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Mirta Alicia Flamini, Claudio Barbeito and Francisco Acuña, designed the experiment. Francisco Acuña and Mirta Alicia Flamini collected the samples. Mirta Alicia Flamini, Claudio Barbeito, Alcira Diaz, Maria Florencia Tano de la Hoz and Francisco Acuña performed the histochemical techniques and the subsequent observation of the slides. Enrique Portiansky, Mirta Alicia Flamini and Francisco Acuña made the selection of images and the subsequent analysis of them. Mirta Alicia Flamini, Claudio Barbeito, Enrique Portiansky and Francisco Acuña, wrote the first draft of the manuscript. All authors reviewed the manuscript.

Histochemistry of the zona pellucida of the ovary of a species with natural polyovulation: *Lagostomus maximus* (Rodentia, Hystricomorpha, Chinchillidae)

Histochemistry of the ZP of *Lagostomus maximus*

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Contents

This study reports the histochemistry and the distribution of glycoconjugates (GCs) in the zona pellucida (ZP) of preantral, secondary, tertiary, polyovulatory and atretic follicles of ovaries from non-pregnant (NPr) and pregnant (Pr) females of *Lagostomus maximus*. GCs were studied using histochemical and lectinhistochemical methods. The viscacha ZP was positive to all the histochemical techniques. In addition, it was observed that the intensity of staining of the ZP was constant in the different follicular stages between both female groups. The lectinhistochemical study revealed that ZP was positive for certain lectins (WGA, RCA-I and CON-A) and that the labeling did not vary between the different follicular stages, but between the two groups of females. By using both histochemical techniques it was established that the GCs present in the ZP label the complexity of the area. These results allow us to increase our knowledge on the biology of the viscacha's ovary, particularly contributing to the study of polyovulation.

Keywords

Glyconjugates, hystricognathi, ovarian follicles, ovulation, plains viscacha

Introduction

Polyovulation is a process that occurs in many mammals, such as the rat (*Rattus norvergicus*), the dog (*Canis familiaris*) and the sow (*Sus scrofa*), which ovulate between 4-15 oocytes per reproductive cycle (McNatty et al., 2005). However, in some species, the number of ovulated oocytes increases markedly. Van der Horst & Gillman (1941) reported that females of the species *Elephantulus myurus* ovulates between 50 to 120 oocytes with implantation of only two blastocysts (one per each uterine horn). In later years, an even greater polyovulation phenomenon was observed in *Lagostomus maximus* (Weir, 1971).

This last species is a model of great interest due to its particular reproductive characteristics, which include the highest known polyovulation in mammals (200 to 800 oocytes) and, in addition, a poly-implantation of around 12 blastocysts, of which only two of them are delivered (Weir, 1971; Flamini et al., 2011). The ovaries of viscacha are atypical within mammalian females (Weir, 1971), even within its own suborder hystricognathi (Hautier et al., 2011). The histological details of the organ were first reported by Weir in 1971. Subsequently, other characteristics were described, such as a low level of apoptosis in different ovarian follicles and corpora lutea (Jensen, Willis, Albamonte, Espinosa, & Vitullo, 2006; Jensen, Willis, Leopardo, Espinosa, & Vitullo, 2008) and the presence of abundant interstitial tissue (Gil et al., 2007).

The morphological study of the ovaries at macro and microscopic levels was deepened (Flamini, Barbeito, Gimeno, & Portiansky, 2009). These authors described the presence of folds of tissue that increase the surface of the organ, facilitating the polyovulation previously described (Weir, 1971). They also classified the different stages of follicular development: primordial, primary, preantral, secondary, tertiary, polyovulatory and atretic, based on the classification criteria for follicles of bovine origin (Rodgers, Lavranos, Van Wezel, & Irving-Rodgers, 1999). Flamini et al (2009) described a ZP in preantral, secondary, tertiary, polyovulatory and atretic follicles.

Zona pellucida is a highly glycosylated extracellular matrix that surrounds the oocyte and the preimplantatory embryo (Sinowatz, Koelle, & Toepfer-Petersen, 2001). In mouse it is constituted almost exclusively by three glycoproteins: ZP1, ZP2 and ZP3 (Gupta et al., 2007). Recently, a fourth glycoprotein (ZP4) was discovered in some species (Lefièvre et al., 2004; Goudet, Mugnier, Callebaut, & Monget, 2008; Izquierdo-Rico et al., 2009; Stetson et al., 2015). Zona pellucida poses multiple roles during oogenesis, fertilization and pre-implantation development (Yanagimachi, 1994; Florman & Ducibella, 2006). Studies in ovaries of mice and bitches have shown that GCs from the ZP undergo a series of cytochemical changes after maturation, ovulation and fertilization of the oocytes (Shimizu & Yamada, 1986; Wassarman, 1999; Parillo, Zelli, Supplizi, Fagioli, & Gargiulo, 2005).

Despite the unique characteristics of the ovaries of the plains viscacha, many aspects of their reproductive biology are unknown. In the same way, the histochemical reactivity of the ZP, among other structures, has not been analyzed in this species. The aim of the present study was to identify the carbohydrate pattern of the ZP and its variations in different follicular stages of non-pregnant and pregnant viscachas, using histochemical and lectinhistochemical techniques.

Materials and methods

Animals

For this study 14 adult female viscachas, body weight ranged between 4 and 5.5 kg, captured at ECAS (Estación de Cría de Animales Silvestres, province of Buenos Aires Ministry of Agribusiness) were used. At ECAS, this species lives in wild state without reproductive control. Cages-traps placed at noon and removed the next day in the morning were located at the entrance of viscacha's caves. The periods of March-April (with females in estrus and recent pregnancy), July-August (with females with full-term pregnancies and births) and December-January (females in anestrous and postpartum) were selected for capturing females. The election of periods was based on studies conducted in previous years at our laboratory (Flamini et al., 2009). Females were classified as: non-pregnant (NPr) and pregnant (Pr).

Females were anesthetized with a combination of 5% ketamine (50 mg/kg) and 2% xylazine (8 mg/kg) (Ketanest, Scott Cassara Laboratory, Bs. As, Argentina), administered intramuscularly. After reaching a deep plane of anesthesia intracardiac perfusion was performed, using physiological solution for the first lavage and then 4% paraformaldehyde in 0.1 M phosphate buffer. This method followed international recommendations for animal testing (Van Zutphen, Baumans, & Beynen, 1999; Zuñiga, Tur Marí, Milocco, & Piñeiro, 2001). The working protocol was previously approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the School of Veterinary Sciences of the National University of La Plata (FCV-UNLP), code 52-4-15T.

Samples processing

The complete genital system was removed from each female. The ovaries were processed for inclusion in paraffin and then serially cut in 3 µm thick sections.

Histology

For general morphology of the ovary, sections were deparaffinized in xylol and hydrated using decreasing concentrations of ethanol until submerged in distilled water. For routine histological staining, sections were immersed in Harris hematoxylin, turned in tap water and washed in distilled water. Then, they were submerged in alcoholic eosin. Finally, samples were dehydrated in increasing concentrations of ethanol, rinsed with xylol and mounted with natural Canada balsam (Álwik, Bs. As, Argentina).

Histochemistry

Some samples were also submitted to histochemical procedures for GCs identification, as detailed in Table 1. Sections were stained either with (1) PAS (periodic acid-Schiff's reagent) to demonstrate periodate-reactive vicinal diols and glycogen; (2) α-amylase digestion before PAS reaction for a control of GCs presence with oxidizable vicinal diols; (3) PA/Bh/KOH/PAS (periodic acid-borohydride reduction-saponification-periodic acid-Schiff reaction): this method was carried out at 2 h oxidation at room temperature with 1% periodic acid (PA). The aldehydes generated by the initial oxidation were reduced to Schiff-unreactive primary alcohols with sodium borohydride (Bh). Following saponification (KOH), only sialic acids with O-acyl substituents at C7, C8, or C9 (or which had two or three side-chains O-acyl substituents) were PAS positive; (4) KOH/PA*/Bh/PAS (saponification-selective periodic acid-borohydride reduction-periodic acid-Schiff reaction) for neutral sugar characterization; (5) AB pH 0.5 (Alcian Blue 8GX pH 0.5) to demonstrate highly sulfated GCs; (6) AB pH 1.0 (Alcian Blue 8GX, pH 1.0) to demonstrate GCs with O-sulfate esters and (7) AB pH 2.5 (Alcian Blue 8GX, pH 2.5) to demonstrate GCs with carboxyl groups (sialic acid or uronic acid). Finally, samples were mounted with natural Canadian balsam (Álwik, Bs. As, Argentina).

Small intestine sections of *L. maximus* were used as positive controls (Tano de la Hoz, Flamini, & Díaz, 2014).

Lectinhistochemistry

Sections were mounted on positive slides, deparaffinized, and immersed in absolute ethanol. Then, samples were immersed in 100 volumes hydrogen peroxide (H_2O_2) activated with 3% methanol, for 30 minutes at room temperature to inhibit the activity of the endogenous peroxidase. Subsequently, they were hydrated, washed with PBS and incubated in a humid chamber with 1% bovine serum albumin in PBS for 30 minutes, to block possible non-specific binding. A battery of 7 biotinylated lectins (Lectin Kit BK 1000, Vector Laboratories, Inc., Burlingame, CA, USA) was used (Table 2). The optimal dilution of each lectin was 30 µg /ml except for PNA (10 µg /ml). Incubation with each of the 7 lectins for the detection of specific carbohydrates was carried out in a humid chamber for 1 h at room temperature (Goldestein & Hayes, 1978). Finally, sections were washed and incubated with streptavidin-peroxidase SA-5704 (Vector Laboratories, Inc., Burlingame, CA, USA) at room temperature for 30 minutes. After washing with PBS, samples were revealed with diaminobenzidine (DakoCytomation, Carpinteria, CA, USA). All sections were counterstained with Mayer's hematoxylin, dehydrated and rinsed. They were then mounted with natural Canada balsam (Álwik, Bs. As, Argentina).As negative controls, slices of ovaries incubated only with PBS were used. As positive controls, sections of vagina from *L. maximus* were used (Flamini, Díaz, Barbeito, & Portiansky, 2012).

Analysis of the samples

Of each ovary, no less than 15 follicles in which the ZP was evident were observed; among them: preantral, secondary, tertiary, polyovulatory and atretic follicles. The intensities of the different techniques were classified according to the following semiquantitative scale: 0, unlabeled; 1, light

mark; 2, moderate mark and 3, strong mark, according to the criterion used in previous works (Liquori et al., 2012; Barbeito, Ortega, Matiller, Gimeno, & Salvetti, 2013; Boonzaier, Van der Merve, Bennett, & Kotze, 2013; Mastrodonato, Mentino, Liquori, & Ferri,2013; Plaul, Barbeito, & Díaz, 2016), including some carried out in the species under study (Flamini et al., 2012; Tano de la Hoz, Flamini, & Díaz, 2016).

The evaluation of each reaction intensity was based on estimates from two independent observers.

For capturing images, a digital video camera (Olympus DP-73, Japan) integrated to an image analysis software (cellSense, Olympus, Japan) was used. Images were saved in TIFF format for later analysis.

Results

General morphology

The ovaries of the viscacha are dorsoventrally flattened, having multiple folds, each of which has a medulla (MZ) and a cortical zone (CZ). Within the latter, follicles at different stages of development (F), interstitial glands (IG) and corpora lutea (CL) are observed (Fig. 1).

Histochemistry

Using the histochemical techniques PAS, α-amylase-PAS and K(OH)/PA*/Bh/PAS, a strong ZP labeling of all the ovarian follicles of both groups of studied females was observed (Fig. 2A). These techniques revealed the presence of GCs with oxidizable vicinal diols and O-acyl sugars. GCs with sialic acid substituted in C7 to C9 and O-acyl sugars were determined with the PA/Bh/KOH/PAS technique. In this case, the reaction was similar to that observed with the previous techniques, either for NPr and Pr females. In NPr the reaction was moderate (Fig. 2B). With the use of the Alcian blue dye at

different pH, a moderate stain was observed for pH 0.5 and pH 1.0 (Fig. 2C), while it was intense at pH 2.5 (Fig. 2D), in both groups. Results are summarized in Table 3.

Lectinhistochemistry

No differences in the lectin binding pattern between different follicular stages of the same reproductive stage were observed. Nevertheless, the location of the glycoside residues present at the ZP of the ovarian follicles of NPr and Pr viscachas was variable. Absence of reaction was observed for both groups of females when PNA, SBA, DBA and UEA-I lectins were used. In contrast, when the WGA lectin was applied, the reaction was moderate and intense for NPr and Pr females, respectively (Fig. 3A-B). The RCA-I lectin exhibited a weak reaction at the ZP of the follicles of NPr females while it was absent in Pr viscachas (Fig. 3C-D). The lectin CON-A, however, showed a negative mark in NPr females while it was weak in the Pr animals (Fig. 3E-F). Results obtained from each lectin are summarized in Table 4.

Discussion

In the present study we investigated the histochemical characteristics of the ZP of the ovarian follicles of *Lagostomus maximus*, and its variations in NPr and Pr females, using histochemical and lectinhistochemical techniques. The results obtained at the ZP using the histochemical PAS and AB at different pH techniques, do not differ from those described by studies carried out in other mammals species such as: *Mus musculus* (Shimizu & Yamada, 1986; Kaufman et al., 1989), *Mesocricetus aureatus* (Delgado & Zoller, 1987), *Sus scrofa scrofa* (Parillo, Diverio, Todini, & Fagioli, 2001), *Melanorivulus punctatus* (Cassel, Mehanna, & Ferreira, 2013), and even in other hystricomorphic rodents such as *Myocastor coypo* (Felipe, Cavodevila, & Callejas, 1999). The α-amylase-PAS, K(OH)/PA*/Bh/PAS and PA/Bh/KOH/PAS a techniques were used to study the presence of sugars,

usually in organs that produce different types of mucins, since they allow differentiating certain features of a specific portion of carbohydrates (McManus, 1948; Lev & Spicer, 1964; Reid, Culling, & Dunn, 1973; Pearse, 1985; Volz, Reid, Park, Owen, & Dunn, 1987). In *L. maximus* these techniques were applied to organs of the digestive system (Tano de la Hoz et al., 2014, 2016; Tano de la Hoz, Flamini, Zanuzzi, & Díaz, 2017). So far, there have been no studies referred to the ZP of the ovary of mammals using these techniques. Therefore, we cannot compare the results obtained in the ovaries of the viscacha with that of other species.

From our results we can establish that the ZP of the viscacha ovarian follicles is composed by several GCs. Among them, there are GCs with oxidizable vicinal diols, O-acyl sugars, substituted sialic acid in C7 to C9, O-sulfated esters and carboxylic groups. Lectinhistochemical analysis showed that ZP do not react to PNA, SBA, DBA, UEA-I lectins, neither in NPr nor Pr females. This indicates the absence of the glycosidic residues: β-D-Gal (β1-3)>D-GalNAc, α-D-GalNAc, β-D-GalNAc, α-D-GalNAc and L-Fuc. In sheep, sows, goats (Parillo, Stradaioli, Dall´Aglio, & Supplizi, 1996), and cows (Supplizi, Monaci, Stradaioli, Greve, & Parrillo, 1996), ZP was also negative for these lectins. In contrast, in a study conducted in rats (Barbeito et al., 2013), ZP of females in pro-oestrus stage do not react with DBA and UEA-I lectins but was positive for PNA and SBA. Nevertheless, in mice only UEA-1 show negative reaction (Shimizu & Yamada, 1986). This observation shows that although the glycoproteins forming ZP are similar in different species, differences in the saccharides that are part of them should be considered.

The positive staining observed with the WGA lectin indicates that ZP of the ovarian follicles of both groups could contain N-acetylglucosamine (GlcNAc). This glycosidic residue is also present in the ovarian follicles ZP of other mammals such as the rat (Barbeito et al., 2013; Avilés, Martinez-Menarguez, Castells, Madrid, & Ballesta, 1994), the mouse (Shimizu & Yamada, 1986), and the wild boar (Parillo et al., 2001). This residue is related to the process of initial recognition of the sperm-ovule, where the superficial enzyme of the male gamete1,4-galactositransferase (GalTase)- binds to

GlcNAc present in one of the three constitutive glycoproteins of the mouse ZP, a species in which fertilization of mammals has been best characterized (Miller, Decker, & Shur, 1993). It has also been determined that removal of GlcNAc residues from glycoprotein ZP3 blocks the binding of sperm (Shur & Hall, 1982; Lopez et al., 1985; Miller, Macek, & Shur, 1992). The presence of this residue in the viscacha ZP could indicate that it plays a similar role during the initial recognition stage between the sperm and the ovule. On the other hand, when using the RCA-I lectin, the presence of the glycosidic residues of β-galactose in the ZP of the ovarian follicles of NPr females but not of Pr viscachas was observed. This type of residue is involved in the oocyte-sperm interaction in different species (Bennoff, 1997; Shalgi & Raz, 1997; Tulsiani, Yoshida-Komiya, & Araki, 1997). Probably the presence of this residue has a similar role in the viscacha, favoring the interaction between both gametes.

The CON-A lectin is specific for β-D-Man and α-D-Glc residues. It has been proposed that β-D-Man is a key molecule in the interaction between oocyte and sperm in humans (Miranda et al., 1997; Maegawa et al., 2002). Studies where this same lectin was used, showed the presence of mannose residues in the ZP of human ovarian follicles (Lucas et al., 1994; Maymon et al., 1994; Talevi, Gualtieri, Tartaglione, & Fortunato, 1997), rat, hamster, rabbit, cat, bitch (Skutelsky, Ranen, & Shalgi, 1994), cows (Nicolson, Yanagimachi, & Yanagimachi, 1975; Amari et al., 2001), mouse (Shimizu & Yamada, 1986) and sows (Yonezawa et al., 2005). The second glycosidic residue identifiable with this lectin is α–D-Glc. Glucose plays a fundamental role during fertilization in the rat and hamster (Niwa & Iritani, 1978; Dravl & Meizel, 1981), although in other species such as guinea pig and bovine, fertilization is inhibited in the presence of this residue (Hyne & Edwards, 1985; Parrish, Susko-Parrish, & First, 1989). In our study it was observed that the ovarian follicles ZP of pregnant viscachas could present both residues. Considering the role of these glucosidic residues in the above

mentioned animals, we might assume that they participate during fertilization of *L. maximus*. In viscacha, follicular development occurs during pregnancy (Flamini et al., 2011), reaching a pseudo-ovulation process that leads to the formation of secondary luteal bodies (Jensen et al., 2006, 2008). The hormonal differences that exist in both groups, generated by the quantity of progesterone-producing luteal bodies during pregnancy, could explain the differences in the glycosylation of the ZP components determined with this lectin. The observed activity of glycosyltranferases within the uterus and oviduct is concurrent with the hormonal changes of the animal (Tulsiani et al., 1997). This suggests that the enzymes may be directly or indirectly regulated by the sex hormones. In an in vitro study with canine oocytes, it was observed that the genes that code for the glycoproteins forming the ZP are regulated by progesterone and estradiol (Kempisty et al., 2015). In *L. maximus*, the levels of progesterone could modulate the activity of the glycosyltranferases of the oocyte, generating a variation in the glycosidic residues that are present in the ZP of NPr and Pr females.

The positive labeling observed with the WGA lectin, which also identifies residues of N-acetylneuraminic acid (sialic acid) and with the histochemical technique PA/Bh/KOH/PAS, which recognizes the same saccharide, allows to establish that sialic acid is present in the ZP of *L. maximus*. This observation coincides with that found in the human (Maymon et al., 1994), rat (Barbeito et al., 2013), golden hamster, rabbit, cat, bitch, sow (Skutelsky et al., 1994), mouse (Shimizu & Yamada, 1986), Rhesus monkey, cow and in the hystricomorphic guinea pig rodent (Soupart & Noyes, 1964) ovarian follicles ZP. This glycosidic residue seems to play a fundamental role during the fertilization process. A study performed in adult mice suggested that sialic acid would remain, in part, associated with glycoproteins until the sperm-oocyte interaction has taken place (Tadano & Yamada, 1978). In

humans this same residue would participate in the sperm-ZP junction (Ozgur, Patankar, Oehninger, & Clark, 1998).

Identification of characteristic residues at the ZP, their distribution and variation in their expression is relevant to understand key processes such as specific recognition among gametes, inhibition of polyspermia, sperm capacitation and fertilization. Knowledge concerning the natural polyovulation process is still scarce in the plain viscacha. Therefore, it is important to delve in the physiology the ovary to understand this process and thus, propose this species as a model that will allow to understand alterations related to the ovulation such as infertility, polycystic ovarian syndrome, both in species of medical and veterinary interest (Rodríguez et al., 2013; Ortega et al., 2016), and carry out reproductive biotechnological studies.

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Conflict of Interests

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Author Contributions

All authors have contributed to this article as follows: Mirta Alicia Flamini, Enrique Leo Portiansky and Claudio Gustavo Barbeito designed the study; Francisco Acuña, María Florencia Tano de la Hoz, Alcira Ofelia Díaz, and Mirta Alicia Flamini performed the experiments and analyzed data; Francisco Acuña, Claudio Gustavo Barbeito, Mirta Alicia Flamini, and Enrique Leo Portiansky drafted the manuscript. All the authors have read and approved the final manuscript as submitted.

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Histochemical techniques	Interpretation	Reference
PAS	GCs with oxidizable vicinal diols and/or glycogen	McManus, 1987
α-amilasa-PAS	GCs with vicinal oxidizable diols	Pearse, 1985
K(OH)/PA*/Bh/PAS	GCs with oxidizable vicinal diols and O-acyl sugars	Volz et al., 1987
PA/Bh/KOH/PAS	GCs with sialic acid substituted in C7 to C9 and O-acyls	Reid et al., 1973
AB pH 0.5	Highly sulfated GCs	Lev & Spicer, 1964
AB pH 1	GCs with O-sulphated esters	Lev & Spicer, 1964
AB pH 2.5	GCs with carboxylic groups and/or with O-sulphated esters	Lev & Spicer, 1964

Table 1. Histochemical techniques used for the visualization and identification of glycoconjugates (CGs).

AB, alcian blue; Bh, borohydride; KOH, potassium hydroxide; PA, periodic acid; PA*, selective periodic acid oxidation; PAS, Schiff's Periodic Acid.

Lectin	Acronym	Affinity
GRUPO I		Glc/Man
Concanavalia ensiformis	Con- A	β-D-Man; α-D-Glc
GRUPO II		GlcNAc
Triticum vulgaris	WGA	β-D-GlcNAc; NeuNAc
GRUPO III		GalNAc/Gal
Dolichos biflorus	DBA	α-D-GalNAc
Glycine maximus	SBA	α-D-GalNAc; β-D-GalNAc
Ricinus communis	RCA-I	β-Gal
Arachis hypogaea	PNA	β-D-Gal (ß1-3)> D-GalNAc
GRUPO IV		L-Fuc
Ulex europaeus	UEA-I	L-Fuc

Table 2. Lectins used, acronyms and glycosidic affinities.

Glc, glucose; Man, mannose; Gal, galactose; GalNAc, N-acetylgalactosmine; GlcNAc, acetylglucosamine; NeuNAc, N-acetylneuraminicacid (sialic acid); L-Fuc, L-fucose.

Histochemical technique	NPr (n=5)	Pr (n=9)
PAS	3	3
α-amilasa-PAS	3	3
K(OH)/PA*/Bh/PAS	3	3
PA/Bh/KOH/PAS	2	3
AB pH 0.5	2	2
AB pH 1	2	2
AB pH 2.5	3	3

Table 3. Histochemical analysis of the ZP in the ovaries of *Lagostomus maximus*. Semi-quantitative scale: 0, unlabelled; 1, light mark; 2, moderate mark and 3, intense mark. NPr: Non-Pregnant; Pr: Pregnant.

Lectin	NPr (n=5)	Pr (n=9)
WGA	2	3
PNA	0	0
SBA	0	0
DBA	0	0
UEA-I	0	0
RCA-I	1	0
CON-A	0	1

Table 4. Glycosylation pattern of the ZP of *Lagostomus maximus*. Semi-quantitative scale: 0, unlabelled; 1, light mark; 2, moderate mark and 3, intense mark.

Figure 1. *Lagostomus maximus*, general morphology of ovary. The cortical zona (CZ) and MZ can be observed. The CZ contains ovarian follicles (F) in different developmental stages, interstitial glands (IG) and corpora lutea (CL). Scale bar = 500 μm.

Figure 2. *Lagostomus maximus*, histochemical characterization of ZP for both groups of females. (A) Labeling of ZP of NPr and Pr females using PAS, α-amilasa-PAS, K(OH)/PA*/Bh/PAS techniques. (B) Labelling of ZP of NPr using the PA/Bh/KOH/PAS technique. (C) Staining of ZP in NPr and Pr females using the AB, pH 0.5 and pH1 techniques. (D) Labelling of ZP of both female groups using the AB, pH 2.5 technique. In all cases, arrow points to the ZP. Abbreviations: ZP, zonapellucida; NPr, non-pregnant; Pr, pregnant. Scale bar = 100 μm.

Figure 3. *Lagostomus maximus*, lectinhistochemical labelling of ZP of both female groups. Images are showing ovarian follicles incubated with WGA (A-B), RCA-I (C-D) and CON-A (E-F) of NPr (A, C, E) and Pr females (B, D, F). In all cases, arrow points to the ZP. Abbreviations: ZP, zonapellucida; NPr, non-pregnant; Pr, pregnant. Scale bar = 100 μm.