Recombinant human zona pellucida protein C produced in Chinese hamster ovary cells binds to human spermatozoa and inhibits sperm–zona pellucida interaction

Recombinant human zona pellucida protein C expressed in Chinese hamster ovary cells associates to the acrosomal region of human spermatozoa and inhibits sperm–zona pellucida interaction in the hemizona assay. Recombinant human zona pellucida protein C may be a useful tool toward the development of diagnostic methods for male factor infertility and the elucidation of the molecular basis of fertilization. (Fertil Steril® 2008; 90:879–82. ©2008 by American Society for Reproductive Medicine.)

Sperm binding and penetration through the egg's extracellular matrix, called zona pellucida (ZP), are essential steps of mammalian fertilization. The human ZP is composed of four main glycoproteins, called ZPA, ZPB, ZPC, and ZP1 (1–3). Nucleotide sequence analysis identifies human ZPC* as the homologue of murine ZP glycoprotein 3 (mZP3 [1]); the results of several investigations have demonstrated the ability of mZP3 to associate to the sperm surface and to participate in molecular events related to sperm-ZP binding and acrosomal exocytosis (4).

Clinical studies performed in the human model have revealed that defective sperm–ZP interaction and disordered ZP–induced acrosomal exocytosis are major causes of fertilization failure in a high proportion of infertile patients (5, 6). However, the diagnosis of these pathologies is limited by the scarcity of human oocytes. To circumvent this problem, human ZP proteins have been generated in vitro with use of recombinant DNA technology. In particular, ZPC has been expressed in prokaryotic and eukaryotic systems (7–14). Recombinant human ZP protein C (rec-hZPC) produced in Chinese hamster ovary (CHO) cells consistently has been shown to induce the acrosomal loss of human spermatozoa (7, 8, 15, 16); in addition, rec-hZPC has been reported to bind to sperm components in solid-phase interaction assays (17, 18).

The aim of the present study was to further analyze the biologic activity of rec-hZPC expressed in CHO cells by assessing its ability to associate to the sperm surface and to compete with the native protein in a sperm-ZP interaction assay.

All human samples used in the study were obtained under the donor's written consent, and experimental protocols were approved by the Institutional Review Board at the Instituto de Biología y Medicina Experimental. Recombinant human ZP protein C was produced and purified from CHO cell culture, as described (10). This 55 to 65 kd protein is secreted into the culture medium and remains soluble, suggesting that native conformation is maintained. Previous studies have shown that rec-hZPC produced in this system induces the acrosomal exocytosis in capacitated human spermatozoa by mechanisms similar to those described for solubilized native ZP (15, 16).

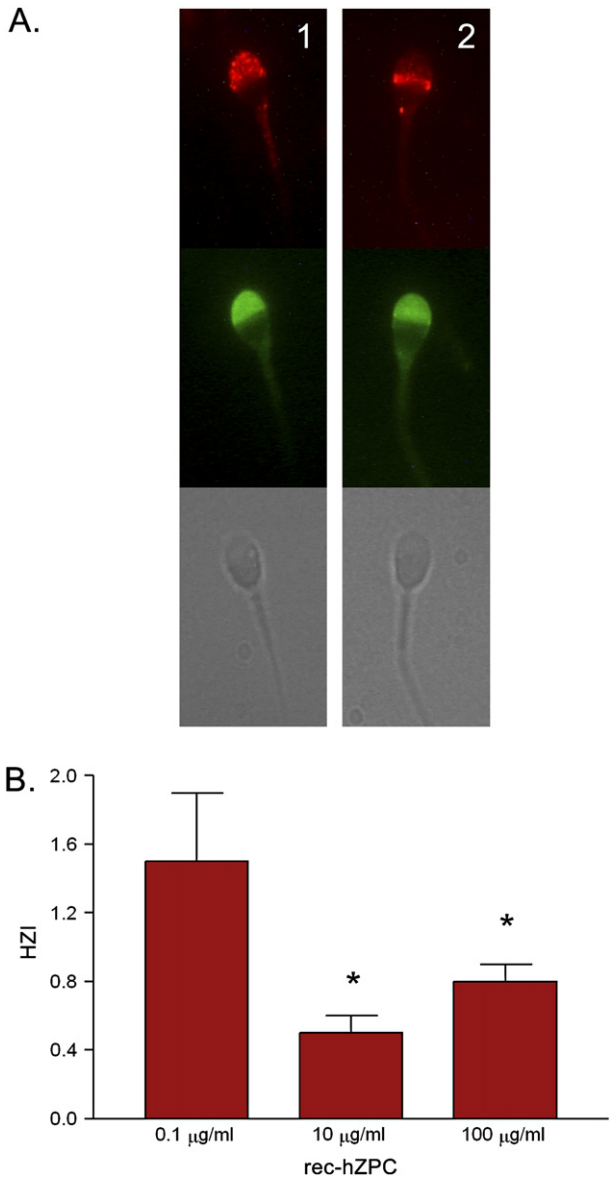
Initially, the ability of the rec-hZPC used in the present study to promote the acrosomal loss in capacitated human spermatozoa was verified. Human semen samples from normozoospermic donors were subjected to the swim-up procedure to select a subpopulation of highly motile cells (19). Selected spermatozoa were incubated under capacitating conditions in a Ca^{2+}-containing medium for 18 hours followed by an additional hour of incubation in the presence of rec-hZPC or phosphate-buffered saline solution (PBS; control). Cells were washed, fixed, permeabilized, stained with *Pisum sativum* agglutinin conjugated to fluorescein (FITC-PSA), mounted, and examined under fluorescence microscopy (20). Incubation with rec-hZPC significantly induced the acrosomal exocytosis in capacitated spermatozoa (100 μg/mL rec-hZPC = 27% ± 3% acrosome-reacted cells, 10 μg/mL rec-hZPC = 27% ± 4%, PBS = 17% ± 3%; mean ± SEM; n = 4; values for both amounts of rec-hZPC $P<.01$ vs. PBS, Student Newman-Keuls multiple-comparison test).

To assess the association of rec-hZPC to receptor sites on the sperm plasma membrane, we incubated cells with the recombinant protein and subjected them to immunolocalization studies. Spermatozoa incubated for 5 hours under

Received March 8, 2007; revised and accepted June 25, 2007.
Supported by grants from the World Health Organization (grant No. 97175) and the Agencia Nacional de Promoción Científica y Tecnológica (PICT2000 0509583 to M.H.V.L.). C.M.B. and M.H.V.L. are career members of the National Research Council of Argentina (CONICET).
Reprint requests: Mónica H. Vazquez-Levin, Ph.D., Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, 1428ADN, Buenos Aires, Argentina (FAX: 54-11-4786-2564; E-mail: mhvaz@dna.uba.ar).
Also named ZP3 (symbol report: ZP3. Human Genome Organization [HUGO]. Gene Nomenclature Committee, April 25, 2005. World Wide Web. URL: http://www.gene.ucl.ac.uk/nomenclature/data/get_data.php?hgnc_id=13189 [September 6, 2005]).

FIGURE 1

Binding of rec-hZPC to human spermatozoa. (**A**) Assessment of rec-hZPC association to surface sperm components by indirect immunofluorescence. Motile human spermatozoa were incubated for 5 hours under capacitating conditions and exposed to rec-hZPC (100 μg/mL) or PBS (control) for an additional hour. The unbound protein was removed by washing with PBS. Cells were fixed, spotted onto microscope slides, incubated with rabbit anti-rec-hZPC (1:300 [10]) and anti-rabbit IgG labeled with Cy3 (1:500; Chemicon International, Temecula, CA). Staining with FITC-PSA was performed to assess the acrosomal status. Slides were mounted and analyzed with use of fluorescence microscopy. A total of 100 cells were scored in each condition; n = 5 semen samples. Specific binding of rec-hZPC was calculated by subtracting the percentage of stained spermatozoa in the control (PBS) condition from the value obtained in cells exposed to the recombinant protein. Representative immunofluorescence patterns are shown. *Top:* immunodetection of rec-hZPC; *middle:* FITC-PSA staining; *bottom:* bright-field images. (**B**) Evaluation of rec-hZPC ability to interfere with human sperm-ZP interaction in the hemizona assay. Motile human spermatozoa were incubated for 5 hours under capacitating conditions and exposed for 1 hour to rec-hZPC (treated) or PBS (control). After washing, spermatozoa were resuspended in medium and incubated for 4 hours with human hemizonae. Sperm cells tightly bound to the outer face of each hemizona were counted, and the hemizona index (HZI) was calculated (HZI = Number of treated spermatozoa bound per hemizona/Number of control spermatozoa bound to the counterpart hemizona). Results are expressed as mean ± SEM; n ≥ 7 hemizona; *$P<.05$ versus 0.1 μg/mL, Student Newman-Keuls multiple-comparison test.



Marín-Briggiler. Biological activity of recombinant human 2PC. Fertil Steril 2008.

capacitating conditions and exposed for 1 hour to rec-hZPC showed binding of the recombinant protein over the head; in 25% ± 3% of the cells, a specific staining was localized over the acrosomal cap (Fig. 1A, 1, top), and in 2% ± 1% a signal on the equatorial segment was detected (Fig. 1A, 2, top). Staining with FITC-PSA revealed that all spermatozoa with bound rec-hZPC had an intact acrosome (Fig. 1A, 1 and 2, middle). When the first antibody (rabbit anti-rec-hZPC) was either omitted or replaced by antibodies produced against other rec-hZP proteins (anti-rec-hZPA and anti-rec-hZPB), no labeling on the sperm head was detected. The percentage of spermatozoa exhibiting binding to rec-hZPC was comparable with the proportion of sperm cells from fertile men with the ability to bind to whole ZP (21). A previous report described the association of agarose beads coated with rec-hZPC produced in a reticulocyte in vitro transcription/translation system to the head of capacitated human spermatozoa, but the subcellular localization of binding sites could not be determined (22). In agreement with our findings, a recent study reported the binding of baculovirus and *Escherichia coli*–expressed human ZPC to the acrosomal cap and the equatorial region of 16-hour capacitated spermatozoa (23); however, the percentages of cells on each pattern were different in both studies, possibly because of differences in the experimental designs used.

Evaluation of the acrosomal status of spermatozoa incubated for 5 hours under capacitating conditions and exposed for an additional hour to rec-hZPC showed no significant changes in the acrosomal exocytosis compared with controls (100 μg/mL rec-hZPC = 11% ± 3% acrosome-reacted cells; PBS = 11% ± 1%; n = 3). These observations were not surprising, considering that using the same experimental design Calvo et al. (24) reported the need of at least an 8-hour incubation to trigger human sperm follicular fluid–induced acrosomal exocytosis. A similar capacitation time was required to achieve maximal acrosomal exocytosis in response to a rec-hZPC produced in human ovarian teratocarcinoma cells (11).

If rec-hZPC binds to native ZP sperm receptors, it should interfere with human sperm–ZP interaction in vitro. To evaluate this possibility, spermatozoa were preincubated for 1 hour with rec-hZPC or PBS, washed, and subjected to the hemizona assay (25) in a Ca^{2+}-containing medium, as described (20). A decrease in the number of sperm bound to the ZP was observed when cells were exposed to rec-hZPC (10 and 100 μg/mL); under these conditions, the hemizona index values were significantly lower than that obtained with 0.1 μg/mL rec-hZPC ($P<.05$; Fig. 1B). These results are in agreement with previous findings from use of either solubilized ZP glycoproteins (26) or rec-hZPC produced in mammalian cells (11). The diminished binding levels observed could not be associated to either a noticeable negative effect of rec-hZPC on sperm progressive motility (100 μg/mL rec-hZPC = 74% ± 1% grade a cells [19], 7% ± 1% grade b cells; PBS = 73% ± 1% grade a cells, 11% ± 2% grade b cells; n = 3) or an increased acrosomal exocytosis (100 μg/mL rec-hZPC = 10% ± 3% acrosome-reacted cells, evaluated at the end of the assay; PBS = 10% ± 2%; n = 3). The recombinant protein may have blocked native ZP receptor sites on the sperm surface. In addition, rec-hZPC may have had a priming effect on native ZP-induced acrosomal exocytosis, as previously described for progesterone and human follicular fluid (27).

Results from the present study demonstrate for the first time that rec-hZPC produced in CHO cells is able to associate to human sperm components located on the acrosomal region; these binding sites would correspond to native ZPC sperm receptors, because the associated protein had the ability to interfere with sperm-ZP interaction. In cells incubated overnight under capacitating conditions, binding of rec-hZPC to specific sites triggered the acrosomal exocytosis. Altogether, these findings indicate that rec-hZPC expressed in this system is a biologically active glycoprotein.

Availability of rec-hZPC with biologic activity is of great relevance to study the molecular mechanisms underlying human fertilization. In addition, rec-hZPC may be useful toward the development of protocols to diagnose and treat male factor infertility, as well as for designing novel immunocontraceptive strategies. Sperm-ZP interaction assays have a high predictive value for IVF outcome (28), but they involve the use of a biologic material available in limited quantities. As an alternative, sperm could be challenged with rec-hZPC immobilized in a solid-phase support (17, 29). Eventually, sperm selection based on their ability to interact with rec-hZPC could be used as a tool to improve fertilization rates in IVF procedures.

Acknowledgments: The authors especially thank Lorena Capece, M.Sc., for her technical assistance.

Clara I. Marín-Briggiler, Ph.D.[a]
María F. Gonzalez-Echeverría, M.Sc.[b]
Jeffrey D. Harris, Ph.D.[c]
Mónica H. Vazquez-Levin, Ph.D.[a]
[a] *Instituto de Biología y Medicina Experimental (IBYME), National Research Council of Argentina (CONICET), Buenos Aires, Argentina;* [b] *Centro Médico Fertilab, Buenos Aires, Argentina; and* [c] *Zonagen Inc., The Woodlands, Texas (present address: University of California, Department of Molecular and Cell Biology, Berkeley, California)*

REFERENCES

1. Harris JD, Hibler DW, Fontenot GK, Hsu KT, Yurewicz EC, Sacco AG. Cloning and characterization of zona pellucida genes and cDNA from a variety of mammalian species: the ZPA, ZPB and the ZPC gene families. DNA Seq 1994;4:361–93.
2. Hughes DC, Barratt CLR. Identification of the true human orthologue of the mouse Zp1 gene: evidence for greater complexity in the mammalian zona pellucida? Biochim Biophys Acta 1999;1447:303–6.

3. Lefièvre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, et al. Four zona pellucida glycoproteins are expressed in the human. Hum Reprod 2004;19:1580–6.
4. Wassarman PM, Jovine L, Litscher ES. A profile of fertilization in mammals. Nat Cell Biol 2001;3:59–64.
5. Liu DY, Baker HWG. Defective sperm–zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. Hum Reprod 2000;15:702–8.
6. Liu DY, Baker HW. Disordered zona pellucida–induced acrosome reaction and failure of in vitro fertilization in patients with unexplained infertility. Fertil Steril 2003;79:74–80.
7. Van Duin M, Polman JEM, De Breet ITM, Van Ginneken K, Bunschoten H, Grootenhuis A, et al. Recombinant human zona pellucida protein ZP3 produced by Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. Biol Reprod 1994;51:607–17.
8. Brewis IA, Clayton R, Barratt CL, Hornby DP, Moore HD. Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. Mol Hum Reprod 1996;2: 583–9.
9. Chapman NR, Kessopoulou E, Andrews P, Hornby D, Barratt CLR. The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa in vitro. Biochem J 1998;330:839–45.
10. Harris JD, Seid CA, Fontenot GK, Liu HF. Expression and purification of recombinant human zona pellucida proteins. Protein Expr Purif 1999;16:298–307.
11. Dong KW, Chi TF, Juan YW, Chen CW, Lin Z, Xiang XQ, et al. Characterization of the biologic activities of a recombinant human zona pellucida protein 3 expressed in human ovarian teratocarcinoma (PA-1) cells. Am J Obstet Gynecol 2001;184:835–44.
12. Martic M, Moses EK, Adams TE, Liu de Y, Gook DA, Garrett C, et al. Recombinant human zona pellucida proteins ZP1, ZP2 and ZP3 co-expressed in a human cell line. Asian J Androl 2004;6:3–13.
13. Chakravarty S, Suraj K, Gupta SK. Baculovirus-expressed recombinant human zona pellucida glycoprotein-B induces acrosomal exocytosis in capacitated spermatozoa in addition to zona pellucida glycoprotein-C. Mol Hum Reprod 2005;11:365–72.
14. Caballero-Campo P, Chirinos M, Fan XJ, González-González ME, Galicia-Chavarría M, Larrea F, et al. Biological effects of recombinant human zona pellucida proteins on sperm function. Biol Reprod 2006;74: 760–8.
15. Bray C, Son JH, Kumar P, Harris JD, Meizel S. A role for the human sperm glycine receptor/Cl^- channel in the acrosome reaction initiated by recombinant ZP3. Biol Reprod 2002;66:91–7.
16. Bray C, Son JH, Meizel S. A nicotinic acetylcholine receptor is involved in the acrosome reaction of human sperm initiated by recombinant human ZP3. Biol Reprod 2002;67:782–8.
17. Tanphaichitr N, Haebe J, Leader A, Carmona E, Harris JD, da Silva SM, et al. Towards a more precise assay of sperm function in egg binding. J Obstet Gynaecol Can 2003;25:461–70.
18. Furlong LI, Harris JD, Vazquez-Levin MH. Binding of recombinant human proacrosin/acrosin to zona pellucida (ZP) glycoproteins. I. Studies with recombinant ZPA, ZPB, and ZPC. Fertil Steril 2005;83: 1780–90.
19. World Health Organization. WHO laboratory manual for the examination of human semen and semen–cervical mucus interaction. 4th ed. New York: Cambridge University Press, 1999.
20. Marín-Briggiler CI, Vazquez-Levin MH, Gonzalez Echeverría F, Blaquier J, Tezón JG, Miranda PV. Strontium supports human sperm capacitation but not follicular fluid–induced acrosome reaction. Biol Reprod 1999;61:673–80.
21. Liu DY, Garrett C, Baker HW. Low proportions of sperm can bind to the zona pellucida of human oocytes. Hum Reprod 2003;18:2382–9.
22. Whitmarsh AJ, Woolnough MJ, Moore HDM, Hornby DP, Barratt CLR. Biological activity of recombinant human ZP3 produced in vitro: potential for a sperm function test. Mol Hum Reprod 1996;2: 911–9.
23. Chakravarty S, Kadunganattil S, Bansal P, Sharma RK, Gupta SK. Relevance of glycosylation of human zona pellucida glycoproteins for their binding to capacitated human spermatozoa and subsequent induction of acrosomal exocytosis. Mol Reprod Dev. Published online May 7, 2007.
24. Calvo L, Vantman D, Banks SM, Tezón JG, Koukoulis G, Dennison L, et al. Follicular fluid–induced acrosome reaction distinguishes a subgroup of men with unexplained infertility not identified by semen analysis. Fertil Steril 1989;52:1048–54.
25. Burkman LJ, Coddington CC, Franken DR, Kruger TF, Rosenwaks Z, Hodgen GD. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. Fertil Steril 1988;49:688–97.
26. Franken DR, Henkel R, Kaskar K, Habenicht UF. Defining bioassay conditions to evaluate sperm/zona interaction: inhibition of zona binding mediated by solubilized human zona pellucida. J Assist Reprod Genet 1996;13:329–32.
27. Schuffner AA, Bastiaan HS, Duran HE, Lin ZY, Morshedi M, Franken DR, et al. Zona pellucida–induced acrosome reaction in human sperm: dependency on activation of pertussis toxin–sensitive G_i protein and extracellular calcium, and priming effect of progesterone and follicular fluid. Mol Hum Reprod 2002;8:722–8.
28. Oehninger S, Franken DR, Sayed E, Barroso G, Kolm P. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta-analysis. Hum Reprod Update 2000;6:160–8.
29. Vazquez MH, Phillips DM, Wassarman PM. Interaction of mouse sperm with purified sperm receptors covalently linked to silica beads. J Cell Sci 1989;92:713–22.