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Exposure of Thawed Frozen Bull Sperm to a Synthetic Peptide Before Artificial Insemination Increases Fertility

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ABSTRACT: We evaluated the effect on fertility of *in vitro* exposure of thawed frozen bull sperm to synthetic FertPlus® peptide prior to artificial insemination (AI). The peptide represented a 60–amino acid sequence within rat prosaposin. Commercial cryopreserved semen was from three Holstein bulls. Onset of estrus in groups of Holstein nulliparous heifers was synchronized via injection of prostaglandin F_2-α, and heifers were scheduled for AI 8–24 hours after estrus was detected. Semen was thawed, diluted to 2.4 × 10^6 sperm/ml with buffer, and split to provide control and exposed aliquots (0 or 30 μM peptide) that were incubated at 37°C for 10 minutes and then were held at 32°C. The two aliquots of semen then were used on an alternate basis 2–65 minutes later to inseminate females. Each AI (one per female) involved the deposit of ~250,000 sperm into each uterine horn. This procedure for AI was used to reduce the pregnancy rate with control semen to below the maximum value for a given bull and to facilitate detection of any beneficial effect of the peptide. For each bull, ~32 heifers were inseminated with control semen, and ~32 heifers were inseminated with peptide-exposed semen. Pregnancy was evaluated ultrasonically ~60 days after AI. After excluding one group of heifers with unusually low fertility, averaged across all animals, a 29% increase in pregnancy rate resulted from exposure of sperm to peptide ($P < 0.04$; one-tailed chi-square test; means were 48 vs. 62%). Pregnancy rates for the three bulls for control and peptide-exposed semen, respectively, were 42 and 62%, 44 and 64%, and 56 and 61%; means in the first two pairs of values tended to differ ($P \simeq 0.10$). These observations should be confirmed with sperm from other bulls used in a more conventional manner. However, with insemination of a limiting number of cryopreserved sperm, brief exposure of the thawed bull sperm to FertPlus® peptide appeared to improve fertility dramatically.

Key words: Enhancement of fertility, bovine sperm, prosaposin.
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Subfertility is a major problem for andrologists working with domestic animals or humans and has an impact on food production in the United States of more than $1.5 billion annually (Gerrits et al, 1979). Molecules that appear to promote binding of sperm to the zona pellucida or to the perivitelline membrane or to promote penetration of these egg investments have been extensively studied in rodent models and pigs. Usually, this is in the context of an individual ligand and receptor combination. Some authors (Töpfer-Petersen et al, 1995; Thaler and Cardullo, 1996; Tanphaichitr et al, 1998) have highlighted the need for multiple and synergistic ligands. It is evident that sperm are exposed to and modified by specific proteins secreted at multiple sites within the testis, excurrent duct system, or both (Amann et al, 1993; Yanagimichi, 1994; Cornwell and Hann, 1995). Many of these molecules are highly conserved across mammalian species, and some are thought to promote fertility (Killian et al, 1993; Jean et al, 1995; Bellin et al, 1996; Boué and Sullivan, 1996; Klinefelter et al, 1997).

Prosaposin and traces of saposins are found in luminal fluids from the rat rete testis, efferent ducts, and epididymis (Sylvester et al, 1989; Igdoura and Morales, 1995). Based on immunocytochemical studies, prosaposin (or some saposin[s]) is associated with the tail of step 19 rat spermatids and their residual bodies after phagocytosis by Sertoli cells (Sylvester et al, 1984, 1989; Igdoura et al, 1993; Igdoura and Morales, 1995) but not with rat sperm in the epididymis (Sylvester et al, 1989; Hermo et al, 1992). Hence, saposin epitope(s) were undetectable on epididymal rat sperm. However, fragments representing other portions of the prosaposin molecule (i.e., that described by Hammerstedt et al, 1997) might have been present.

Possible roles for prosaposin secreted into male reproductive fluids and found in seminal plasma (Hiraiwa et al, 1993) were unknown until Hammerstedt et al (1997) demonstrated that a fragment of prosaposin was involved in the binding of sperm to the avian perivitelline membrane. They described a 60–amino acid sequence thought to contain epitopes within prosaposin that were active in sperm–egg binding. This region is highly conserved among species studied (chicken, human, mouse, and rat). The sequence is presented in a companion paper (Amann

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et al, 1999), which shows that for many males (human, boar, and bull), *in vitro* binding of sperm to an egg membrane substrate is increased by prior exposure to synthetic FertPlus®. (This peptide technology was developed by BioPore, Inc., under exclusive worldwide license from the Penn State Research Foundation, holder of pertinent patents. Trademarks are property of BioPore, Inc.)

The objective of the present study was to determine if exposure of sperm from a prototype mammal to synthetic FertPlus® peptide improved fertilizing potential of the sperm. Cryopreserved bull sperm were utilized because sensitive and robust fertility trials, using sperm from bulls of known fertility, are practical and cost effective in a commercial setting.

Experimental Design

The split-sample study had a 2 × 3 × 2 design intended to compare the following factors: 1) pregnancy rates achieved with sperm exposed to 0 (control) or 30 μM FertPlus® peptide, 2) semen from three bulls, and 3) two AI technicians. For each bull, all semen was from one freeze code, but seven pools of thawed semen were prepared, and each pool was used to inseminate, on an alternate basis, up to six control heifers and up to six treated heifers. Each technician inseminated, in turn, one control heifer and one treated heifer. A random subgroup from each of six groups of Holstein heifers was allocated to this study, and inseminations for each bull used heifers from two or three groups. Given that a limited number of heifers would be inseminated, we set $\alpha = 0.10$ to minimize the probability of a false-negative conclusion. The design anticipated insemination of ~32 treated heifers and ~32 control heifers with semen from each bull. For data pooled across all bulls, this was expected to afford a >65% chance ($P < 0.10$) of detecting a 15% increase in fertility. Inseminations were made from October through March in an outdoor setting.

We wanted to facilitate detection of an improvement in fertility associated with exposing sperm to FertPlus® peptide, if indeed there was a biological effect. Hence, we used the following: 1) bulls with average fertility; 2) thawed frozen sperm, held at 37 and 32°C for 15–75 minutes before AI; 3) a limiting number of sperm per AI, namely, 500,000 total sperm, so that the insemination dose likely was below the asymptote of the response curve for fertility as a function of sperm/insemination; 4) an insemination procedure that involved deposition of one-half the insemination dose into each uterine horn (Seidel et al, 1996, 1997), rather than entirely in the uterine body, as is conventional with cattle; and 5) only nulliparous Holstein heifers, whose oocytes typically have a high fertilizing potential and whose reproductive systems usually support pregnancy, in order to minimize the female effect on the outcome.

Materials and Methods

Bulls

We identified three Holstein bulls with average fertility and whose genetic attributes were acceptable to the commercial dairy owning the females to be inseminated (Duo Dairy, Loveland, Colorado). Frozen semen (egg yolk citrate extender) was donated by ABS Global, Inc., and Select Sires, Inc., from one freeze code per bull. Estimated relative conception rates for bulls A, B, and C were (for 1997) −2, +2, and −3 percentage units, compared to a national average. Representative straws of semen were thawed and were used to prepare duplicate pools of semen. Concentration of sperm in each pool was determined using a hemacytometer, and resulting data were used to calculate the standard dilution necessary to provide 500,000 total sperm in the 0.21 ml deliverable from the 0.25-ml plastic straws used for inseminations.

Semen Handling

The FertPlus® peptide (BioPore Inc., State College, Pennsylvania) used to treat thawed sperm was a rat sequence (60 amino acids long; Hammerstedt et al, 1997) prepared and stored at −20°C (Amann et al, 1999). The desired mass of peptide (to provide 30 μM solution) was transferred into a series of polycarbonate tubes and lyophilized therein. Tubes were capped and held at −20°C until used. The buffer used to dilute bull semen was a modified Tyrodes solution (bTALP) containing 3.0 mg/ml bovine serum albumin in the following concentrations of these substances: sodium chloride, 95.0 mM; HEPES, 40.0 mM; lactic acid (60% syrup), 25 mM; sodium bicarbonate, 10.0 mM; glucose, 5.0 mM; potassium chloride, 3.0 mM; pyruvic acid, 2.0 mM; magnesium chloride, 0.40 mM; and dibasic sodium phosphate, 0.30 mM.

To minimize cold shock to thawed sperm in the outdoor arena used for preparation of insemination doses, heater blocks adjusted to 32 or 37°C were used to prewarm buffer or tubes and to hold tubes containing sperm suspensions until they were used for AI. Straws to be filled with sperm were prewarmed under a heat lamp, which also warmed the immediate work area, whereas insemination guns and sheaths were prewarmed on a 32°C table. The first semen was thawed ~15 minutes before the first scheduled AI on a given evening.

As appropriate, semen in two straws was thawed by immersion in 37°C water for ~30 seconds and pooled in a prewarmed 12 × 75 mm tube. An aliquot was transferred to a 15-ml tube and diluted to 2.4×10^6 sperm/ml by addition (at ~15-sec intervals) of bTALP in eight aliquots of increasing volume, each designed to reduce glycerol concentration by ~10% of the preceding concentration. For bulls A, B, and C, the final dilutions were 1:13.8, 1:25.6, and 1:9.5. Aliquots of 1.66 ml were transferred into an empty (control) or peptide-containing tube; the latter contained lyophilized material to make the suspension 30 μM peptide. Tubes were capped, inverted several times, and incubated for 10 minutes at 37°C, during which they were inverted another one to three times. Both tubes then were held at 32°C until a subaliquot of the appropriate sperm suspension was aspirated into a 0.25-ml plastic straw immediately before AI of a heifer. The straw was placed into an embryo transfer gun and covered with a side-opening sheath (IMV International, Minneapolis, Minnesota) designed to minimize trauma to the uterine lining. AI occurred within 0.5–8.0 minutes after semen was placed into a straw. Pairs of straws were thawed and processed as above to enable successive inseminations of all scheduled

heifers (for 17 of 21 pools, all AIs were completed by ≤70 minutes after semen was thawed).

Approximately 1 hour after completing the last insemination on a given evening, the residual suspension in each tube (held at 20–32°C) was evaluated (at 37°C, with a phase-contrast microscope) to determine the percentage of motile sperm. With only three exceptions (50 or 55%), all samples had ≥60% motile sperm. It was considered meaningless to try to compare the percentage of motile sperm for a given suspension with pregnancy outcome.

Heifers

Animals were maintained in a commercial setting consistent with National Institutes of Health/U.S. Department of Agriculture guidelines for cattle in an agricultural setting, had continuous access to fresh water, and received appropriate amounts of fresh feed. Virgin Holstein heifers (13–15 months old; 350–450 kg each) were assigned in groups (80–95 heifers per group) to research by the cooperating dairy farm. Each animal received an intramuscular injection of prostaglandin F_2-α (4 ml Lutalyse™, which equals 20 mg prostaglandin F_2-α) and a second injection of 5 ml Lutalyse™ 12 days later. This treatment results in ~75% of animals exhibiting estrus in a relatively synchronized manner (onset of estrus is spread over 3 days) and ovulating ~28 hours after first detected in estrus. Starting 1 day after the second injection of prostaglandin F_2-α, all animals were observed at ≤12-hour intervals, and those heifers detected in estrus were shifted to a holding pen. Each evening (5:30–8:40 PM), animals who were in estrus 8–24 hours earlier were moved randomly into a cattle chute, positioned, identified, and inseminated with sperm of the appropriate treatment. For each transcervical insemination, one of two experienced technicians deposited ~0.1 ml (~250,000 sperm) of the appropriate sperm suspension into each uterine horn (Seidel et al, 1996). On most evenings, some of the heifers in estrus were used for this study, and others were used for other studies.

Animals were returned to group housing and subjected to ultrasonic examinations for pregnancy on days 29–31 and 59–61 after the day of AI. On days 59–61, animals having a viable fetus were classified as pregnant, whereas those lacking a fetus were classified as nonpregnant. In addition, data were scrutinized to identify animals with a viable embryo on days 29–31 but nonpregnant on days 59–61.

Statistical Analyses

Pregnancy data pooled across all three bulls were evaluated by one-tailed Chi-square analysis (using Fisher-Yates correction) to test the significance ($\alpha = 0.10$) of the increase in pregnancy rate after AI using sperm exposed to the peptide. Replicate pool of semen within bull or technician within bull was not considered. Data for each bull also were evaluated on a per-bull basis, as were pregnancy rates achieved by the two technicians for all inseminations (pooled across bulls).

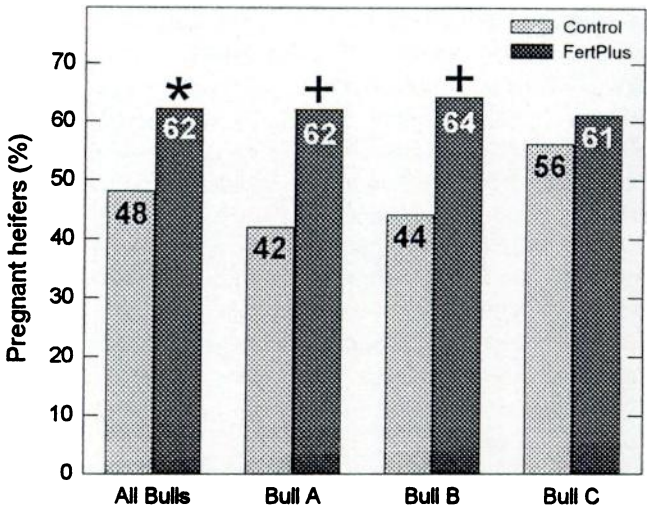

FIG. 1. Effect on pregnancy rate of exposure of thawed bull sperm to 0 (control) or 30 μM FertPlus® peptide for 10 minutes at 37°C, followed 3–65 minutes later by artificial insemination (AI) of ~250,000 sperm deep in each uterine horn. See text for details. Pregnancy data for 180 heifers, representing 24 heifers per group for bull A and ~33 heifers per group for bull B and for bull C, based on ultrasonic evaluations on day ~60 after AI. Significance was designated as follows: *, $P < 0.04$; and +, $P \simeq 0.10$.

Results

Effect of Exposure of Thawed Sperm to FertPlus® Peptide

Data for 197 heifers were available, but two heifers were excluded retrospectively because they were detected in estrus 7–11 days after the experimental AI and were rebred (by farm employees). Occurrence of such short cycles is unrelated to the outcome of any insemination at the earlier estrus. Hence, data were available for 98 heifers inseminated with control sperm and for 97 heifers inseminated with sperm exposed to FertPlus® peptide.

For the sixth group of heifers inseminated, in the cases of both animals used for this study ($n = 17$) and those used for other studies ($n = 29$), there was a very low pregnancy rate. This could be explained by an abrupt change in feeds available to the animals; the animals lost weight over the first 3 weeks after AI. Mean pregnancy rate for this sixth group of heifers was approximately one-half that for the other five groups used in this and concurrent studies (30 vs. 51%; $n = 297$ heifers). Hence, data for these 17 heifers (used only with semen from bull A) were excluded. (If data for this sixth group of heifers had not been excluded, pregnancy rates with control or peptide-exposed sperm for the combined data for all three bulls would have been 46 and 59% [$P \simeq 0.05$], and those for bull A would have been 38 and 52% [$P \simeq 0.19$].)

For data combined across bulls, pregnancy rate was increased by 14 percentage units ($P < 0.04$), or 29%, when sperm were exposed to peptide prior to AI (Fig. 1).

Further, for bulls A and B, pregnancy rate tended to be increased ($P < 0.12$ and $P < 0.09$, respectively). Similar benefit from exposure of sperm to FertPlus® peptide was evident for heifers first detected in estrus ~10 or ~22 hours before AI. Hence, we concluded that exposure of sperm to 30 μM peptide prior to AI increased pregnancy rate.

Pregnancy loss between days 29–31 and days 59–61 was summarized by bull and by treatment. Neither factor seemed important; such loss occurred in one control animal and three treated animals. On average, >97% of the heifers pregnant on days 29–31 were pregnant on days 59–61.

Effect of Interval Between Thawing and AI and Effect of Technician

We classified pregnancy outcome within seminal treatment (but across bulls and technicians) as a function of the size of the interval between thawing semen and actual AI. For semen used 17–25 minutes, 26–40 minutes, 41–55 minutes, 56–70 minutes, or 71–81 minutes after thawing (n = 24, 70, 55, 26, and 5 heifers, respectively), the pregnancy rates were 46, 56, 64, 42, and 60%. There was no decrease in pregnancy rate associated with the size of the interval after thawing semen, although known and unknown factors likely confound this summary.

Pregnancy rates obtained by the two technicians were not different (both averaged 55%; $P \simeq 0.95$). For both technicians, pregnancy rates obtained with treated or control semen were similar.

Discussion

These initial data convincingly demonstrate that the pregnancy rate for thawed sperm from some bulls can be increased by exposure to FertPlus® peptide shortly before AI. This is consistent with data showing that the percentage of sperm binding to an egg membrane substrate *in vitro* can be increased by exposure to this peptide for some, but not all, bulls or boars (Amann et al, 1999). The power of the present experiment was insufficient to detect small changes in pregnancy rate. Hence, bull C might be a male for whom the magnitude of the benefit is a few percentage units and below the detection limit of the present study. Alternatively, he might be a male whose sperm do not benefit from exposure to this peptide, at least at the concentration used. Available data do not allow a conclusion as to which alternative is correct.

With both turkey (Donoghue, unpublished) and chicken (Gill et al, unpublished) sperm, brief exposure to this same peptide prior to AI resulted in significant increases in fertility (measured as percentage of eggs laid providing a living young). Although this is the first synthetic peptide demonstrated to have such an effect in mammals or birds, other unrelated materials have been shown to increase the *in vitro* fertilization rates achieved with bull sperm (Funston and Seidel, 1995; Henault et al, 1995) or the hemizona penetration rates achieved with human sperm (Jean et al, 1995).

The chemically synthesized peptide used in this study incorporates the A–B intervening sequence of prosaposin, as well as a few amino acids normally included in saposins A and B (Hammerstedt et al, 1997; Amann et al, 1999). Prosaposin has been evolutionarily conserved, especially in the A–B intervening sequence. Hence, if some portion of the prosaposin molecule normally is involved in the fertilization process and can be present in suboptimal amounts on some sperm, it is not surprising that the rat sequence was effective in this study with bull sperm. The differential response to synthetic FertPlus® peptide of the populations of sperm representing different males, both *in vitro* (Amann et al, 1999) and *in vivo* (Fig. 1), is evidence that 1) the native molecule might have a role in fertilization, at least under some circumstances; and 2) exposure to the synthetic molecule can enable many sperm to overcome deficiencies in one or more attributes (lacking "enough") so that the combined effective amount is raised sufficiently to increase fertility (see discussion in Amann and Hammerstedt, 1993). Different males will differ in the extent to which exposure of their sperm to this peptide increases fertility, just as the response to any drug depends, in part, on the individual.

If data reported herein are confirmed, the FertPlus® peptide could be used to improve fertility of certain subfertile males. Alternatively, there might be an application appropriate for normal males. It is known that for bulls of normal fertility, the minimum number of sperm required to obtain maximum pregnancy rates extends over an eleven-fold range (a function of the bull; den Daas et al, 1998). For some bulls, use of the peptide might reduce the number of sperm required to obtain maximum fertility and enable greater exploitation of valuable germ plasm.

We do not know the mechanism of action or the role of FertPlus® peptide in increasing pregnancy rate. Based on *in vitro* observations, it is unlikely that it is via a role as a motility stimulant. A role in alteration of sperm binding to the oviductal epithelium cannot be excluded, but Amann et al (1999) suggested that the primary role was in increasing the number of sperm binding to the outer egg investment (zona pellucida for mammals). Speculations in the companion paper (Amann et al, 1999) might serve as a basis for future research. On the other hand, amelioration of subfertility might be better served by establishing that this synthetic peptide indeed is beneficial with many males of species of interest.

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References

Amann RP, Hammerstedt RH. In vitro evaluation of sperm quality: an opinion. *J Androl* 1993;14:397–406.

Amann RP, Hammerstedt RH, Shabanowitz RB. Exposure of human, boar or bull sperm to a synthetic peptide increases binding to egg-membrane substrate. *J Androl* 1999;20:34–41.

Amann RP, Hammerstedt RH, Veeramachaneni DNR. The epididymis and sperm maturation: a perspective. *Reprod Fertil Dev* 1993;5:361–381.

Bellin ME, Hawkins HE, Oyarzo J, Vanderboom RJ, Ax RL. Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. *J Anim Sci* 1996;74:173–182.

Boué F, Sullivan R. Cases of human infertility are associated with the absence of P34H, an epididymal sperm antigen. *Biol Reprod* 1996; 54:1018–1024.

Cornwall GA, Hann SR. Specialized gene expression in the epididymis. *J Androl* 1995;16:379–383.

den Daas JHG, de Jong G, Lansbergen LMTE, van Wagtendonk-de Leeuw AM. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. *J Dairy Sci* 1998;81:1714–1723.

Funston RN, Seidel GE Jr. Gonadotropin-releasing hormone increases cleavage rates of bovine oocytes fertilized in vitro. *Biol Reprod* 1995; 53:541–545.

Gerrits RJ, Blosser TH, Purchase HG, Terrill CE, Warwick EJ. Economics of improving reproductive efficiency in farm animals. In: Hawk HW, ed. *Animal Reproduction.* New York: John Wiley and Sons; 1979: 413–421.

Hammerstedt RH, Cramer PG, Barbato GF. A method and use of polypeptide in sperm–egg binding to enhance or decrease fertility. International Patent Publication Number WO/97/25620. Geneva: World Intellectual Property Organization; 1997:1–42.

Henault MA, Killian GJ, Kavanaugh JF, Griel LC Jr. Effect of accessory sex gland fluid from bulls of differing fertilities on the ability of cauda epididymal sperm to penetrate zona-free bovine oocytes. *Biol Reprod* 1995;52:390–397.

Hermo L, Morales C, Oko R. Immunocytochemical localization of sulfated glycoprotein-1 (SGP-1) and identification of its transcripts in epithelial cells of the extratesticular duct system of the rat. *Anat Rec* 1992;232:401–422.

Hiraiwa M, O'Brien JS, Kishimoto Y, Galdzicka M, Fluharty AL, Ginns EI, Martin BM. Isolation, characterization, and proteolysis of human prosaposin, the precursor of saposins (sphingolipid activator proteins). *Arch Biochem Biophys* 1993;304:110–116.

Igdoura SA, Hermo L, Rosenthal A, Morales CR. Nonciliated cells of the rat efferent ducts endocytose testicular sulfated glycoprotein-1 (SGP-1) and synthesize SGP-1 derived saposins. *Anat Rec* 1993;235: 411–424.

Igdoura SA, Morales CR. Role of sulfated glycoprotein-1 (SGP-1) in the disposal of residual bodies by Sertoli cells of the rat. *Mol Reprod Dev* 1995;40:91–102.

Jean M, Dacheux J-L, Dacheux F, Sagot P, Lopes P, Barriere P. Increased zona-binding ability after incubation of spermatozoa with proteins extracted from spermatozoa of fertile semen. *J Reprod Fertil* 1995; 105:43–48.

Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod* 1993;49:1202–1207.

Klinefelter GR, Laskey JW, Ferrell J, Suarez JD, Roberts NL. Discriminant analysis indicates a single sperm protein (SP-22) is predictive of fertility following exposure to epididymal toxicants. *J Androl* 1997; 18:139–150.

Seidel GE Jr, Allen CH, Johnson LA, Brink Z, Holland, MD, Cattell MB. Insemination of heifers with very low numbers of frozen spermatozoa. *J Anim Sci (Suppl 1)* 1996;74:235.

Seidel GE Jr, Allen CH, Johnson LA, Holland MD, Brink Z, Welch GR, Graham JK, Cattell MB. Uterine horn insemination of heifers with very low numbers of non-frozen and sexed spermatozoa. *Theriogenology* 1997;48:1255–1264

Sylvester SR, Morales C, Oko R, Griswold MD. Sulfated glycoprotein-1 (saposin precursor) in the reproductive tract of the male rat. *Biol Reprod* 1989;41:941–948.

Sylvester SR, Skinner MK, Griswold MD. A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. *Biol Reprod* 1984;31:1087–1101.

Tanphaichitr N, Moase C, Taylor T, Surewicz K, Hansen C, Namking M, Bérubé B, Kamolvarin N, Lingwood CA, Sullivan R, Rattanachaiyanont M, White D. Isolation of antiSLIP1-reactive boar sperm P68/62 and its binding to mammalian zona pellucida. *Mol Reprod Dev* 1998;49:203–216.

Thaler CD, Cardullo RA. The initial molecular interaction between mouse sperm and the zona pellucida is a complex binding event. *J Biol Chem* 1996;271:23289–23297.

Töpfer-Petersen E, Calvete JJ, Sanz L, Sinowatz F. Carbohydrate- and heparin-binding proteins in mammalian fertilization. *Andrologia* 1995;27:303–324.

Yanagimichi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* Vol 1, 2nd ed. New York: Raven Press; 1994:189–317.