Binding of Lectins to the Zona Pellucida of In Vitro Matured Pig Oocytes and Sperm-Oocyte Interaction In Vitro

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Abstract: *Immature pig oocytes cultured for 36 h in a modified tissue culture medium 199B were freed from cumulus cells and treated for 30 min with fluorescein isothiocyanate-labelled lectins. When examined under fluorescence illumination, Ricinus communis agglutinin (RCA-I) and Lens culinaris agglutinin (LCA) bound to all oocytes, with the strongest fluorescence in either the outer region of the ZP (RCA-I) or throughout the ZP (LCA). However, Bandeiraea simplicifolia lectin-I (BS-I) and Ulex europaeus agglutinin-I (UEA-I) bound with either strong or weak intensity mainly to the outer and inner regions of the ZP, respectively, in ~70–90% of oocytes examined. Bandeiraea simplicifolia lectin-II (BS-II) bound to various regions of the ZP with different intensities in only 60% of oocytes examined. At 2 h after insemination in vitro, significantly fewer spermatozoa were bound to the ZP of lectin-treated oocytes than untrearted control oocytes. Of the lectins, RCA-I and LCA most inhibited sperm binding. At 12 h after insemination, penetration rates were similar in control oocytes and those treated with BS-I or BS-II, but penetration rates were lower in oocytes treated with UEA-I than those in untreated controls, and an almost complete block of sperm penetration was observed in oocytes treated with RCA-I or LCA. The incidence of monospermy was similar in untreated oocytes and those treated with BS-I or UEA-I, but it was higher in oocytes treated with BS-II. These results suggest that β-D-galactose and α-D-mannose residues in the pig ZP may act as primary sperm receptors and/or inducers of the sperm acrosome reaction and β-D-N-acetylglucosamine residues may be involved in the block of polyspermy. The variability in binding of BS-II to the ZP, which correlated with monospermy/polyspermy may reflect differences in the maturation state of individual oocytes. In future, this lectin might provide a means of monitoring maturation in vitro, leading to the development of improved culture systems.*

Key words: *Pig, Oocyte, Zona pellucida, Lectin-binding, Gamete interaction.*

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The zona pellucida (ZP), a relatively thick extracellular coat that surrounds mammalian oocytes, is known to mediate critical steps in fertilization including species-specific binding of spermatozoa, inducing the sperm acrosome reaction and preventing penetration of excess spermatozoa (reviewed in [1, 2]). The mammalian ZP typically consists of a few glycoproteins which have different polypeptide chains and oligosaccharides [3–8] and evidence indicates that these oligosaccharides play an important role in sperm-oocyte recognition in all mammalian species (reviewed in [9–11]). Lectins are cell-agglutinating and sugar specific proteins or glycoproteins of non-immune origin that precipitate glycoconjugates having saccharides of appropriate complementarity [12]. Because of these properties, plant lectins have been used to help characterize the carbohydrate moieties of glycoproteins in the ZP of several mammalian species [13–17] including pigs [18]. Treatment of oocytes with various lectins blocks sperm binding to the ZP in various mammalian species [13, 19–21].

In the pig, the structure and function of carbohydrate chains of the pig ZP glycoproteins have been analyzed [22, 23]; in addition, the distribution of various lectin-binding sites in the ZP of follicular cumulus-oocyte complexes has been evaluated utilizing a post-embedding histochemical approach [17]. However, there is little information on the relationship between sugar residues of the pig ZP and sperm-oocyte interaction. The

Table 1. Lectins used for identifying carbohydrate components in the zona pellucida of *in vitro* matured pig oocytes

Lectin origin	Common name	Acronym	Major sugar specification
Bandeiraea simplicifolia		BS-I	α□-D-galactose
Bandeiraea simplicifolia		BS-II	β-D-N-acetylglucosamine
Ricinus communis	Castor bean	RCA-I	β-D-galactose
Lens culinaris	Common lentil	LCA	α-D-mannose
Ulex europaeus	Gorse	UEA-I	α-L-fucose

present study was undertaken to examine 1) the distribution of sugar residues in the ZP of pig oocytes matured *in vitro* utilizing fluorescein isothiocyanate (FITC)-labelled lectins and 2) the ability of spermatozoa to bind to and penetrate oocytes treated with various lectins.

Materials and Methods

Medium

The basic medium used for manipulation of oocytes and spermatozoa was tissue culture medium (TCM) 199 with Earle's salts (Gibco Lab., Grand Island, NY, USA) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate and 10% (v/v) fetal calf serum (FCS; Gibco). This medium, essentially the same as TCM-199B used by Wang *et al.* [24] except that calcium lactate was omitted, has been designated modified TCM-199B (mTCM-199B).

Collection and maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution containing 75 μg/ml penicillin G and 50 μg/ml streptomycin sulfate at 35–37°C. Oocytes were aspirated from antral follicles of 2–5 mm in diameter with an 18-gauge needle attached to a 10-ml disposable syringe. Oocytes surrounded by compact cumulus and with an evenly granulated cytoplasm were selected and washed 3 times with maturation medium, mTCM-199B (pH 7.4) supplemented with 10 IU/ml eCG and 10 IU/ml hCG. Ten to fifteen oocytes were transferred to a 100-μl drop of the same medium, which had previously been covered with warm paraffin oil in a polystyrene culture dish (35 × 10 mm; Falcon No. 1008, Becton Dickinson Labware, Lincoln Park, NJ, USA) and equilibrated in an atmosphere of 5% CO_2 in air for a minimum of 3 h, and were cultured for 36 h at 39°C under the same atmospheric conditions. It has been reported that 71% of oocytes cultured under these conditions matured to the metaphase II stage [24].

Treatment of oocytes with fluorescein isothiocyanate (FITC)-labelled lectins

After culture, oocytes were placed in Dulbecco's phosphate buffered saline (D-PBS; Sigma Chemical Co., St. Louis, MO, USA) containing 0.1% (w/v) hyaluronidase from bovine testis (Sigma) and freed from cumulus cells by repeated passage through a fine pipette with an inside diameter of about 200 μm. The denuded oocytes were washed 3 times with D-PBS and fixed for 30 min with 3% (w/v) paraformaldehyde (Sigma) in D-PBS at room temperature. Fixed oocytes were washed 3 times with D-PBS and incubated for 30 min with 1 μM FITC-labelled lectins in D-PBS at room temperature. The lectins used in the present study are shown in Table 1.

Examination of binding capacity and distribution of lectins in the ZP

After treatment with FITC-labelled lectins, oocytes were washed 3 times with D-PBS and placed in the centre of 4 vaseline-paraffin (1:9) spots on a glass slide. After being compressed gently with a cover-slip, the slides were sealed with colourless nail polish. To evaluate the binding capacity and distribution of FITC-labelled lectins in the ZP, oocytes were examined under a fluorescent microscope (DIAPHOT-TMD-EFZ: Nikon, Tokyo, Japan) equipped with 100 W mercury lamp and DM 510 filter (Nikon), at a magnification of × 100 or × 200. The binding capacity of FITC-labelled lectins in the ZP was classified as a positive or negative fluorescence. The degree of positive fluorescence was classified semi-quantitatively as strong or weak. In preliminary experiments, the specificity of the lectins was confirmed by demonstrating that inclusion of excess specific sugars completely inhibited lectin binding (data not shown).

Preparation of oocytes and spermatozoa for in vitro fertilization

Oocytes freed from cumulus cells after maturation culture were incubated for 30 min with 1 μM FITC-labelled lectins in D-PBS at room temperature and washed 3 times with fertilization medium, mTCM-199B (pH 7.8)

Table 2. Binding of lectins to the zona pellucida (ZP) of *in vitro* matured pig oocytes[a]

Lectin[b] (1 μM)	No. of oocytes examined	% (mean ± SEM) of oocytes with ZP stained		
		Total	With strong fluorescence[c]	With weak fluorescence[c]
BS-I	43	88.6 ± 1.8[d]	56.4 ± 2.4[d]	43.6 ± 2.4[d]
BS-II	76	60.2 ± 7.9[e]	30.5 ± 4.3[e]	69.5 ± 4.3[e]
RCA-I	40	100.0[d]	100.0[f]	0.0[f]
LCA	50	100.0[d]	100.0[f]	0.0[f]
UEA-I	78	68.4 ± 3.4[e]	34.2 ± 2.4[d]	65.8 ± 2.4[d]

[a]Experiments were repeated four times. [b]Refer to Table 1 for lectin origin. [c]Percentage of oocytes with ZP stained. [d, e, f]Values with different superscripts within each column differ significantly, $P<0.01$.

supplemented with 2.92 mM hemicalcium lactate and 10 mM caffeine sodium benzoate (Sigma), which had previously been equilibrated for about 3 h in an atmosphere of 5% CO_2 in air at 39°C. Then 10–15 oocytes were put into a 50-μl drop of the same medium in a polystyrene culture dish (35 × 10 mm; Falcon) and kept in a CO_2 incubator (5% CO_2 in air at 39°C) for about 30 min until spermatozoa were added.

Frozen ejaculated spermatozoa (3 pellets each with a 100-μl in volume) were thawed in 2 ml of caffeine-free fertilization medium at 39°C for 1 min. After thawing, 6 ml of the same medium were added and spermatozoa were washed 3 times by centrifugation at 550 × g for 5 min each. The final sperm pellet was resuspended in the same medium as used for washing to give a concentration of 10^7 spermatozoa/ml. A 50-μl aliquot of the sperm suspension was introduced into a 50 μl drop of fertilization medium that contained oocytes for insemination, thus producing a mixture with final concentrations of 5×10^6 spermatozoa/ml and 5 mM caffeine.

Examination of sperm binding and penetration of lectin-treated oocytes

At 2 h post-insemination, some oocytes were removed and passaged through a fine pipette with an inner diameter of about 250 μm 30 times to remove the weakly attached spermatozoa from the surface of the ZP. Then, oocytes were fixed for 30 min in 3% (v/v) paraformaldehyde, stained for 3–5 min with 10 μg/ml bis-benzimide (Hoechst 33342; Sigma) in D-PBS to label attached spermatozoa and placed on a glass slide. The number of spermatozoa firmly bound to the ZP was counted using a fluorescent microscope with DM 400 filter (Nikon) at a magnification of × 200.

The remaining oocytes were cultured further with spermatozoa until 12 h post-insemination when all spermatozoa attached to the ZP were removed by repeated passage through a fine pipette with an inner diameter of about 200 μm. Then the oocytes were mounted, fixed for about 72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined for evidence of sperm penetration under a phase-contrast microscope at a magnification of × 200 or × 400. Oocytes were considered penetrated when they had one or more decondensing sperm nuclei and corresponding sperm tail(s).

In an additional experimental protocol, unfertilized oocytes were preincubated in *Bandeiraea simplicifolia* lectin-II (BS-II) as described earlier, evaluated for fluorescence and grouped according to intensity of fluorescence (none, weak and strong). These three groups of lectin-treated oocytes plus untreated control oocytes were inseminated and then evaluated 12 h later.

Statistical analysis

All proportional data were subjected to arc-sine transformation. The transformed values were analyzed using one-way ANOVA. When ANOVA revealed a significant treatment effect, each treatment was compared by Fisher's protected LSD test.

Results

As shown in Table 2, higher ($P<0.01$) proportions of oocytes showed fluorescence of the ZP after treatment with *Bandeiraea simplicifolia* lectin-I (BS-I; 88.6 ± 1.8%), *Ricinus communis* agglutinin (RCA-I; 100%) and *Lens culinaris* agglutinin (LCA; 100%) than after treatment with *Bandeiraea simplicifolia* lectin-II (BS-II; 60.2 ± 7.9%) and *Ulex europaeus* agglutinin-I (UEA-I; 68.4 ± 3.4%). All of the oocytes treated with RCA-I exhibited strong fluorescence in the outer region of the ZP (Fig. 1A)

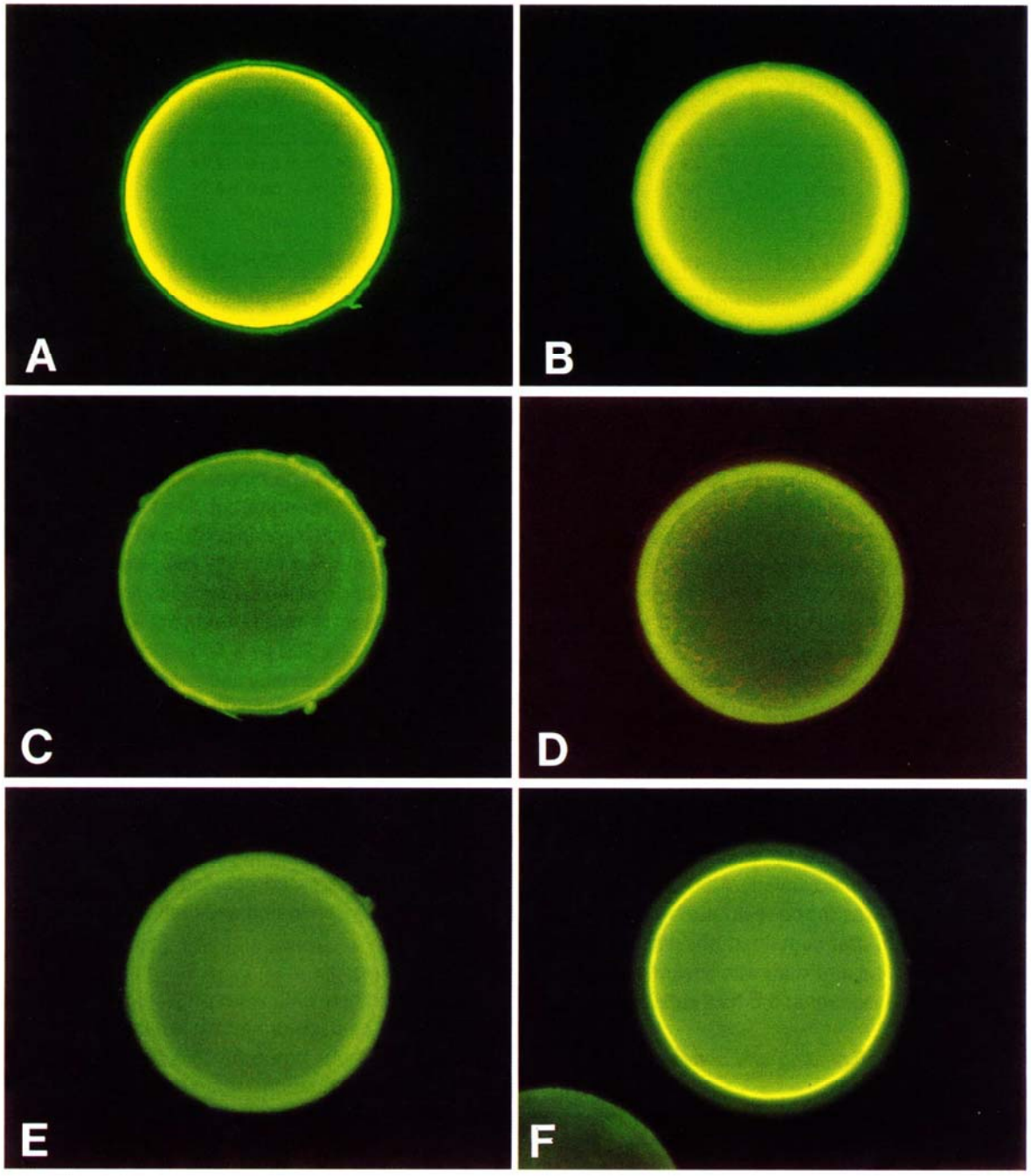

Fig. 1. Fluorescence micrographs of lectin-binding to the zona pellucida (ZP) of *in vitro* matured pig oocytes. Cumulus-free oocytes were treated for 30 min with 1 μM fluorescein isothiocyanate-labelled lectins. *Ricinus communis* agglutinin (A) and *Lens culinaris* agglutinin (B) bound mainly to the outer region of the ZP and throughout the ZP, respectively, showing strong fluorescence. *Bandeiraea simplicifolia* lectin-I (C) and *Ulex europaeus* agglutinin (D) bound mainly to the outer and inner regions of the ZP, respectively, showing strong or weak fluorescence. However, *Bandeiraea simplicifolia* lectin-II bound throughout the ZP with weak fluorescence (E) and, in some case, only to inner (F) or outer region of the ZP with strong fluorescence. × 253.

while those treated with LCA exhibited strong fluorescence throughout the ZP (Fig. 1B). BS-I bound mainly to the outer region (Fig. 1C) and UEA-I bound mainly to the inner region of the ZP (Fig. 1D), with either strong or weak fluorescence. In contrast, about 40% of oocytes treated with BS-II did not exhibit any fluorescence in the ZP. In oocytes with the ZP stained, 2 different distributions of fluorescence were observed: 70% had weak fluorescence mostly throughout the ZP (Fig. 1E) but, in the remaining 30%, BS-II bound mostly to the inner (Fig. 1F), and occasionally to the outer, regions of the ZP with strong fluorescence.

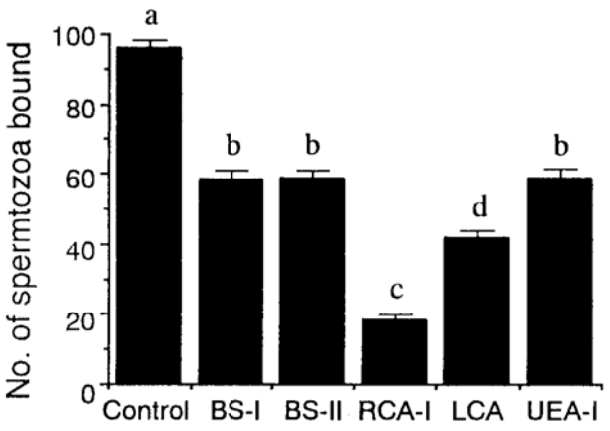

Fig. 2. Binding of spermatozoa to the zona pellucida of lectin-treated, *in vitro* matured pig oocytes. Cumulus-free oocytes were treated for 30 min with 1 μM fluorescein isothiocyanate-labelled lectins, then incubated for 2 h with spermatozoa; experiments were repeated 4 times using a total of 40 oocytes in each treatment. Bars with different superscripts differ significantly (P<0.01) in the numbers of bound spermatozoa. BS-I: *Bandeiraea simplicifolia* lectin-I; BS-II: *Bandeiraea simplicifolia* lectin-II; RCA-I: *Ricinus communis* agglutinin; LCA: *Lens culinaris* agglutinin; UEA-I: *Ulex europaeus* agglutinin.

As shown in Fig. 2, significantly fewer (P<0.01) spermatozoa were bound to the ZP of lectin-treated oocytes than untreated oocytes evaluated 2 h after insemination. Of the lectins used, RCA-I and LCA inhibited sperm binding to the ZP more than BS-I, BS-II and UEA-I.

As shown in Table 3, when oocytes were pretreated with BS-I or BS-II, the penetration rate (~80%) did not differ significantly from that of untreated oocytes (~91%). However, in oocytes treated with UEA-I the penetration rate was significantly (P<0.01) lower (~67%) than in untreated controls. An almost complete block of sperm penetration was observed in oocytes treated with RCA-I or LCA. Although the incidence of monospermy did not differ between untreated oocytes (~21%) and those treated with BS-I or UEA-I (~27–30%), it was significantly (P<0.01) higher in those treated with BS-II (~36%). The mean number of spermatozoa in penetrated oocytes was significantly (P<0.01) lower in all lectin-treated oocytes (~1–2 spermatozoa/oocyte) compared with untreated controls (~3 spermatozoa/oocyte).

When oocytes treated with BS-II were divided into 3 groups on the basis of intensity of fluorescence (none, weak fluorescence throughout the ZP or strong fluorescence in either the inner or outer region of the ZP), very high penetration rates (~82–91%) were obtained in all groups of oocytes whether treated with lectins or not (Table 4). However, the highest rate of monospermy (~47%) and the lowest number of spermatozoa in penetrated oocytes (1.7 ± 0.3 spermatozoa/oocyte) were observed in oocytes with strong fluorescence (P<0.05).

Discussion

The results of the present study indicate that: (1) at least 5 different carbohydrate residues, with various distributions, are present in the ZP of pig oocytes matured *in vitro*; (2) β-D-galactose(β-Gal) and α-D-mannose (α-Man) residues are found primarily in the outer region of

Table 3. Sperm penetration *in vitro* of *in vitro* matured lectin-treated pig oocytes[a]

Lectin[b] (1 μM)	No. of oocytes examined	%[c] (mean ± SEM) of oocytes penetrated		No. (mean ± SEM) of spermatozoa in penetrated oocytes
		total	Monopermy	
Control	54	90.7 ± 5.7[d]	21.2 ± 2.1[d]	3.0 ± 0.4[d]
BS-I	51	79.5 ± 4.1[de]	27.0 ± 2.0[de]	2.0 ± 0.3[e]
BS-II	54	78.2 ± 7.6[de]	35.9 ± 3.5[e]	1.7 ± 0.3[e]
RCA-I	56	0.0[f]	–	–
LCA	54	1.7 ± 1.7[f]	1.7 ± 1.7[f]	1.0 ± 0.3[f]
UEA-I	51	67.0 ± 3.7[e]	29.6 ± 2.4[de]	1.6 ± 0.2[e]

[a]Experiments were repeated four times. [b]Refer to Table 1 for lectin origin. [c]Percentage of oocytes examined. [d, e, f]Values with different superscripts within each column differ significantly, P<0.01.

Table 4. Correlation between sperm penetration *in vitro* and different intensities of *Bandeiraea simplicifolia* lectin-II (BS-II) binding to the zona pellucida of *in vitro* matured pig oocytes[a]

Intensity of BS-II binding	No. of oocytes inseminated	%[b] (mean ± SEM) of oocytes penetrated		No. (mean ± SEM) of spermatozoa in penetrated oocytes
		Total	Monospermy	
Control	52	86.6 ± 3.4	28.3 ± 1.3[c]	2.9 ± 0.3[c]
Strong	68	81.5 ± 4.3	46.5 ± 3.5[d]	1.7 ± 0.3[d]
Weak	60	87.5 ± 5.1	24.6 ± 3.1[c]	2.4 ± 0.4[c]
No binding	69	90.6 ± 5.7	9.6 ± 3.7[e]	3.1 ± 0.5[c]

[a]Experiments were repeated four times. [b]Percentage of oocytes inseminated. [c, d, e]Values with different superscripts within each column differ significantly, $P<0.05$.

the ZP and throughout the ZP, respectively, and appear to play roles as sperm receptors both for initial binding to the ZP and for sperm penetration; and (3) binding of BS-II to β-D-*N*-acetylglucosamine (β-Glc*N*Ac) residues especially in the inner region of the ZP may increase the incidence of monospermic fertilization.

Each lectin is considered to bind to specific carbohydrate residues [25]; in the ZP of mammalian oocytes these lectins may provide the key for the species specificity of gamete interaction [17]. Consistent with earlier investigations of mouse oocytes [14], differences in the intensity and distribution of binding of 5 different lectins to the pig ZP were observed in the present study, reflecting variations in the content or availability of different carbohydrate residues. RCA-I and LCA exhibited the strongest binding and were found in the outer region of the ZP and throughout the ZP in all oocytes examined, respectively. This suggests the presence of many glycoconjugates with β-Gal and α-Man residues in their terminal regions in the pig ZP. The abundant distribution of oligosaccharides with terminal Gal residues has been demonstrated in the pig ZP [4, 5, 26–28], along with β-Gal and α-Man residues which have been observed in most mammalian ZP including pig [17] and human [29]. On the other hand, BS-I and UEA-I bound to the ZP with a reduced intensity in about 70–90% of oocytes examined in the present study. In an earlier study, histochemical analysis of lectin binding in follicular oocytes in paraffin wax-embedded ovaries was unable to demonstrate the presence of α-D-galactose (α-Gal) and α-L-fucose (α-Fuc) residues in the pig ZP [17]. Taken together, these results suggest that glycoconjugates with α-Gal and α-Fuc residues are not abundant in the pig ZP. Under the present experimental conditions, BS-II bound to the different regions of each ZP with variable intensity. The possibility that this variability in the distribution of β-Glc*N*Ac residues in the pig ZP reflects differences in *in vitro* maturation of individual oocytes needs to be examined further.

The carbohydrate residues of the ZP glycoproteins are known to play an important role in sperm binding to the ZP (reviewed in [9, 10]). Gal [30, 31], Man [32] or Glc*N*Ac [33–36] residues of the mouse ZP may function as sperm receptors. Furthermore, Fuc, Man and methyl-Man residues of the rat ZP [37] and Fuc and Gal residues of the hamster, guinea-pig and human ZP [38] may participate in sperm binding. The pig ZP is composed of three structurally and immunologically distinct glycoproteins designated as ZP1, ZP3α and ZP3β [4, 5]. It has been suggested that the ZP3 family plays a major role in binding of spermatozoa to the ZP [39, 40] as well as in inducing spermatozoa to undergo the acrosome reaction [41]. In the present study, when oocytes were treated with BS-I or BS-II, the number of spermatozoa bound to the ZP was significantly lower when compared with untreated control oocytes but penetration rates remained high. These results suggest that α-Gal and β-Glc*N*Ac residues in the pig ZP3 participate in sperm-oocyte binding but not in inducing the acrosome reaction. In contrast, binding of RCA-I or LCA to the ZP inhibited both binding and penetration of spermatozoa to the ZP, suggesting that β-Gal and α-Man residues may participate in both sperm-oocyte recognition and induction of the acrosome reaction.

Unlike the other lectins investigated, binding of BS-II to the ZP exhibited a variable pattern which differed with each oocyte; although the penetration rate of lectin-treated oocytes was the same as that of untreated control oocytes, the incidence of monospermic fertilization was significantly higher. These results may indicate that the distribution of β-Glc*N*Ac residues within the pig ZP reflects the degrees of oocyte maturity achieved *in vitro*. The lectin-binding pattern of immature rat oocytes has been shown to differ from that of mature oocytes, both in the binding capacity for certain lectins and in the distribution of lectin receptors within the ZP [16]. Furthermore, *N*-acetylglucosaminidase localized in cortical granules of mouse oocytes, appears to be responsible

for the loss in sperm-binding activity that constitutes the zona block to polyspermy [34]. Since, in the present study, the highest incidence of monospermy was obtained in oocytes with the greatest binding of BS-II to the ZP, the β-Glc*N*Ac residues may be involved primarily in preventing polyspermy. Perhaps BS-II could be used to assess the relative maturity of pig oocytes cultured *in vitro* to induce maturation. This could lead to improved culture conditions that could promote more uniform and consitent maturation.

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