte morphology on the expression and differential cellular distribution of *zona pellucida* glycoproteins, as a potential way to recognize a low fertilization potential of oocytes characterized by inferior morphology or traits of degeneration.

In several studies, the *zona pellucida* has been found to be permanently modified during oocyte maturation *in vivo* and *in vitro* (5, 9, 17, 23). Therefore, when only gametes of high quality are analyzed, the expression of ZP glycoproteins throughout the *zona* structure may differ significantly in morphology-dependent and/or maturation-stage-dependent manner. In the case of analysis involving only oocytes of lower morphological quality, the expression of ZP proteins may be restricted to oocyte cytoplasm (1, 10, 14-16). As observed in this study, the expression of both ZP3 and ZP4 glycoproteins was mostly restricted to cytoplasm and very limited in the *zona pellucida*. However, in the case of ZP4, a specific *zona* distribution of this protein was found. Similar results were obtained in a porcine

model by Kempisty et al. (15). Moreover, the partial signal from the expression of ZP4 glycoprotein in the canine *zona* (25% of all analyzed oocytes) may suggest that this protein, detected in only a few mammalian species, also plays a significant role during fertilization or represents the remains of its higher expression during early stages of oogenesis. Similar results were obtained by Kempisty et al. (16), who analyzed the effect of P4 and E2 on the expression of ZP glycoproteins. They found that in control oocytes (oocytes with the best morphology without any stimulation) the expression of ZP4 was significantly higher, as compared to that of ZP3. The results of these analyses performed on canine COCs may suggest a new important role of ZP4 in enigmatic canine fertilization. The low expression of ZP3 obtained in this study may be partially explained by results of Pökkylä et al. (26). They demonstrated that changes in ZP glycoprotein abundance, as well as the main *zona* anomalies, may be associated with sequence variations in genes coding ZP2 and ZP3 glycoproteins. Jiménez-Movilla et al. (12) used the hamster oocyte *zona* as a model, showing that a differential expression and partial distribution of *zona* glycoproteins throughout the *zona* structure is reflected by a variable expression of *zona* components (ZP glycoproteins), which is especially visible during each maturation stage of hamster COCs. In view of our results, we may suggest that not only the maturation stage of COCs, but also the morphology of female gametes has a significant influence on the distribution and structure of the *zona*. The partial expression of *zona* glycoproteins throughout the *zona pellucida* may also be explained by the results obtained by Lunn et al. (21). Using a low vacuum scanning electron microscope (LVSEM) to observe the *zona* structure of feline and canine oocytes, they found that in both species the *zona* was characterized by spherical or elliptical pores that narrowed centripetally splitting into several smaller and deep pores. Additionally, the *zona pellucida* differed significantly in a size-dependent manner. It was also observed that a different diameter of *zona* pores may significantly influence the developmental competence of oocytes, especially as related to the fertilization rate and the frequency of polyspermy (2, 30, 34).

A low developmental competence of morphologically abnormal oocytes has been well recognized by several studies. However, the expression and cellular distribution of canine ZP glycoproteins in these oocytes still requires further investigations. The results obtained in this study may suggest that the low fertilization potential of canine oocytes of abnormal quality may be associated with a decreased expression of ZP glycoproteins and with their specific location between oocyte cytoplasm and the *zona*. This study also shows that ZP4 glycoprotein may play a significant role during fertilization in canine species because of its partial expression observed within the *zona pellucida*.

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