Topographical localisation of glucidic residues and their variations in the canine zona pellucida during folliculogenesis

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Summary

In the present ultrastructural study, horseradish peroxidase-labelled lectins, in conjunction with antiperoxidase antibody and protein A-gold, were used to characterise and localise the oligosaccharide sequences of zona pellucida glycoproteins at different stages of follicular development in the canine ovary. Deacetylation and sialidase digestion were also performed before lectin cytochemistry. The zona pellucida of oocytes present in unilaminar primary follicles reacts with WGA- and RCA-I-lectins. The zona pellucida of oocytes present in bilaminar and trilaminar secondary follicles displays positivity to WGA, RCA-I, Con-A, UEA-I, and sialidase/SBA. This labelling pattern persists in the zona pellucida of oocytes present in antral tertiary follicles with the exception of WGA and RCA-I reactive sites which are differently distributed throughout the zona pellucida. The topographical distribution of these carbohydrates is not uniform throughout the zona pellucida, indicating the regionalization of oligosaccharide chains within three concentric bands of the zona matrix: an inner surface close to the oocyte plasmamembrane, an intermediate portion and an outer layer in contact with the follicular cells. Our results demonstrated variations in the presence and distribution of the carbohydrate residues in the canine zona pellucida during different stages of follicular growth. We also observed the presence of vesicles in both the ooplasm and granulosa cells, showing a similar lectin binding pattern to that of the zona pellucida.

Introduction

The molecular composition of mammalian zona pellucida (ZP) varies among different species, consisting of three to four major acidic glycoproteins (Grootenhuis et al. 1996). The protein components of these glycoproteins are highly homologous among species; conversely the carbohydrate contents of the N- and O-linked oligosaccharides show interspecific differences, and these may relate to species-specific binding of sperm to egg (Chapman et al. 2000). Mouse spermatozoa bind to α-Gal residues or β-GlcNAc (Miller et al. 1992) at the non-reducing end of the O-linked chain of ZPC protein. L-fucose has been shown to be involved in sperm-ZP-recognition in guinea-pig, hamster, rat and human oocytes (Chapman et al. 2000) while in pigs, an N-linked glycans of ZPC have a significant role in sperm-egg interaction (Yonezawa et al. 1997). These studies provide strong evidence that the glycan portion of ZP glycoproteins is the ligand for spermatozoa.

Lectin histochemistry has been employed as a tool to characterize the oligosaccharide chains of the ZP glycoproteins in a variety of mammalian species (Roux & Kan 1991, Avilés et al. 1994, 2000, Parillo et al. 1996, 1998, 1999, 2000, 2001, 2003, Parillo & Verini Supplizi 1999, 2001, Verini Supplizi et al. 1996, Chapman et al. 2000, Blackmore et al. 2004). These lectin studies have revealed species-dependent variations in the expression and distribution of sugar moieties throughout the ZP. They have also provided evidence showing variations in the presence and distribution of carbohydrate residues in the ZP during different stages of follicular growth (Roux & Kan 1991, Parillo & Verini Supplizi 1999, 2001, Parillo et al. 2001, Avilés et al. 2000, Blackmore et al. 2004). It has been suggested that these modifications might be the cause of the changes in the chemical composition and biological properties of the ZP during folliculogenesis.

In the present ultrastructural study we employed lectin binding in conjunction with enzymatic degradation to analyse the topographical localisation of the glucidic determinants and to assess the possible modifications occurring in the canine ZP during folliculogenesis. The knowledge of detailed ZP chemical composition could

form the basis for future studies on IVM-IVF and immunocontraception in dogs.

Materials and methods

Ovarian tissue collection

Canine (*Canis familiaris*, $n = 3$) ovaries were obtained from sexually mature bitches (3–6 years old; estrus phase) which had undergone ovariectomy.

Cytochemical labelling

Ovarian tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2 h at room temperature. Subsequently, they were processed for electron microscopy according to the partially modified procedures previously described by Scala *et al.* (1992) and Menghi *et al.* (1996a). The samples were dehydrated in a series of 50%, 70% and 90% acetone and embedded in Bioacryl resin (Bio-optica, Milano-Italy). Ovarian follicles were first located in 1 μm-thick sections of Bioacryl-embedded specimens by light microscopy. Thereafter, ultrathin sections (about 80 nm thick) were placed on parlodion-coated 200 mesh nickel grids. The nickel grids were preincubated at room temperature for 10 min in 0.5 M TBS (Tris Bufferd Saline) pH 7.4 containing 0.25% BSA (Bovine Serum Albumin) and then incubated at room temperature for 3 h with horseradish-peroxidase (HRP) conjugated lectins diluted in TBS pH 7.4 plus 1% BSA. The lectins used, their carbohydrate specificity and the optimal dilutions are reported in Table 1. After lectin incubation, the sections were rinsed with 0.05 M TBS containing 1% BSA and reacted with anti HRP antibody (raised in rabbits and diluited 1:100 in TBS-1% BSA) at room temperature for 1 h. Then, the sections were washed with 0.05 M TBS-1% BSA and treated

Table 1. Lectins used and their carbohydrate specificities.

Source of lectin	Acronym	Lectin specificity[a]	Lectin dilutions
Arachis hypogaea	PNA	β-D-Gal-(1 → 3)-D-GalNAc	1:500
Ulex europaeus	UEA-I	α-L-Fuc	1:100
Lotus tetragonolobus	LTA	α-L-Fuc	1:100
Dolichos biflorus	DBA	α-D-GalNAc	1:500
Glycine max	SBA	α-D-GalNAc > β-D-GalNAc	1:500
Triticum vulgare	WGA	GlcNAc > sialic acid	1:500
Canavalia ensiformis	Con-A	α-D-Man > α-D-Glc	1:500
Ricinus communis	RCA-I	β-D-Gal-(1 → 4)-D-GlcNAc	1:500

[a] β-D-Gal = β-D-galactose; α-D-Gal = α-D-galactose; D-GalNAc = D-N-acetylgalactosamine; β-D-GalNAc = β-D-N-acetylgalactosamine; α-D-GalNAc = α-D-N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; α-L-Fuc = α-L-fucose; α-D-Man = α-D-mannose; α-D-Glc = α-D-glucose.

with protein A gold (10–20 nm), diluted 1:50 in 0.1 M TBS pH 7.4 plus 1% BSA and 0.05% Tween 20, at room temperature for 1 h. Finally, after counterstaining with uranyl acetate and lead citrate, the sections were examined with a Philips EM 208 electron microscopy.

All the HRP-labelled lectins, the anti-HRP antibody and protein A gold were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Enzymatic treatment

Before WGA, PNA, SBA, RCA-I and DBA lectin cytochemistry, the tissue sections were incubated with sialidase (neuraminidase type V, from *Clostridium perfringens*) at a concentration of 0.86 U/ml in acetate buffer pH 5.5 containing 10 mM $CaCl_2$ for 16 h at 37 °C.

In order to remove sialic acid residues with O-acetyl groups, the sections were pretreated with 0.5% KOH in 70% ethanol for 30 min at room temperature before enzymatic digestion (Menghi *et al.* 1996b).

Cytochemical controls

The control for the specificity of lectin cytochemistry was carried out either by adding the specific competing sugar at a concentration of 0.2–0.4 M in the lectin solutions or by omitting the anti-peroxidase antibody or lectin HRP conjugates. Controls for enzymatic digestion were performed by substituting the enzyme with its corresponding buffer.

Results

Follicles were classified according to Fayrer-Hosken *et al.* (2000). In our study we examined the oocytes present in the unilaminar primary follicles, bilaminar and trilaminar secondary follicles (preantral follicles with one, two or three layers of granulosa cells around the oocyte, respectively) and the oocytes present in tertiary antral follicles ($\varnothing \leq 0.5$ mm) with multi-layers of granulosa cells around the oocyte.

A semi-quantitative analysis of lectins distribution has been performed based on a subjective evaluation.

Zona pellucida

The nascent ZP of oocytes present in the unilaminar primary follicle reacted intensely with WGA- (Figure 1a,b) and RCA-I-lectins (Figure 2). Sialidase digestion did not modify lectin labelling. The other lectins tested resulted negative.

The ZP of oocytes present in bilaminar and trilaminar secondary follicles exhibited different patterns of binding with WGA, Con-A, UEA-I, RCA-I and SBA.
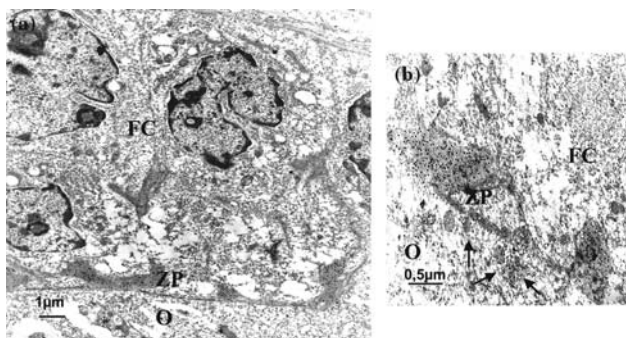
Figure 1. Unilaminar primary follicle. WGA staining. (a) Numerous binding sites are present in the nascent ZP. (b) The gold particles are also located in the vesicles (arrows) present in the ooplasm. ZP, zona pellucida; O, ooplasm; FC, follicular cell.
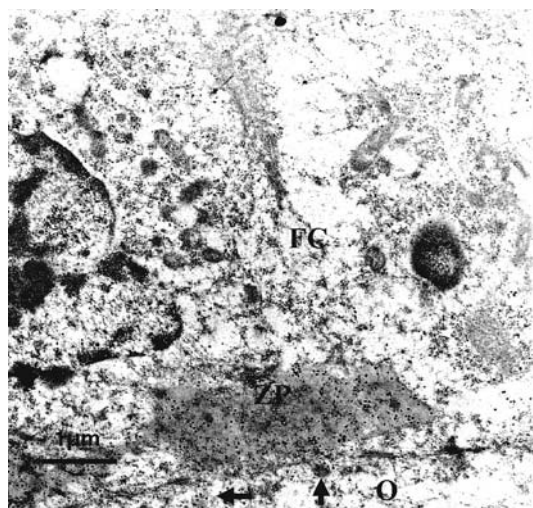

Figure 3. Bilaminar secondary follicle. WGA staining. The binding sites are intensely distributed in the inner ZP and moderately in the central and outer regions. ZP, zona pellucida; O, ooplasm; FC, follicular cell.

Figure 2. Unilaminar primary follicle. RCA-I staining. The ZP shows an intense labeling. The gold particles are also localised in the vesicles (arrows) present in the ooplasm. ZP, zona pellucida; O, ooplasm; FC, follicular cell.

Figure 4. Bilaminar secondary follicle. Con-A staining. A few labelling sites are slightly distributed in the ZP preferentially in the inner layer . The vesicles (arrows) present in the ooplasm and in the follicular cell are also labelled. ZP, zona pellucida; O, ooplasm; FC, follicular cell.

In particular, there appeared to have a higher concentration of WGA-binding sites (Figure 3) in the inner portion of the ZP as compared to the central and outer regions; sialidase digestion did not modify this labelling pattern. Con-A (Figure 4) and UEA-I showed a slight reactivity preferentially located in the inner layer of the ZP. RCA-I reactive sites were moderately detected in the internal boundary of the ZP and weakly in the central and external parts (Figure 5). Cleveage of sialic acid enhanced the concentration of the gold particles mainly in the central and external regions of the ZP (Figure 6). SBA, after enzymatic degradation, preferentially labelled the inner and the outer bands of the ZP (results not shown). No labelling was observed with PNA, DBA and LTA (results not shown).

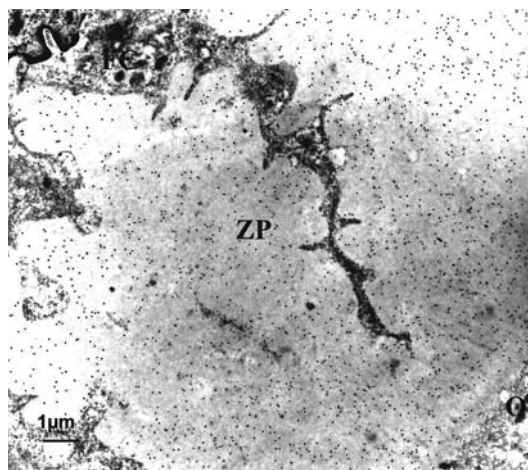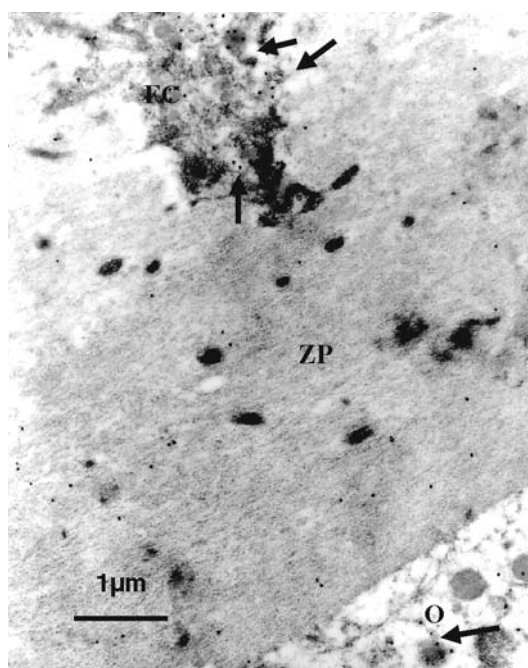The above labelling pattern persisted in the ZP of oocytes present in tertiary antral follicles, with the exception of WGA-binding sites present preferentially in the outer region (Figure 7), RCA-I-binding sites which were uniformly distributed over the entire thickness of the ZP (Figure 8) and sialidase/RCA-I-binding sites located mainly in the inner region (Figure 9).

In all cases deacetylation treatment did not modify the positivity obtained with sialidase/WGA/RCA-I/SBA.
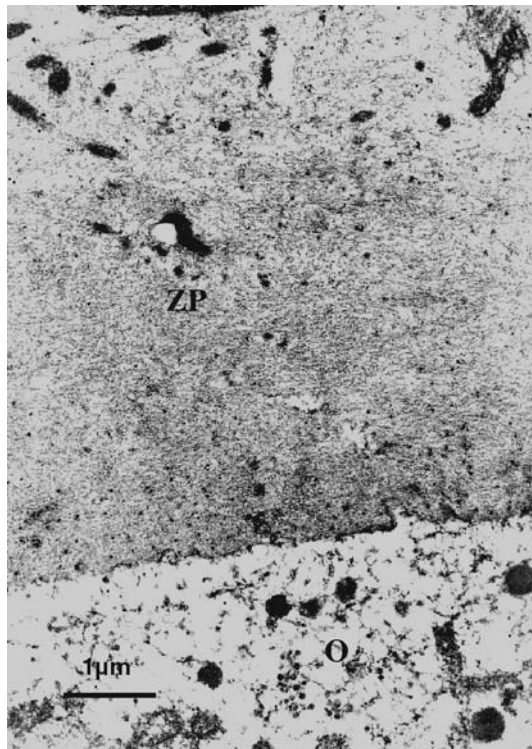
Figure 5. Bilaminar secondary follicle. RCA-I staining. The labelling is moderate in the inner layer and it is weak in the intermediate and outer portions of the ZP. ZP, zona pellucida; O, ooplasm.
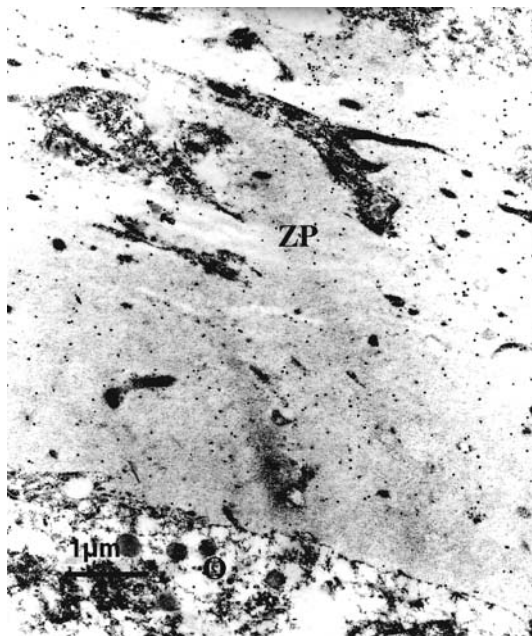

Figure 6. Bilaminar secondary follicle. Sialidase/RCA-I staining. Sialidase degradation prior to RCA-I staining enhances the labelling of gold particles mainly in the central and external regions of the ZP. ZP, zona pellucida; O, ooplasm.

Ooplasm

A large population of vesicles that were reactive to WGA (Figure 1b), RCA-I (Figures 2 and 8), Con-A
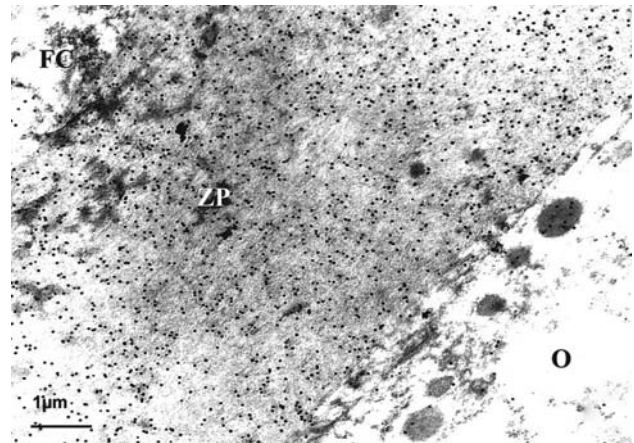

Figure 7. Antral tertiary follicle. WGA staining. The binding sites are intensely and uniformly distributed in the entire thickness of the ZP. ZP, zona pellucida; O, ooplasm; FC, follicular cell.
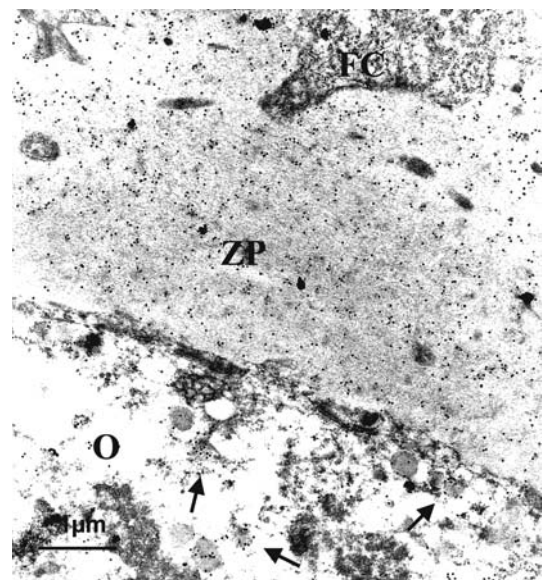

Figure 8. Antral tertiary follicle. RCA-I staining. The reactive sites are moderately and evenly distributed over the ZP. The vesicles (arrows) present in the ooplasm are also reactive. ZP, zona pellucida; O, ooplasm; FC, follicular cell.

(Figure 4), UEA-I and sialidase/SBA were evidenced in the ooplasm. Other cellular organelles, such as mitochondria and endoplasmic reticulum, were devoid of any labelling.

Granulosa cells

The cytoplasm of the granulosa cells contained numerous vesicles which displayed reactivity with WGA (Figure 10a), RCA-I (Figure 10b), Con-A (Figure 4), UEA-I and sialidase/SBA.

Controls

The control tissue sections did not show any appreciable reactivity, confirming the specificity of the lectin labellings (Figure 11).
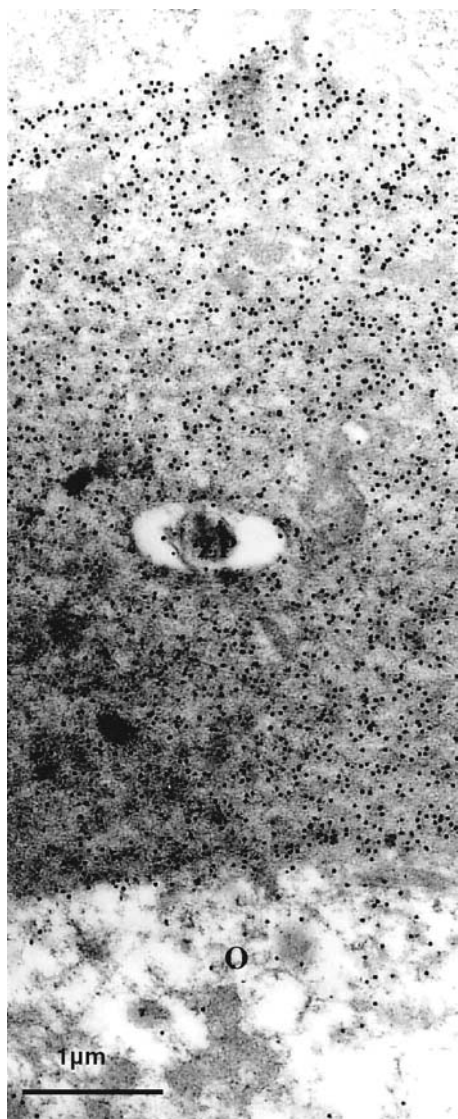
Figure 9. Antral tertiary follicle. Sialidase/RCA-I staining. Sialidase degradation prior to RCA-I staining promotes a uniform and heavy labelling of gold particles in the entire thickness of the ZP. ZP, zona pellucida; O, ooplasm.
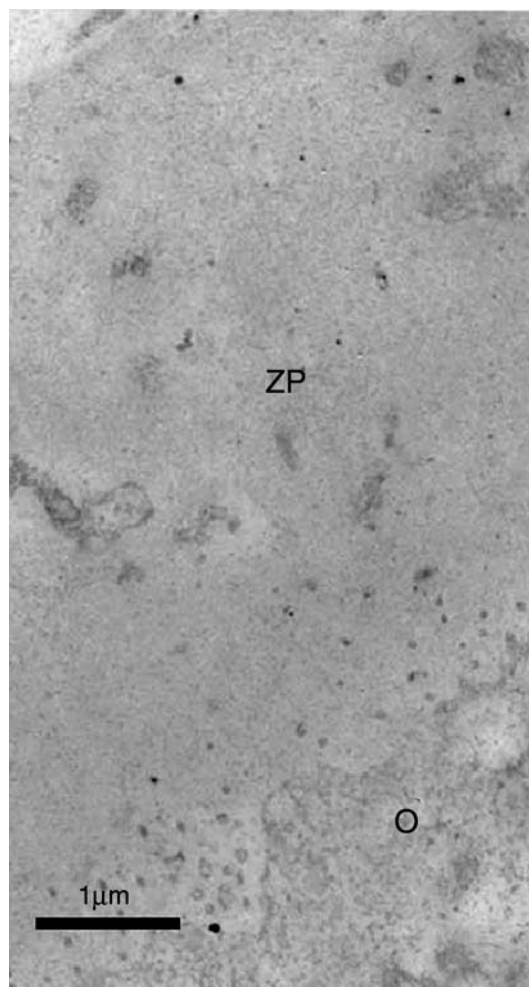

Figure 10. Follicular cells. WGA (a) and RCA-I (b) stainings. The labelling sites are localised in vesicles (arrows) present in the cyto-plasm of follicular cells. FC, follicular cell.

Figure 11. Antral tertiary follicle. Control section incubated with RCA-I with 0.4 M D-Gal. Note the absence of reactivity in the ZP. ZP, zona pellucida; O, ooplasm.
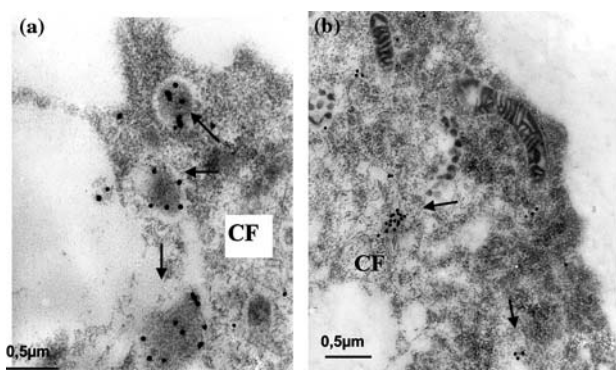
Discussion

The canine ZP begins to form in unilaminar primary follicles within the intercellular spaces between the granulosa cells adjacent to the oocyte. At this stage the nascent ZP only reacts with WGA- and RCA-I-lectins, indicating the presence of GlcNAc and Galβ1-4Glc-NAc residues. These carbohydrates probably pertain to O-linked oligosaccharides since Con-A-lectin, which binds α-Glc/Man preferentially in complex N-linked oligosaccharides, results negative. In addition, sialidase digestion does not alter RCA-I and WGA reactivity suggesting the absence of sialyl residues in the ZP at this stage of follicular development.

The ZP of oocytes present in secondary bilaminar and trilaminar follicles is well developed and com-pletely circumferential. It displays a positivity to WGA-, RCA-I-, Con-A-, UEA-I- and SBA-lectins indicating the occurrence of GlcNAc, Galβ1-4GlcNAc, α-Glc/Man, α-Fuc and β-GalNAc. The topographical distribution of these carbohydrates is not uniform

throughout the ZP indicating the regionalization of oligosaccharide chains within three concentric bands of the zona matrix: an inner surface close to the oocyte plasmamembrane, an intermediate portion and an outer layer in contact with the follicular cells. In the internal zone, we identified above all GlcNAc, Galβ1-4GlcNAc and sialic acid (α-2-6)-β-GalNAc detected by WGA, RCA-I and sialidase/SBA sequence; in addition, we observed the presence of scarce amounts of α-Glc/Man and α-Fuc residues detected by Con-A- and UEA-I-lectins. In the outer ZP we revealed the occurrence of GlcNAc and Galβ1-4GlcNAc residues, although their concentration was lower than that observed in the inner region, and also the dimer sialic acid (α-2-6)-β-GalNAc. The most significative difference in the glucidic content between the inner and outer ZP at the bilaminar and trilaminar primary follicle stage, is the presence of the trisacchride sialic acid (α-2-3,6)-Gal-β-(1-4)GlcNAc revealed by sialidase/RCA-I sequence and also the absence of α-Glc/Man and α-Fuc residues in the outer ZP. The glucidic composition of the central portion of the ZP is similar to the external ZP with the exception of the disacchaide sialic acid (α-2-6)-β-GalNAc which is not expressed in the intermediate region.

The ZP of oocytes present in antral follicles displays a distribution pattern of the α-Glc/Man, α-Fuc and sialic acid-β-GalNAc reactive sites similar to the ZP of oocytes at the bilaminar/trilaminar follicle stage. Conversely, GlcNAc and subterminal Galβ1-4GlcNAc residues result mainly localised in the outer and in the inner region of the ZP, respectively; the terminal Galβ1-4GlcNAc residues are uniformly distributed in the entire thickness of the ZP . Taken together, these lectin cytochemical findings, obtained in the ZP of bilaminar/trilaminar follicles and in the ZP of antral oocytes, suggest that O-linked oligosaccharides are restricted in the outer and central portions of the ZP whereas both O- and N-linked oligosacchrides are confined mainly in the inner layer. In our previous histochemical study, carried out in the canine ZP with a light microscope, Con-A and UEA-I-binding sites were not evidenced (Parillo & Verini Supplizi 1999), probably due to the scarce amounts of α-Glc and α-Fuc residues. Fucosyl moieties were identified by UEA-I but not by LTA which has the same nominal specificity for α-fucose as UEA-I. Similar discrepancies are frequent between lectins with the same monosaccharide specificity (Damjanov 1987) and, probably in this case, the fucose visualized by UEA-I seems to be involved in α 1,2 linkage with D-Gal whereas fucose α 1,4 linked to GlcNAc recognized by LTA is not detected (Spicer & Schulte 1992).

The results obtained from our study agree with previous histochemical investigations (Roux & Kan 1991, Avilés *et al.* 1994, 2000, Verini Supplizi *et al.* 1996, Parillo *et al.* 1996, 1998, 2001, 2003, Parillo & Verini

Supplizi 1999, 2001, El-Mestrah & Kan 2001, 2002) regarding the asymmetric distribution of some glyco-molecules through the ZP. It has been suggested (El-Mestrah & Kan 2002) that the different topographical localisation of the various lectin reactive-sites on the zona matrix indicates a different possible role of the glucidic determinants in the process of sperm-zona binding. Indeed, the Authors suggested that the glyco-molecules localised in the outer region of the ZP may be involved in the initial stages of spermatozoon-ZP interaction, whereas the carbohydrates confined in the inner layer may participate in the establishment of a block to polyspermy.

Our study shows that some glucidic residues, such as α-Glc/Man, α-Fuc, β-GalNAc and sialic acid, are not expressed in the ZP of the unilaminar primary follicles, but appear in the later stage of zona maturation. Similar findings have also been reported in the rat (Avilés *et al.* 2000). The Authors hypothesized that the expression of the carbohydrates depends on the temporal activation or inactivation of specific glycosyltransferases during the secretion of the zona matrix.

Canine ZP is particularly rich in sialic acid moieties also evidenced in several other mammalian species (Avilés *et al.* 1994, 2000, Parillo *et al.* 1996, 1998, 1999, 2001, 2003, Parillo & Verini Supplizi 1999, 2001 Verini Supplizi *et al.* 1996, Roux & Kan 1991, Chapman *et al.* 2000). Because they are negatively charged, sialic acids are involved in the binding and transport of positively charged compounds which play a role in the hydration of the ZP. Sialic acids are also essential components of receptors. In addition, the presence of an α-2-3 linkage, that is relatively rigid, in conjuction with the more freely rotating α-2-6 linkage, imparts viscoelastic properties to the ZP that may facilitate the penetration of spermatozoon during fertilisation. The failure of deacetylation to increase the affinity of the ZP for WGA, RCA-I and SBA before sialidase digestion indicates the absence of sialic acids with O-acetyl groups. Sialic acids lacking O-acetyl esters, detected mainly in the outer surface of the canine ZP, may allow selective glycolysis and proteolysis by the acrosomal enzymes of spermatozoa (Chapman *et al.* 2000).

In the present cytochemical work, a large number of vesicles, showing a lectin labeling pattern resembling that of the ZP, were observed in the ooplasm and in the cytoplasm of granulosa cells. It has been hypothesized that these aggregates seen in the ooplasm and follicular cells are implicated in the synthesis and final secretion of glycosylated ZP proteins as intermediary vehicles (Avilés *et al.* 1994, 2000, El-Mestrah & Kan 2001). This allows us to suggest that both the oocyte and the surrounding granulosa cells are involved in the synthesis and secretion of the glycocomponents of the canine ZP in agreement with the findings of other Authors (Barber *et al.* 2001, Blackmore *et al.* 2004). In particular, Blackmore *et al.* (2004), evidenced the presence of ZPA

mRNAs in the ooplasm of developing primary and secondary follicles. In contrast, mRNAs of ZPB and ZPC genes were localised in the granulosa cells of activated follicles. This is similar to ZP synthesis observed in the rabbit (Lee & Dumbar 1993) but it is different in the mouse, in which only the oocyte is responsible for the synthesis of ZP glycoproteins (Avilés *et al.* 1994).

In conclusion, the present cytochemical research shows variations in the presence and distribution of the carbohydrate residues in the canine ZP during different stages of follicular growth, in agreement with other functional studies (Oehninger *et al.* 1991, Blackmore *et al.* 2004), suggesting that the chemical composition and the biological properties of the mammalian ZP are modified during folliculogenesis to prepare the oocyte for fertilisation. Further and detailed quantitative analysis are required to elucidate ZP glucidic modification during oocyte maturation in the dog.

Moreover, as postulated by Blackmore *et al.* (2004), canine oocytes in activated primary follicles seem to direct the synthesis of the ZP by granulosa cells, probably through secretion of TGF-β superfamily molecules.

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