

Binding characteristics of sperm with recombinant human zona pellucida glycoprotein-3 coated beads

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Background & objectives: An inability or decreased ability of spermatozoa to bind to the zona pellucida (ZP), an extracellular glycoproteinaceous matrix surrounding egg, is one of the plausible causes of idiopathic infertility. It will be clinically useful to distinguish this condition from other causes of infertility. An assay system, investigating binding of human sperm with ZP glycoprotein may prove useful in this regard. We attempted to develop a simple assay system to analyse the binding of capacitated human spermatozoa to human zona pellucida glycoprotein-3 (ZP3) using baculovirus-expressed recombinant human ZP3 coated beads.

Methods: Recombinant baculovirus-expressed ZP3 was purified, labelled with biotin and coated on streptavidin sepharose beads. An *in vitro* assay system was optimized to study binding of capacitated human sperm to ZP3 coated beads.

Results: A higher percentage of baculovirus-expressed recombinant human ZP3 coated beads showed significant ($P<0.05$) binding of capacitated human sperm as compared to beads coated with fetuin. An inhibition in the binding of sperm to ZP3 coated beads was observed in presence of cold recombinant human ZP3. Further, prior incubation of ZP3 coated beads with monoclonal antibodies (MAbs) against ZP3 but not against ZP2 resulted in the decrease in number of sperm bound to bead.

Interpretation & conclusion: An *in vitro* assay system to study the binding of human sperm to ZP3-primary sperm receptor was established, which may be useful to determine the functional competence of spermatozoa.

Key words Baculovirus-expression system - human zona pellucida glycoprotein-3 - monoclonal antibody - spermatozoa - streptavidin sepharose beads

In mammals, fertilization is a well-orchestrated event culminating in the union of two highly specialized cells *i.e.*, the spermatozoon and the egg. Binding of sperm after going through a series of changes in female reproductive tract, termed 'capacitation', to the zona pellucida (ZP) of the oocyte is an important

step for successful fertilization. ZP not only plays a crucial role in fertilization by serving as a species-selective substrate for sperm binding but also acts as an agonist for regulated exocytosis of the spermatozoon's acrosomal vesicle¹. Inability or decreased ability of spermatozoa binding to the ZP is considered as one

of the plausible causes of idiopathic infertility. It may be clinically useful to distinguish this condition from other causes of infertility².

The ZP in mammals is composed of 3 to 4 biochemically distinct highly sulphated glycoproteins. The ZP matrix of mice, pigs and rabbits is comprised of 3 glycoproteins whereas 4 glycoproteins constitute the ZP matrix of rats, monkeys and humans³⁻⁵. In humans, these glycoproteins have been designated as ZP1, ZP2, ZP3 and ZP4. In mice as well as humans, ZP3 has been shown to be the primary sperm receptor⁶⁻⁸. Our group has reported expression of recombinant human ZP3 in prokaryotic (*Escherichia coli*) and eukaryotic (insect cells using baculovirus expression) system⁷. Both non glycosylated and glycosylated forms of recombinant human ZP3 binds to the anterior head of the capacitated human spermatozoa⁸. No binding of recombinant human ZP3 however, was observed in the anterior head region of the acrosome reacted spermatozoa⁸. Our group has also reported the development of highly specific monoclonal antibodies (MAbs) against recombinant human ZP3, which recognize native human ZP3⁹. Employing, one of the specific MAb against recombinant human ZP3, recently purification of native human ZP3 from human egg has been successfully achieved¹⁰. Binding studies employing purified native human ZP3 revealed similar binding profile as baculovirus-expressed recombinant human ZP3¹¹.

In the present study, employing baculovirus-expressed recombinant human ZP3 coated on streptavidin sepharose beads, we developed a simple assay system for analysis of binding of capacitated human spermatozoa to ZP3. In addition, the efficacy of the ZP3 specific MAbs to inhibit binding of sperm to ZP3 coated sepharose beads has also been evaluated.

Material & Methods

Purification of baculovirus-expressed recombinant human ZP3 and its biotinylation: Cloning and expression of recombinant human ZP3 (aa residues 1-424) as polyhistidine tagged fusion protein in the baculovirus-expression system has been reported earlier⁷. For large scale production of the recombinant protein, a suspension culture of 50 X 10⁶ *Spodoptera frugiperda* (*Sf21*) insect cells (BD Pharmingen, San Diego, CA, USA) growing in a Spinner bottle (Thermolyne, Barnstead International, Iowa, USA) was incubated with the ZP3 recombinant virus at a multiplicity of infection (MOI) of 3 at 42 rotations per minute (rpm) on a biological stirrer (Thermolyne Cellgro, USA) at

27°C. After 96 h of incubation, cells were pelleted at 1000 × g for 15 min, and recombinant ZP3 purified using Ni-NTA resin (Qiagen GmbH, Hilden, Germany) as described previously⁷. The purified recombinant protein in 20 mM Tris (pH 7.4) was assessed for protein concentration using bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA) using bovine serum albumin (BSA; Sigma-Aldrich Inc., St. Louis, MO, USA) as the standard.

For biotinylation, 1 mg of the purified ZP3 or Fetuin (taken as negative control in the study; Sigma-Aldrich Inc. USA) was extensively dialyzed against biotin labelling buffer (0.1 M NaHCO₃, 0.1 M NaCl, pH 8.0) and conjugated to NHS-LC-biotin (Pierce, USA). The NHS-LC-biotin used here had spacer arm of 13.5 Å. The conjugation reaction was performed at room temperature (RT) for 1 h by incubating the protein with freshly prepared NHS-LC-biotin at a 1:20 molar ratio, with end-to-end shaking. Post-incubation, unreacted sites were blocked with 0.2 M glycine in 0.1 M NaHCO₃, 0.1 M NaCl, pH 8.0, for 30 min at RT and the biotin labelled protein was extensively dialyzed against dialysis buffer (0.1 M Tris, 0.2 M NaCl, pH 8.0) for 24 h. The concentration of the biotinylated protein was determined by BCA. To assess the quality of biotinylated ZP3, it was resolved on 0.1 per cent SDS- 10 per cent PAGE followed by Commassie blue staining as described previously⁷. In addition, resolved protein was processed for Western blot analysis after transferring protein to nitrocellulose membrane of 0.45 µm (Advanced Microdevices Pvt Ltd., Ambala Cantt, India), employing avidin-HRPO conjugate (1:3000; Pierce), as described elsewhere⁷.

Coating of streptavidin sepharose beads with biotinylated recombinant human ZP3 and fetuin: Streptavidin sepharose beads (6% beads suspension, mean particle size 34 µm, 25 µl; GE Healthcare Biosciences AB, Uppsala, Sweden) were washed twice with 0.1 M Tris, 150 mM NaCl (pH 7.4; TBS) and incubated with varying amount of biotinylated ZP3 at RT for 2 h with intermittent shaking. In addition, sepharose beads were incubated with BSA or biotinylated fetuin as negative controls under similar conditions. After incubation, coated beads were pelleted at 1000 × g, washed twice with TBS, resuspended in 100 µl of TBS and stored at 4°C till further use. Coating of recombinant human ZP3 on beads was confirmed by Western blot as described above.

Human spermatozoa and capacitation: All experiments using human spermatozoa were carried out after

informed consent and following the clearance from the Institutional Bio-safety and Human Ethical Committees. Semen samples were collected from healthy voluntary donors and subjected to liquefaction at RT for 30 min. Aliquots (0.5 ml) of semen were layered over a two-step Percoll density gradient¹². The gradient layered with semen was centrifuged at 500 × g for 30 min at RT. The pellets comprising of >90 per cent motile spermatozoa were pooled and washed thrice with 5 ml of Biggers-Whitten-Whittingham medium¹³ (BWW; pH 7.4) supplemented with 0.3 per cent BSA (Sigma Aldrich Inc., embryo tested) by centrifugation at 500 × g for 10 min. The sperm pellet was resuspended in BWW medium supplemented with 2.6 per cent BSA at a concentration of 10×10^6 sperm/ml and incubated in aliquots of 1 ml for 6 h at 37°C in a humidified chamber with 5 per cent CO₂ in air for capacitation.

Binding of ZP3 or fetuin coated beads with capacitated human sperm: ZP3 or fetuin coated beads suspension (20 µl/reaction) was washed once with 500 µl of BWW medium and resuspended in 25 µl of BWW medium + 0.3 per cent BSA. To the ZP3 coated bead suspension, capacitated human sperm (1×10^6) in 25 µl of BWW medium + 0.3 per cent BSA were added and incubated at 37°C in humidified chamber with 5 per cent CO₂ for 15 min followed by 30 min at 4°C. After incubation, 500 µl of BWW medium + 0.3 per cent BSA was added to the reaction mixture and beads were centrifuged at 50 × g for 2 min at 25°C. Supernatant was discarded to remove unbound sperm. Pellet was resuspended in 100 µl of 3 per cent glutaraldehyde (Sigma Aldrich Inc.) in 50 mM PBS, pH 7.4, and incubated at RT for 40 min for fixing of the spermatozoa bound to beads. After incubation, beads were washed once with PBS and spotted on clean glass slides and number of sperm bound to the coated beads were counted microscopically (Nikon, Chiyoda-ku, Tokyo, Japan) under bright field using 40X objective. In all the binding experiments, at least 100 beads were scored for each reaction and the experiment was repeated four-five times with semen sample from three different male donors to rule out any ambiguity. For each group, the percentage of beads showing binding with sperm were determined by counting the number of beads showing binding with one or more capacitated sperm. Number of sperm bound per bead was calculated by total number of bound sperm divided by the number of counted beads in different fields. Additionally, data have been convoluted to represent number of sperm bound per bead showing binding of sperm.

To study the specificity of binding of spermatozoa with ZP3, ZP3 or fetuin coated beads, were pre-incubated with varying amounts of purified ZP3 specific MAbs generated as described previously⁹. ZP3 coated beads pre-incubated with human ZP2 specific MAbs served as an internal control. Subsequently, these beads were washed once with 50 mM PBS and used for binding of sperm as described above.

Statistical analysis: Results were expressed as mean ± SEM of 4-5 different experiments using semen samples from at least three different donors. The statistical analysis was done by comparing the means of the either uncoated or fetuin coated beads with ZP3 coated beads group or within two different experimental groups by using one way ANOVA followed by Newmans-Keuls multiple comparison test employing CCSTAT Quick Statistics For Optometrist (Version 3.5, India). $P < 0.05$ was considered to be statistically significant.

Results

Characterization of biotinylated baculovirus-expressed recombinant human ZP3: To study the interaction of human ZP3 with capacitated spermatozoa, baculovirus-expressed recombinant human ZP3 was purified by Ni-NTA affinity chromatography and labelled with NHS-LC-biotin. The analysis of biotinylated ZP3 by SDS-PAGE and Western blot revealed a prominent band of ~56 kDa (Fig. 1a and 1b respectively).

Binding of capacitated human sperm with ZP3 coated beads: Streptavidin sepharose beads coated with 2, 5, 10 and 25 µg of biotinylated recombinant human ZP3 were employed to study the binding of capacitated human spermatozoa. Significantly higher number of ZP3 coated beads as compared to uncoated beads or fetuin coated beads showed binding of capacitated human sperm ($P < 0.05$; Table I). Not only the higher number of ZP3 coated beads showed sperm binding but an increase in the number of sperm bound per ZP3 coated bead as compared to control beads was also observed. Beads coated with 10 µg of biotinylated ZP3 showed optimum binding of sperm (Table I). A representative profile of ZP3 coated bead showing binding of sperm as compared to fetuin coated bead is shown in Fig. 2. Majority of the sperm showed binding of the head to the recombinant ZP3-coated beads. Further optimization of the assay, employing different concentrations of ZP3 coated beads revealed that 0.096 per cent bead suspension resulted in optimum binding of spermatozoa to the beads (Table II). A positive correlation was seen between number of beads and number of sperm/bead showing binding.

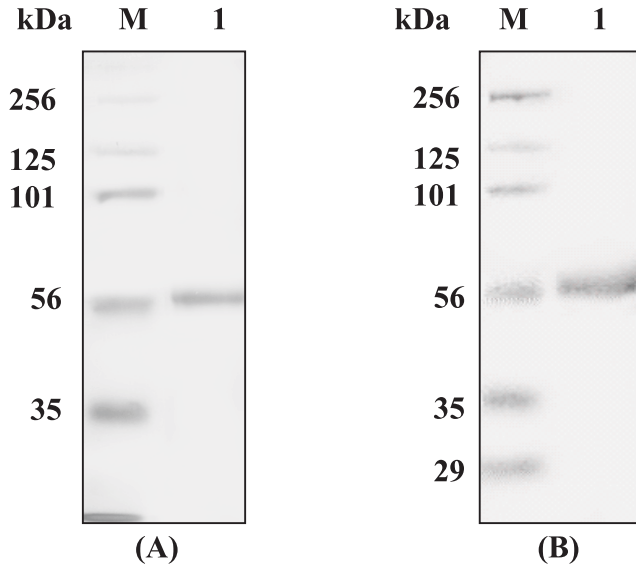


Fig. 1. Analysis of purified recombinant human ZP3 conjugated to NHS-LC-biotin by SDS-PAGE and Western blot. The purified baculovirus-expressed recombinant human ZP3 was conjugated to NHS-LC-biotin, resolved by 0.1 per cent SDS-10 per cent PAGE under reducing conditions and analyzed by Commassie blue staining (Panel A) & Western blot (Panel B) using avidin-HRP conjugate (1:2K). Lanes are represented as M: Molecular weight markers; Lanes 1: Baculovirus-expressed recombinant human ZP3 conjugated to NHS-LC-biotin (5 μ g/lane in Panel A and 2 μ g/lane in Panel B).

Linear regression analysis showed that up to 0.144 per cent of stock ZP3 beads (~2100 beads), sperm binding increased in a linear fashion (Coefficient of Regression, $r=0.9794$; Fig. 3). Binding of capacitated sperm to ZP3 coated beads was specific as a decrease in the percentage of beads showing sperm binding along with the number of sperm bound per bead was observed in presence of unlabelled ZP3 (Table III). Further, prior incubation of ZP3 coated beads with ZP3 specific MAbs (MA 1552 and MA 1558) also resulted in a decrease in percentage

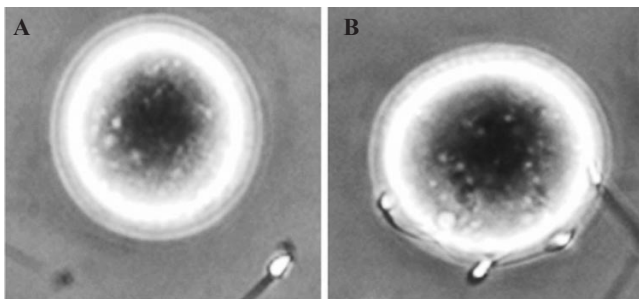


Fig. 2. Representative profile of binding of capacitated human sperm with human ZP3 coated beads. Binding of the capacitated human sperm to the baculovirus-expressed recombinant human ZP3 coated streptavidin sepharose beads along with fetuin coated beads used as negative control, was carried out. Panel A: Binding of sperm to fetuin coated bead (negative control) and Panel B: Binding of sperm to ZP3 (10 μ g ZP3) coated bead.

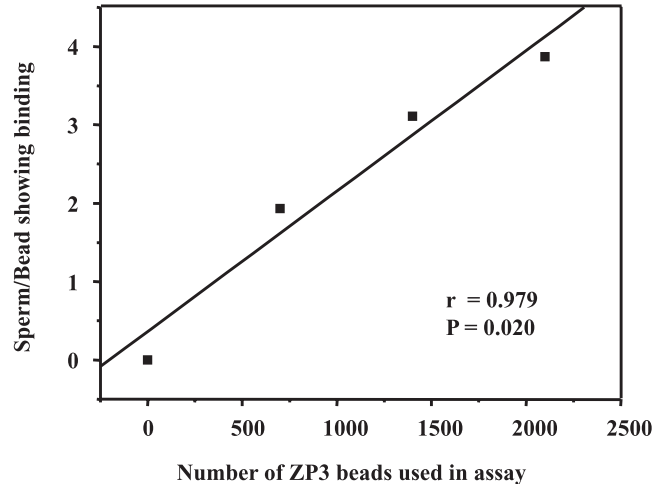


Fig. 3. Linear regression analysis of the ZP3 bead bound sperm as a function of the number of beads used in the assay. Different concentrations of ZP3 coated beads (0.048%, ~700 beads; 0.096, ~1400 beads; 0.144%, ~2100 beads) suspension were allowed to bind with 1×10^6 capacitated human sperm. Regression coefficient was calculated by applying apparent linear fit to data on X-axis (number of ZP3 beads used in the assay) and Y-axis (sperm/bead showing binding) on linearized scales.

of beads showing sperm binding, number of sperm bound per coated bead along with number of sperm bound per bead showing sperm binding (Table III). However, prior incubation of ZP3 coated beads with ZP2 specific MAbs (MA 1615) did not result in any decrease in the binding of sperm to beads suggesting that binding of sperm to ZP3 coated beads is specific. No alteration in the binding of sperm to fetuin coated beads was observed in the presence of various monoclonal antibodies (Table III).

Discussion

Several investigators have successfully studied sperm-ZP interaction employing recombinant proteins in the past and found that recombinant human ZP proteins are functionally active^{7-8,14-16}. In the present study, baculovirus-expressed recombinant human ZP3 was employed which has been shown to have both N- and O-linked glycosylation with high mannose type carbohydrate residues⁷. Native human zona proteins also have high mannose type carbohydrate residues¹⁷. Human sperm have been reported to harbour mannose binding sites on the surface that have been implicated to play a crucial role in sperm-egg interaction¹⁸⁻¹⁹. Binding studies with FITC labelled baculovirus-expressed recombinant human ZP3 with capacitated human spermatozoa revealed similar binding characteristics as native human solubilized isolated zona suggesting that the baculovirus-expressed recombinant human ZP3 can be used to develop assays pertaining to ZP3-spermatozoa interaction⁸.

Table I. Binding characteristics of capacitated human sperm to streptavidin sepharose beads coated with biotinylated baculovirus-expressed recombinant human ZP3

Experimental conditions	% Beads showing sperm binding	No. of sperm bound/coated bead	Sperm/bead showing binding
Streptavidin sepharose beads	8.2 ± 0.9	0.09 ± 0.01	1.17 ± 0.03
Streptavidin sepharose beads coated with 10 µg biotinylated fetuin	7.6 ± 0.8*	0.08 ± 0.01	1.07 ± 0.03
Streptavidin sepharose beads coated with 2 µg biotinylated ZP3	29.6 ± 2.8**	0.49 ± 0.04	1.69 ± 0.09
Streptavidin sepharose beads coated with 5 µg biotinylated ZP3	37.8 ± 1.4**	0.63 ± 0.03	1.67 ± 0.07
Streptavidin sepharose beads coated with 10 µg biotinylated ZP3	41.5 ± 1.4**	0.88 ± 0.02	2.12 ± 0.06
Streptavidin sepharose beads coated with 25 µg biotinylated ZP3	36.3 ± 1.6**	0.79 ± 0.04	2.20 ± 0.08

Values are shown as mean ± SEM of 5 experiments

* $P > 0.05$ as compared to streptavidin coated beads; ** $P < 0.05$ as compared to streptavidin coated beads

Table II. Binding characteristics of capacitated human sperm to different concentrations of streptavidin sepharose beads coated with 10 µg biotinylated baculovirus-expressed recombinant human ZP3

Experimental conditions	Concentration of coated beads (%)	% Beads showing sperm binding	No. of sperm bound/coated bead	Sperm/bead showing binding
Streptavidin sepharose beads	0.06	7.9 ± 1.2	0.11 ± 0.03	1.36 ± 0.13
Streptavidin sepharose beads coated with 10 µg biotinylated fetuin	0.096	11.9 ± 0.7	0.15 ± 0.01	1.44 ± 0.13
Streptavidin sepharose beads coated with 10 µg biotinylated ZP3	0.048	27.6 ± 1.2	0.54 ± 0.03	1.95 ± 0.03
	0.096	38.4 ± 0.7	1.19 ± 0.01	3.11 ± 0.05
	0.144	27.3 ± 2.9	0.98 ± 0.07	3.66 ± 0.13
	0.192	33.3 ± 1.7	0.64 ± 0.05	1.89 ± 0.07

Values are shown as mean ± SEM of 4 experiments

Table III. Binding characteristics of capacitated human sperm to biotinylated recombinant human ZP3 coated streptavidin sepharose beads in presence or absence of inhibitors

Experimental conditions	Competitive inhibitor in equilibrium	% Beads showing sperm binding	No. of sperm bound/coated bead	Sperm/bead showing binding
Streptavidin sepharose beads	-	8.2 ± 0.2	0.09 ± 0.01	1.16 ± 0.05
0.048% Streptavidin sepharose beads coated with 10 µg biotinylated fetuin	-	11.4 ± 0.6	0.14 ± 0.01	1.19 ± 0.06
	MA 1552* (10 µg/ml)	7.7 ± 0.6	0.11 ± 0.01	1.41 ± 0.11
	MA 1558* (10 µg/ml)	8.0 ± 0.7	0.10 ± 0.01	1.24 ± 0.07
	MA 1615* (10 µg/ml)	11.6 ± 0.7	0.14 ± 0.01	1.21 ± 0.07
0.048% Streptavidin sepharose beads coated with 10 µg biotinylated ZP3	-	36.7 ± 0.9	1.07 ± 0.02	2.93 ± 0.03
	Unlabelled ZP3 (300 ng)	24.8 ± 1.2	0.45 ± 0.02	1.81 ± 0.04
	Unlabelled ZP3 (600 ng)	20.6 ± 0.5	0.29 ± 0.01	1.45 ± 0.03
	MA 1552* (2 µg/ml)	30.6 ± 0.8	0.61 ± 0.02	1.99 ± 0.04
	MA 1552* (10 µg/ml)	25.2 ± 0.5	0.44 ± 0.01	1.73 ± 0.02
	MA 1558* (2 µg/ml)	21.1 ± 0.8	0.36 ± 0.02	1.69 ± 0.03
	MA 1558* (10 µg/ml)	21.3 ± 1.1	0.35 ± 0.02	1.62 ± 0.02
	MA 1615* (2 µg/ml)	36.7 ± 0.7	1.05 ± 0.02	2.86 ± 0.02
	MA 1615* (10 µg/ml)	34.3 ± 0.8	0.98 ± 0.04	2.84 ± 0.06

*MA 1552 and MA 1558 have specificity for human ZP3 and MA 1615 for human ZP2; Values are mean ± SEM of 5 experiments

Conventional semen analyses in the *In Vitro* Fertilization (IVF) clinics may not provide accurate information about sperm fertilizing ability since many patients with subtle sperm defects cannot be detected²⁰. More advanced and physiologically relevant sperm function tests are required to detect sperm defects that may lead to failure of fertilization in standard IVF. Sperm-ZP binding and sperm-ZP penetration tests have been suggested to be most powerful indicators for sperm fertilizing ability *in vitro*²¹. Presently available functional tests under these categories include, zona-free hamster oocyte sperm penetration test²², hemizona assay (HZA)²³ and the hypoosmotic swelling (HOS) test²⁴.

Zona-free hamster oocyte penetration test has been developed as an indicator of the ability of human spermatozoa to capacitate, acrosome react and fuse with the vitelline membrane of the oocyte²². However, some reports have suggested that assessment of sperm fertilizing capacity using this test does not have significant clinical benefit in diagnosing infertility^{25,26}. Dead or immotile spermatozoa also penetrate zona-free hamster eggs under certain conditions²⁶. Furthermore, this assay is time consuming, complex and subject to many variables such as capacitation time, sperm concentration and incubation conditions, medium composition and the age of ovum after ovulation²⁷. Hemizona assay has an excellent predictive power for IVF outcome and for the identification of male infertility but it requires technical expertise to use expensive micromanipulators to bisect native human oocyte, which are difficult to obtain owing ethical restrictions. The HOS test can predict the fertilizing capacity of human spermatozoa. A positive HOS test is an indicator of the functional integrity of the sperm membrane as judged by a swollen head and curled tail of spermatozoa when exposed to hypo-osmotic conditions²⁴. However, the clinical utility of the HOS test in assessment of sperm function is still debatable as there are contrasting reports regarding positive correlation between HOS and IVF outcome²⁸. Therefore, an easy as well as reliable tool to assess the fertilizing potential of human spermatozoa will be of immense help.

In the present study, after characterizing the binding characteristics of baculovirus-expressed recombinant human ZP3⁸, attempts were made to optimize an *in vitro* assay system employing biotinylated baculovirus-expressed recombinant human ZP3 coated on streptavidin sepharose beads and capacitated human sperm. Size (mean particle size 34 μm) of streptavidin sepharose beads employed in the present study to coat human

ZP3 was close to human oocytes which may allow accessibility of ZP3 epitopes for binding to spermatozoa. Moreover, previous studies from our group have shown that biotinylated recombinant bonnet monkey (*Macaca radiata*) ZP3 binds with anterior head of the capacitated spermatozoa and did not reveal any binding to the acrosome-reacted spermatozoa²⁹, thereby suggesting that biotinylation of ZP3 does not result in alteration of its biological activity. The optimum binding of capacitated sperm to ZP3 coated beads was observed when beads were coated with 10 μg biotinylated ZP3/reaction. The decrease in the binding of spermatozoa to beads coated with higher amount of human ZP3 (25 μg ZP3/reaction) may be due to steric hinderance. Further, dilution curve with 10 μg ZP3 coated beads revealed a bead suspension of 0.096 per cent exhibiting maximum binding of sperm, which may be attributed to the optimization of beads to spermatozoa ratio. Binding of *Xenopus laevis* sperm with purified vitelline envelope proteins coated on agarose beads have been reported earlier³⁰. Binding of porcine and bovine sperm with baculovirus-expressed recombinant porcine ZP glycoproteins has also been studied using the bead-binding assay format³¹. In murine model ZP3 coated silica beads have been used to study sperm-ZP3 interaction. In this solid phase assay, only one spermatozoa was observed to bind per ZP3-bead via sperm head³².

The observations that the binding of capacitated human spermatozoa to ZP3 coated beads was inhibited in the presence of ZP3 or prior incubation of ZP3 coated beads with ZP3 specific MAbs, strongly suggest that the binding of sperm to ZP3 coated beads is specific. This assay being simple, low cost and easy to handle can be used effectively in IVF clinics to check the functional competence of sperm to bind with ZP of human egg.

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