Lectin Binding Characteristics of Murine Zona Pellucida During Folliculogenesis; the Importance of N-Acetyl Sugar Derivates

Habibi-Rezaei M.[1]*, Hosseinipanah S.M.[2], Fazel A.R.[3]
[1]*School of Biology, College of Science, University of Tehran, Tehran, Iran*
[2]*Department of Anatomy, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*
[3] *Department of Anatomy, Faculty of Medicine, Mashhad University of Medical Sciences, Mashad, Iran*
[*] *Corresponding author, e-mail: mhabibi@khayam.ut.ac.ir*
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Abstract

Here we report the change in glycodecoration of the oocyte during with folliculogenesis. Five peroxidase-conjugated lectins were used to study their ability to bind to the oocyte at the successive developmental stages. During oocyte maturation process sugar parts of the glycoconjugates from zona pellucida undergo directed biochemical changes to provide glycan configuration suitable for their important physiological functions. In this study we report presence of terminal and accessible sugar moieties of the zona pellucida through sharp distinguishable differences against background of the oocyte by using five peroxidase conjugated lectins. Lectin binding features of the successive stages of the developing zona pellucida through staining with lectins revealed the absence of mannose and non-acetylated forms of galactose and glucose termini and conversely importance of the N-acetylated derivates for both of galactose and glucose as a speculative remark on oocyte maturation process. Moreover presentation of the β-anomers precedes α-anomers in the mentioned sugar termini, based on observed DBA (lectin derived from *Dolichos biflorus*) and PNA (lectin derived from *Arachis hypogaea*) binding capacity.

Keywords: folliculogenesis, glycoconjugate, lectin, oocyte, zona pellucida.

1. Introduction

Several studies have revealed the importance of the carbohydrate moieties of the zona pellucida (ZP) glycoproteins in sperm-zona binding (Wassarman 1995; Shalgi & Raz 1997). In essence, the zona pellucida supports communication between oocyte and follicular cells during oogenesis and serves as a "gate-keeper," to regulate interactions between ovulated eggs and free swimming sperms during and following fertilization which can be ascribed to certain of its featured glycoproteins. The zona pellucida is a thick translucent acellular and extracellular coat that surrounds all mammalian eggs and preimplanted embryos. It forms a spherical shell with remarkable uniform thickness (5-10 microns in eutherian mammals). The structure, composition, distribution and function of zona pellucida glycoproteins have been thoroughly investigated (Wassarman & Mortillo 1991; Green 1997). Some studies support the hypothesis that zona pellucida is constituted by several layers of different materials (Nicolson *et al.* 1975), which agrees with the reported scanning electron-microscopic observations (Phillips & Shalgi 1980). Some other reports, on the other hand support the idea that the zona pellucida is homogeneous (Roux & Kan 1991; Kan *et al.* 1994).

The mouse zona pellucida is composed of three major glycoproteins mZP1, mZP2, and mZP3 with molecular mass of 185-200, 120-140 and 83 kDa respectively (Bleil & Wassarman1980). These structurally integrated glycoproteins in the form of filamentous dimerized ZP2 and ZP3 that are cross-linked by ZP1 have been identified to bind with sperm receptors, leading to sperm acrosome reaction (AR) (Mengerink & Vacquier 2001) through G-protein mediated signal transduction mechanism (Loeser & Tulsiani 1999). These glycoproteins are under-extensive post-translational modification during oogenesis (Green 1997) and probably after ovulation. The glycan portion of these glycoproteins is the species-specific ligand for spermatozoa, which is mirrored by heterogeneity between species (Mori *et al.* 1991; Yurewicz *et al.* 1991). These findings encouraged investigations with lectin histochemistry to monitor the zona pellucida-carbohydrate distribution patterns of ovulated oocytes in laboratory rodents (Avile *et al.* 1996). Several lines of evidence suggest that *O*-linked oligosaccharides of mZP3 are the ligands for sperm

that are involved in primary binding and induction of acrosome reaction (AR) rather than mZP3 *N*-linked oligosaccharides or polypeptide (Liu *et al.* 1997; Wassarman 1999). Also, site directed mutagenesis showed that Ser^{332}→Ala and Ser^{334}→ Ala mutations resulted in complete inactivation of ZP3, indicating the importance of *O*-linked oligosaccharides at these sites (Chen 1998). The structures of the *O*-linked oligosaccharides have been proposed by Easton *et al.* (2000). Similarly to the *N*-linked oligosaccharides, *O*-glycans of the murine ZP are heterogeneous and carry the similar terminal structures as the complex-type *N*-glycans. The majority of the glycans are terminated by Galb1–4GlcNAcb1–6[Galb1–3]GalNAc (Easton *et al.* 2000). Extensive studies have been performed on the zona pellucida of the mature ovulated oocytes. Based on the studies on the recognition mechanism which is involved in fertilization, two hypotheses have been proposed. Wassermann (1988) and (1994) proposed that O-linked oligosaccharides of ZP3 with α-galactosyl residues at their non-reducing termini, mediates detective role in mature oocyte accessible surface. The other is a proposal by Shur (1998), that sperm-egg binding is mediated through interactions in which O-linked sugar chain of ZP3 with non-reducing termini as N-acetyl glucosamine (GlcNAc) residues is involved.

Here we report glycan related maturation process of murine oocyts which have been monitored by surveying the zona pellucida with five horse radish peroxidase (HRP)-conjugated lectins for corresponding terminal sugar moieties. The accessible carbohydrate moieties for lectins have been elucidated for developing follicle, as a featured capacitation mechanism for oocyte side which is correlated with the suggested capacitation features of the sperm side.

2.Material and Methods

All chemicals including Alcian blue and horse radish peroxidase (HRP)-conjugated lectins were purchased from Sigma, St. Louis Mo, USA.

2.1 Tissue collection and processing

Sexually mature mouse (strain, NMRI) with average weight (25-30 gr) and age (8-12 weeks) has been used in this study (n=20). After beginning of ovarian cycle which was determined by examining of external genitalia, mice were sacrified with cervical dislocation and ovaries were kicked out as rapidly as possible and fixed in 10% formalin solution. The tissues were dehydrated through graded alcohols and xylene and embedded in composite paraffin blocks by routine procedure (Bancroft & Gamble 2002). All of the ovary slices were prepared at thickness of 5 µm.

2.2 Lectin Histochemistry

The ovary slices were deparafinized and incubated with defined peroxidase-conjugated lectins. Glycoform descriptive lectins were used in this study and their related carbohydrate binding preference and inhibitory sugars are presented in Table 1. Samples were incubated with 20 µg/ml lectins in 0.1 M phosphate buffer (PBS), pH 6.8, containing 1 mM $MnCl_2$ for 120 minutes at room temperature. To prove the specificity of the lectins, control sections were incubated with lectin in a 0.2 M solution of appropriate inhibitory sugars. These solutions were prepared 30 min before applying to the sections. Unbound lectins were removed by rinsing in PBS and bound lectins were incubated in substrate medium containing 0.03% diaminobenzidine (DAB) and 0.006% H_2O_2 for 10 minutes at room temperature. Then sections were counter-stained with a 1.0% solution of Alciane Blue, pH 2.5 for 1 minute (Brooks *et al.* 1997). Oocyte diameter was used as a marker for oocyte maturity (Griffin *et al.* 2006) and confirmed morphological aspects of developing structures were emphasized to distinguish between successive phases of the folliculogenesis throughout of the study.

Table 1- Lectins used, their sugar specificities and related inhibitory sugars.

Lectin abbreviation	*Source*		*Carbohydrate specificity*	*Inhibitory sugar*
	Taxonomic name	Common name		
PNA	Arachis hypogaea	Peanut	ß-D-Gal(1-3)-D-Gal NAc	α-D-Gal
BS1	Bandeiraea simplifolia	Bandeiraea	α-D-Gal	α-D-Gal
ConA	Canavalia ensiformis	Jack bean	α-D-Man>α-D-Glc	α-methyl-D-gluco/manno- pyranoside
DBA	Dolichos biflorus	Horse gram	α-D-Gal NAc	α-D-Gal NAc
WGA	Triticum vulgaris	Wheat germ	ß -D-Glc NAc	ß -D-Glc NAc

Gal, galactose; Man, mannose; Glc, glucose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine.

2.3 Statistics

Under progression, zona pellucida was graded through staining with representative lectin, at; no, very low, low, moderate and high levels of intensities (Gong *et al.* 1997). The minimum sample size was estimated to be 23 based on α=0.05 and β=0.20. For comparing the groups, non-parametric Kruskall-Wallis one-way analysis of variance test was performed using SPSS for windows v.10.05 software.

3. Results

Accessible carbohydrate moieties of the developing oocytes for HRP-conjugated lectins; ConA, BS1, WGA, PNA and DBA, have been elucidated by lectin treatment of the follicles at various stages of their development. Therefore sharp distinguishable differences against background have been documented. The follicular content and glycoconjugate distribution patterns are not affected from size in any case for zona pellucida samples. To confirm the specificity of lectin binding, control sections were co-treated with peroxidase labeled lectins in the presence of appropriate inhibitory sugar. Those samples abolished staining with horseradish peroxidase substrate.

Photomicrographs treating the issue of successive stages of the follicular development through their lectin binding affinities have been monitored from primary to tertiary stages in which later phases of follicular development or antral phases have been emphasized. In the survey from intermediate to mature follicles, the examined lectins can be categorized as; non-interactive including Con A and BS1 (Fig. 1a) and interactives including WGA, PNA and DBA (Fig. 1b). Concanavalin A (Con A) staining failed to produce significant dense micrograph region throughout of the developing process. Also zona pellucida did not present binding site for *Bandeiraea simplicifolia* derived lectin; BS1, along with its maturation pathway. Corresponding saccharid markers for wheat germ agglutinin; WGA, from zona pellucida are stained from early developmental stage to the late stage of the follicles with almost uniform and strong intensity. Three zona pellucida structural layers have been observed by photomicrography. The intensity of peanut agglutinin **(**PNA) staining increased along with maturation of the follicles. In this regard, there was progressive binding of PNA to intermediate follicle from stage I to pre-ovulatory follicle. In the case of lectin localization for the staining, based on *Dolichos biflorus* derived lectin (DBA); the light binding was found in the zona pellucida of the follicle during primary to pre-antral stages. But zona pellucida transformed to obvious strong positive in later stages of the development.

Figure 2 have summarized semi-quantitative and statistically obtained zona pellucida binding behavior against to five end-glycan representative lectins in which at least 23 samples per stage of cell maturation, have been used.

4. Discussion

The zona pellucida is a relatively thick translucent, acellular coat that surrounds the plasma membrane of fully growing mammalian oocytes. This structure performs a variety of important functions during oogenesis, fertilization and pre-implantation development.

Early studies on zona pellucida strongly suggested that it is a typical glycocalyx, which then was supported with several other reports (Camaioni *et al.* 1996). It has been accepted that distribution of sugar moieties varies during oocyte development and that structural alterations of the complex carbohydrates during embryonic development (Bleil & Wassarman 1980) and pathogenesis (Camaioni *et al.* 1996) have been demonstrated in cells as well as oocyte. The involvement of glycoconjugate structures in cell-cell interactions including fertilization has been widely investigated (Hokke *et al.* 1994) and several studies supports a definite role of zona pellucida-associated glycans in egg-sperm interactions (Wassarman 1999; Hokke *et al.* 1994). Therefore fertilization can be described as a binding process, which is initiated by the recognition of certain oligosaccharide moieties of the zona pellucida glycoconjugates. There is also compelling evidence that carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates on the zona pellucida (Sinowatz *et al.* 1998; Jansen *et al.* 2001).

Here we study thedistribution of sugar residues, which are targets for the corresponding lectins, using a simple and reliable strategy. They undergo directed biochemical changes to set glycan

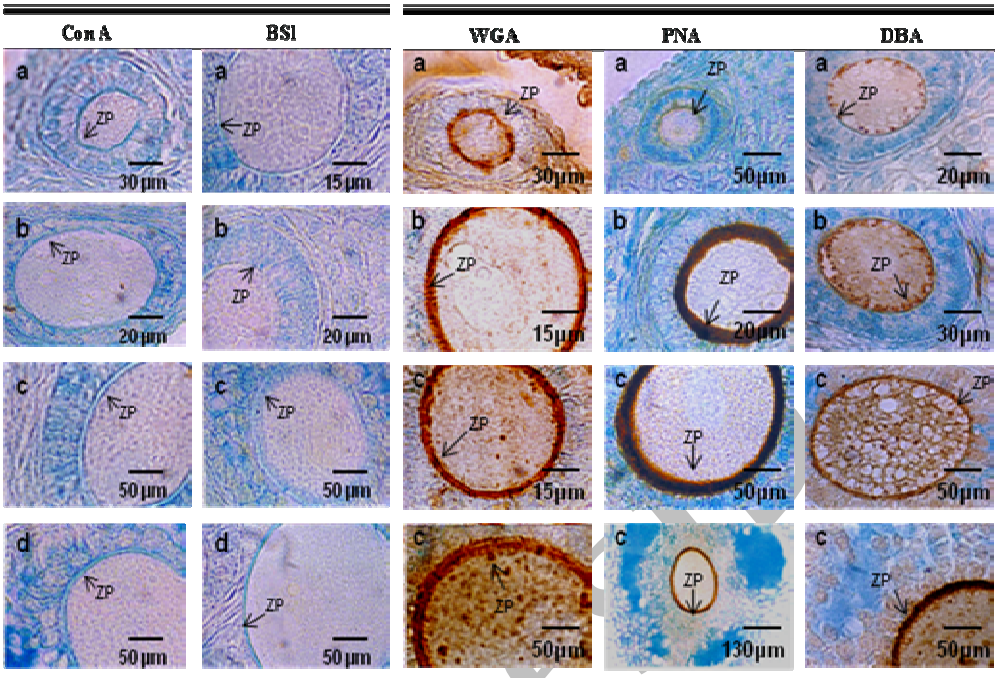

Figure1. Successive stages of the follicular development have been monitored from primary to tertiary stages by emphasizing on later phases of follicular development or antral phases, in two distinguishable groups non-interactive,(a) and interactive lectins (b). Con A (Concanavalin A) staining; a) Intermediate follicle, Stage I, b) Pre-antral follicle, Stage II, c) Antral follicle, Stage II, d) Post-antral follicle, Stage II., BS1 (Bandeirea simplicifolia) staining; a) Intermediate follicle, Stage I, b) Pre-antral follicle, Stage II, c) Antral follicle, Stage II, d) Post-antral follicle, Stage II., WGA (Triticum vulgaris) staining; a) Intermediate follicle, Stage I, b) Secondary follicle, Stage Ш, c) Pre-antral follicle, Stage II, d) Antral follicle, Stage II., PNA (Arachis hypogaea) staining. a) Intermediate follicle, Stage I, b) Pre-antral follicle, Stage II, c) Antral follicle, Stage II, d) Post-antral follicle, Stage IV. and DBA (Dolichos biflorus) binding site during follicular maturation from intermediate to pre-ovulatory follicles: a) Primary follicle, Stage I, b) Pre-antral follicle, Stage II, c) Antral follicle, d) Post-antral follicle, Stage II.; (The magnification for all microphotographs has chosen at ×1000 except at ×400 for steps A and D from PNA).

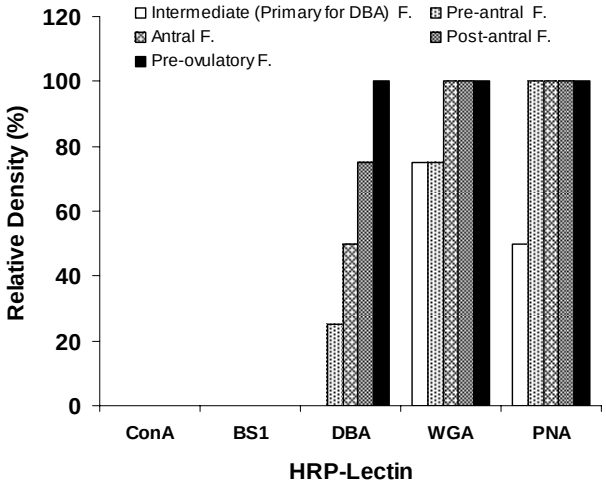

Figure 2. Semi-quantified staining level of the zona pellucida at successive stages of the follicular development (data analysis have been done for at least 23 follicles per stage by using Kruskall-Wallis one-way analysis of variance test). Stages are shown from primary to pre-ovulatory follicle, as following; primary and intermediate follicle (▯), pre-antral follicle (▯), antral follicle (▯), post-antral follicle (▯), and pre-ovulatory follicle (■).

configuration suitable to do their important physiological functions during oocyte development and maturation. From five horseradish peroxidase (HRP)-conjugated lectins which are introduced to follicles at various stages of their development, two of them failed to show significant changes in binding during with the course of follicular maturation. Con A and BS1 did not stain oocytes at none of the developmental stages which reveals the non-significant presence of the corresponding sugars during the follicular maturation process; D-glucose and/or D-mannose end moieties and D-galactose for Con A and BS1, respectively (Fig.1a). This finding is in agreement with the report on non-effective Con A inhibition of sperm-oolema fusion (Gougoulidis *et al.* 1999). Although some reports implicate the necessity of the galactose residues at the nonreducing terminus of *O*-linked oligosaccharides from mouse egg zona pellucida for spermatozoa-ZP binding activity (Bleil & Wassarman 1988) our observation is in accordance with the others who suggest that galactose is unlikely to be related to spermatozoa-ZP recognition (Chiu *et al.* 2003). Based on the present report, WGA was shown to have strong affinity to oocyte envelope, which stained effectively from early; immature intermediate follicle to the late mature; secondary follicle (see Fig. 1b). WGA binding to the zona pellucida shows us the strong presence of N-acetyl-ß-D-glucosamine and/or sialic acid residues along with the all developmental stages from early to the late developing follicles. This observation strictly supports the possible participation of N-acetylglucosamine residues in zona pellucida-sperm interactions. Brooks *et al*. reported that such kind of interactions is perfectly blocked through oocyte treatment with GlcNAc specific lectin (Brooks *et al*. 1997). Shur and Hall (1982) have reported that solubilized polylactosamines inhibit sperm-zona pellucida binding through competing with the surface galactosyltransferase. Galactosyltransferases normally transfer galactose from UDPGal to terminal GIcNAc residues, or to free GIcNAc, to produce N-acetyllactosaminyl linkages (i.e., Gal→ GlcNAc). Their results are interesting in light of the evidence that sperm surface galactosyltransferase is at least one of the sperm receptors for binding to the zona pellucida. Based on this report, the prerequisite to the presence of accessible GlcNAc moieties in the mature oocyte can be resulted. It has been reported that soluble N-acetylglucosamine reduces the binding of human capacitated spermatozoa to zona pellucida (Miranda *et al.* 1997). The number of bound spermatozoa to the zona pellucida is also significantly reduced in the presence of hexosaminidase, which hydrolyzes terminal N-acetylglucosamine (Miranda *et al.* 2000). Morover immunochemical studies reveal that GlcNAc residues dispersed throughout of the zona matrix, and one class of *O*-linked oligosaccharides on ZP3 has been characterized as a small trisaccharide with a terminal GlcNAc (Aviles *et al.* 1997). Although these data further implicate GlcNAc as a participant in sperm binding, whether this specific trisaccharide has exposed to be involved in sperm-binding activity has not yet known. Intense staining of zona structure by HRP conjugated lectin reaction states definite presence of available GlcNAc moieties as a fertilization requisite.

Lectin binding site localization of zona pellucida for two lectins with the ability to recognize both anomers of N-acetyl derivatives of galactose; DBA and PNA, results in a progressive negative to positive in the course of developing process of the follicle. This observation indicates that the corresponding carbohydrate components of oolema from mouse are under vigorous change during follicular maturation. PNA recognizes N-acetyl ß-D-galactosamine (ß-D-GalNAc) at the terminal ß-D-Gal(1-3)-D-GalNAc sequence and DBA recognizes N-acetyl α-D-galactosamine (α-D-GalNAc), presumably indicating the importance of the incorporation either α or β anomers of N-acetylated forms of galactose during with follicle capacitation process. However there is an obvious difference between DBA and PNA series regards in their binding trend presented in Figure 2. The β-anomer of GalNAc is observed even at intermediate stage and achieved to its maximum exposure at pre-antral stage. The α-anomer is not observed at intermediate or primary stage and shows stepwise increase during with successive steps along with the pre-antral, antral, post-antral stages of the follicles and attains maximum value at pre-ovulatory stage of the folliculogenesis.

Trounson and his co-workers suggested that there is a carbohydrate-binding molecule on the sperm that binds GalNAc (Gougoulidis *et al.* 1999) and

others concluded a specific role of N-acetylgalactosamine (GalNAc) moieties and not of galactose (Rivkin *et al.* 2000), during sperm-oolema fusion, which are confirmed in this study. Our observation is in accord also with the interesting report by Loeser and Tulsiani (1999) who have reported that bovine serum albumin (BSA)-based neoglycoproteins which are terminated at multiple positions with a single monosaccharide (GalNAc-BSA, GlcNAc-BSA, or Man-BSA), induce the acrosome reaction in mice. In conclusion, follicle development from early to mature oocyte presumably has been directed to get specific and characterized glycodecoration which is known as capacitated state. Meanwhile, similar complementary process is derived for sperm capacitation in which certain proteins should be presented. The presence of the N-acetylated galactose and glucose as a speculative remark on oocyte maturation have been concluded by lectin-binding character of the successive developmental stages of the zona pellucida which confirms the importance of their presence during follicular maturation.

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