Summary
Over the years, the ZP glycoproteins, constituting the mammalian ZP matrix have been a subject of intense investigations for gaining insight into the interplay between the sperm and the egg during fertilization. These glycoproteins, either alone, in conjunction with each other or with other factors in the physiological milieu, play an important role in different stages of fertilization. Four glycoproteins, hZP1, hZP2, hZP3 and hZP4 comprise the human ZP matrix as advocated recently. Keeping in view the elucidated functions of the different zona proteins in various mammalian species, in the present thesis, attempts have been made to understand the role of human zona proteins in their interaction with spermatozoa at the pre-fertilization stage. To achieve this, the three human zona proteins, hZP2, hZP3 and hZP4 have been expressed in E. coli and baculovirus-expression systems to obtain them in non-glycosylated and glycosylated forms respectively. It has helped in delineating the role of the polypeptide backbone vis-à-vis glycans in mediating different functions. For expression in E. coli, the hZP2, hZP3 and hZP4, excluding their respective N-terminal SS and C-terminal TD, were cloned downstream of T7 promoter in pRSET-A expression vector and expressed as polyhistidine-tagged fusion proteins in BL21[DE3]pLysS host strain of E. coli deficient in the lon and ompT proteases to aid in minimizing the lower molecular weight fragments which may arise due to proteolytic degradation. The expression of all the above recombinant proteins was localized exclusively in the insoluble intracellular fraction of the host cells. Hence, they were purified under denaturing conditions in the presence of 8 M urea, by Ni-NTA affinity chromatography. Subsequently, the purified recombinant proteins were renatured by extensive dialysis in buffer containing oxidized and reduced glutathione to aid in the formation of disulfide bonds. The SDS-PAGE analysis of the purified recombinant hZP2, hZP3 and hZP4 revealed bands of ~90, ~50 and ~65 kDa respectively. The approximate yield of each recombinant protein from an induced 1 litre culture (at shake flask level) of BL21[DE3]pLysS cells was 1 mg. In addition, the hZP2, hZP3 (hZP3(1-424)) and hZP4 including their SS and TD, along with an additional construct of hZP3, including SS but devoid of TD (hZP3(1-348)), were cloned in baculovirus transfer vector, pAcHLT-A and expressed in Sf21 insect cells. The baculovirus-expressed purified recombinant hZP2, hZP3(1-348), hZP3(1-424) and hZP4 had apparent molecular weights corresponding to ~105, ~55, ~65 and ~75 kDa respectively. An average yield of ~250-500 μg of each of the purified baculovirus-expressed recombinant proteins was obtained from transfected Sf21 cells grown in one
spinner flask (50 x 10^6 cells/flask). Characterization of the carbohydrate moieties by lectin binding analysis revealed that all the baculovirus-expressed proteins contained both N-linked glycosides mainly having mannose α 1-3 and/or mannose α 1-6 residues (ConA) and O-linked glycosylation having α-O glycosides of Gal or GalNAc moieties (Jacalin). Some other oligosaccharide residues were also found to be present on these glycoproteins in lesser amounts as demonstrated by weak reactivity with other lectins. The *E. coli*-expressed zona proteins did not react with any of the lectins. These results confirmed that the recombinant proteins expressed in *E. coli* were non-glycosylated while the baculovirus-expressed proteins were glycosylated.

In order to develop a probe to recognize hZP4 and employ in functional assays, a panel of 10 MAbs, MA-1650, -1654, -1657, -1660, -1662, -1665, -1667, -1671, -1673 and -1677 was generated against baculovirus-expressed hZP4. Analysis of reactivity of the MAbs in ELISA and Western blot revealed that the above MAbs reacted only with hZP4 and not with hZP2 and hZP3. The isotype analysis revealed 7 MAbs to be of IgG1, and one each of IgG2a, IgG2b and IgM isotypes. Further analysis by indirect immunofluorescence revealed that MA-1660 reacted with native bonnet monkey ZP (bonnet monkey ZP4 and hZP4 share 92% sequence identity at aa level).

Evaluation of binding characteristics of the *E. coli*- as well as baculovirus-expressed recombinant zona proteins both by direct and indirect binding assays revealed similar results. Both the glycosylated and non-glycosylated hZP3 bound to the capacitated (acrosome-intact) spermatozoa showing two different binding patterns. Majority of the capacitated sperm showed binding of recombinant hZP3 to the equatorial region while a lesser percentage of spermatozoa exhibited the binding of the recombinant protein to the acrosomal cap. The *E. coli*- and baculovirus-expressed recombinant hZP4 also showed similar binding patterns as hZP3 but their distribution was different. In case of hZP4, a higher percentage of spermatozoa showed the binding of the recombinant protein to the acrosomal cap than to the equatorial region. Co-localization of baculovirus-expressed hZP3_{1-424} and hZP4, labeled with different fluorochromes, on the sperm, revealed that although the two proteins may have similar localization sites on sperm, they may bind to different ligands on the sperm surface. Competitive inhibition binding studies, employing 5 and 10 molar excess of the competing zona proteins, further reconfirmed the specificity of binding of these proteins to the human sperm. For example, binding of FITC labeled hZP3 to the capacitated spermatozoa was
inhibited by the unlabeled hZP3, but not hZP2 and hZP4. The binding of the non-glycosylated and glycosylated recombinant hZP3 and hZP4 was however, restricted only to the equatorial region of the acrosome-reacted sperm. Under similar experimental conditions, the recombinant hZP2 expressed both in E. coli and baculovirus, failed to bind to capacitated (acrosome-intact) spermatozoa, but bound to acrosome-reacted sperm at the equatorial region. Since the non-glycosylated and glycosylated recombinant ZP proteins bind with similar binding profiles to capacitated and acrosome-reacted human spermatozoa, it might be speculated that the carbohydrate moieties of the ZP glycoproteins may not be critical in mediating the initial binding event per se between the sperm and the zona, and that the polypeptide backbone of the zona proteins may be sufficient for this event.

When analyzed for their ability to induce acrosome reaction in capacitated human spermatozoa, the baculovirus-expressed recombinant hZP3(1-424) and hZP4 were able to induce significant induction of acrosome reaction in human sperm whereas their corresponding E. coli-expressed counterparts failed to do so. In addition, baculovirus-expressed hZP3(1-348) was found to be equally competent as hZP3(1-424) in inducing acrosome reaction in capacitated sperm, suggesting that the TD may not have a role to play in the process of acrosomal exocytosis mediated by hZP3. The recombinant hZP2 expressed either in E. coli or in baculovirus did not induce acrosomal exocytosis in capacitated sperm. These results reiterate earlier findings attributing hZP3 the primary sperm receptor function and hZP2, the secondary receptor role once the sperm undergoes acrosomal exocytosis. The present investigations also envisage an important role for hZP4 in the sperm receptor function and induction of acrosomal exocytosis. Moreover, these findings suggest that though, the binding of recombinant zona proteins to the sperm may be mediated by their polypeptide backbone alone, the ability to induce acrosome reaction by hZP3 and hZP4 needs the involvement of oligosaccharide residues. In an effort to delineate the signaling pathway by which baculovirus-expressed hZP3(1-424) and hZP4 induce acrosome reaction in human sperm, Pertussis toxin, an inhibitor of Gt protein was employed. The results demonstrated that while hZP3(1-424) follows a Gt protein dependent pathway for bringing about acrosome reaction in human sperm, the mechanism followed by hZP4 is Gt protein independent. The expression of hZP3 in mammalian expression system was also undertaken to further understand the influence of glycosylation that might be taking place in insect
Summary
cells as compared to mammals. Transient transfection studies with pcDNA6/V5-His-B mammalian expression vector harboring cDNA encoding hZP4 in CHO-K1 cells demonstrated its expression in the cell lysate as a doublet corresponding to 60-70 kDa. Further studies are needed to investigate, if hZP3 expressed by CHO-K1 cells is secreted in the culture medium. In an indirect binding assay, using MAbs generated against baculovirus-expressed hZP3(1-424), the cell lysate from CHO-K1 cells transfected with pcDNA-hZP3 showed binding of hZP3 to the human spermatozoa. The hZP3 present in the cell lysate of CHO-K1 cells was also competent to induce significant induction of acrosome reaction when compared with the cell lysate of untransfected CHO-K1 cells.

To ascertain the importance of specific oligosaccharide residues of zona proteins in the interaction with the spermatozoa, recombinant hZP3(1-424) and hZP4 were obtained by growing transfected SJ21 cells in the presence of varying concentrations of tunicamycin to make these proteins deficient in N-linked glycosylation. Further, purified baculovirus-expressed recombinant hZP3(1-424) and hZP4 were treated with mild alkali to selectively remove O-linked glycosylation. Assessment of these partially deglycosylated proteins for their ability to induce acrosome reaction revealed that removal of N-linked glycosylation led to a significant reduction in the acrosome reaction inducing ability of baculovirus-expressed hZP3(1-424) and hZP4. On the other hand, removal of O-linked sugar residues had no significant effect on their ability to induce acrosome reaction, suggesting that in humans, N-linked carbohydrate residues of ZP glycoproteins may be more relevant in mediating acrosomal exocytosis in spermatozoa.

After analysis of the sperm-binding characteristics and acrosome reaction inducing ability of the recombinant human zona proteins, an attempt was made to understand, if the recently described sperm proteasome has a function in mediating binding and/or induction of acrosome reaction in human sperm by the zona proteins. The presence of proteasome-specific inhibitors did not influence the binding of the recombinant baculovirus-expressed hZP2, hZP3(1-424) and hZP4 to the spermatozoa. However, the ability of baculovirus-expressed hZP3(1-424) and hZP4 to induce acrosome reaction in human sperm was significantly inhibited in presence of these inhibitors. This implies that the sperm proteasome may not have a direct role in sperm-egg binding but may influence subsequent events such as zona mediated induction of acrosome reaction.
Summary

These studies will facilitate in furthering our understanding pertaining to the role of zona proteins during the process of human fertilization.