Normal sperm–zona pellucida interaction and fertilization in vitro in α-1,3-galactosyltransferase gene knockout mice

Dear Sir,

Sperm binding to the zona pellucida (ZP) is highly species-specific and one of the most important steps for fertilization in mammals (Bedford, 1977; Yanagimachi, 1994). However, the molecular mechanism of sperm–ZP interaction is poorly understood. In mice it is reported that the ZP contains three major glycoproteins; ZP1, ZP2 and ZP3. ZP3 has been defined as the secondary receptor for binding acrosome intact spermatozoa and also for induction of the acrosome reaction (Wassarman, 1987). ZP2 may be the secondary receptor for binding acrosome-reacted spermatozoa (Bleil et al., 1988). It is believed that carbohydrates may play important role in sperm–ZP interaction (Chapman and Barratt, 1996; Patankar et al., 1997). For example, O-linked oligosaccharides with terminal α-galactosides displayed on ZP3 may be critical for sperm recognition during initial sperm–ZP binding (Flormann and Wassarman, 1985; Bleil, 1991). Shur and others demonstrated that mouse sperm β-1,4-galactosyltransferase recognizes and binds to terminal N-acetylglucosaminyl residues in the ZP (Shur and Hall, 1982; Lopez et al., 1985). Thus it is suggested that mouse sperm surface β-1,4-galactosyltransferase and ZP glycoprotein ZP3 are complementary adhesion molecules that mediate primary gamete binding (Miller et al., 1992, Gong et al., 1995). Addition of either UDP-galactose or anti-galactosyltransferase antibody produced a dose-dependent inhibition of sperm–zona binding (Shur and Hall, 1982; Lopez et al., 1985). Spermatozoa from β-1,4-galactosyltransferase knockout mice are unable to bind to the zona (Shur, 1997).

A group of α-1,3-galactosyltransferase gene knockout mice produced for xenotransplantation research have been reported to be fertile but there was a discrepancy in the transmission of the targeted gene to offspring of crosses of heterozygotes (Tearle et al., 1996). There was an 11% reduction in transmission of the targeted gene but no reduction in the proportion of homozygous targeted offspring. This suggested that the gene knockout conveyed some disadvantage but that the knockout phenotype was not the problem. Lack of the enzyme or its product might reduce the rate of successful sperm–oocyte interaction. However it has been reported that the α-1,3-galactosyltransferase gene is not expressed in male germ cells (Johnston et al., 1995). Also, Thall et al. (1995) reported normal fecundity in their α-1,3-galactosyltransferase gene knockout mice. To determine whether this enzyme is critical for mouse sperm–oocyte interaction, sperm–ZP binding and penetration, and fertilization in vitro were studied in the Tearle et al. (1996) α-1,3-galactosyltransferase gene knockout mice and wild type mice.

The α-1,3-galactosyltransferase gene knockout mice were produced as described previously (Tearle et al., 1996). Briefly, the gene knockout mice were generated by targeting exon 9 of the mouse α-1,3-galactosyltransferase gene. The construct used for homologous recombination contained 9.5 kb of 129 bp mouse genomic DNA encompassing exons 8 and 9 of α-1,3-galactosyltransferase gene, into which a 1.4 kb Sal fragment containing the neo® resistance gene and termination codons in all three reading frames had been inserted. Embryonic stem cells were electroporated and homologous recombinants detected by Southern analysis. Targeted cells were microinjected into CBA×C57BL6 F2 blastocysts and transferred to pseudo-pregnant mice. Progeny were genotyped by polymerase chain reaction and galactosyltransferase expression determined by flow cytometric analysis using fluorescein isothiocyanate (FITC) conjugated to Vicia villosa isolectin B4.

Oocytes were obtained from mice treated with pregnant mare’s serum gonadotrophin (5 IU) 48 h and human chorionic gonadotrophin (5 IU) 17 h previously. Mouse spermatozoa were collected from the cauda epididymis and capacitated in modified minimal essential medium supplemented with 0.3% bovine serum albumin for 2 h. Oocytes (n = 15–20) were inseminated with 3–5×10⁶ motile spermatozoa/ml in 1 ml culture medium in one well of a four-well multidish (Nunclon, Roskilde, Denmark) and incubated at 37°C in 5% CO₂ in air. Fertilization was determined by examining for pronuclei after 7–8 h and cleavage after 18–20 h.

In order to count the actual number of spermatozoa bound to and penetrating into the ZP, separate experiments were performed in which 2×10⁵/ml spermatozoa were incubated with 16–17 oocytes for 2 h at 37°C in 5% CO₂. Oocytes were then washed in fresh medium with a wide-bore glass pipette to dislodge loosely adherent spermatozoa and the number of spermatozoa bound to the ZP was counted using an inverted phase contrast microscope. It was difficult to count the number accurately when there were >100 spermatozoa bound per oocyte. Accordingly, oocytes binding >100 spermatozoa (30% total) were recorded as 100. After counting the number of spermatozoa bound on the ZP, all spermatozoa on the surface of the ZP were removed by repeated pipetting of the oocytes using a fine glass pipette with a diameter slightly smaller than the diameter of the oocyte. The number of spermatozoa which had penetrated in the ZP was counted by optical microscopy. According to this technique was developed to study human sperm–ZP interaction (Liu and Baker, 1994). Spermatozoa with heads embedded in the ZP or perivitelline space cannot be removed by further pipetting.

The significance of differences in fertilization rates and proportions of ZP penetrated between the knockout and wild-type mice and between the cross (knockout×wild-type) inseminations were determined by χ² tests. Differences in the mean numbers of spermatozoa bound to and penetrating the ZP were examined by non-parametric analysis of variance (Friedman test).

There were no significant differences in the numbers of
spermatozoa bound to or penetrating into the zona pellucidae, or in the proportions of zonae pellucidae penetrated between the gene knockout and wild-type mice, or between the cross insemination groups at 2 h after insemination (Table I). There was no unusual phenomenon or pattern of sperm–zona binding between the knockout and wild-type mice. Furthermore, there were similar proportions of oocytes fertilized in the knockout, wild-type and cross insemination groups (Table II). All the oocytes had >100 spermatozoa bound to the ZP 2 h and 4 h after insemination in the fertilization experiments. The overall polyspermy rate averaged 10% and there was not significant difference between the insemination groups.

The results confirm the conclusion of Thall et al. (1995) that the fertilization process is similar in the knockout and wild-type mice since there was similar ability of spermatozoa to bind to and penetrate the ZP and fertilize in all combinations of gametes from knockout and wild-type mice. There were similar numbers of spermatozoa tightly bound to and penetrating into the ZP at 2 h after insemination between the gametes of knockout, wild-type and knockout/wild-type cross inseminations. Therefore, the fertilization process for the gene knockout mice does not appear to be different from that of the wild-type mice.

Although it is possible that mouse spermatozoa may have more than one type of receptor responsible for binding to the ZP and loss of one may be compensated by others, it is clear from the present study that mouse α-1,3-galactosyltransferase and terminal α galactosyl residues on ZP3 do not play a critical role in fertilization. However, the scale of this study and that of Thall et al. (1995) are not sufficiently large to exclude the possibility that the enzyme contributes to other processes or forms part of an interacting network involved in ZP interaction.

Acknowledgements

The technical assistance of Helen Barlow is gratefully acknowledged.

References


De Yi Liu and H.W.Gordon Baker
University of Melbourne, Department of Obstetrics and Gynaecology, Royal Women’s Hospital, Carlton 3053, Victoria, Australia

Martin J.Pearse and Anthony J.F.d’Apice
Immunology Research Centre, Department of Clinical Immunology, St Vincent’s Hospital, Fitzroy 3052, Victoria, Australia