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Induction of Acrosome Reactions of Canine Sperm by Homologous Zona Pellucida

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ABSTRACT

In this study the induction of the acrosome reaction of canine sperm by homologous zona pellucida (ZP) was examined. Twelve semen samples obtained from 6 normal beagle dogs were evaluated after sperm incubation in vitro with canine capacitation medium (CCM). Washed sperm were preincubated at 37°C in 5% CO_2 in air for 4 and 7 h prior to experimental treatment. Sperm were co-incubated for 1 min with intact oocytes collected from canine ovaries. Half of the oocytes were then fixed, and the bound sperm were assessed for acrosome reactions through use of a polyclonal antisperm antiserum and indirect immunofluorescence. The remaining oocytes were incubated in sperm-free medium for an additional 1-h period, and the acrosomal status of sperm bound to the ZP was evaluated similarly. The percentage of acrosome-reacted sperm on the ZP increased significantly during the 1-h incubation period. In other experiments, capacitated canine sperm were incubated with heat-solubilized ZP for 1 h and their acrosomal status was determined using fluoresceinated *Pisum sativum* lectin. The percentages of acrosome-reacted sperm increased significantly in ZP solution compared with controls. These data demonstrate that intact and solubilized canine ZP are capable of inducing acrosome reactions of canine sperm.

INTRODUCTION

Mammalian spermatozoa must undergo the physiological changes of capacitation in the female reproductive tract in order to penetrate oocytes [1]. Sperm capacitation is followed by the acrosome reaction, which involves the breakdown and fusion of the sperm plasma membrane and outer acrosomal membrane covering the anterior portion of the sperm head [2, 3]. Spermatozoa bind tightly to the zona pellucida (ZP) prior to penetration of the oocyte. Sperm-ZP binding may involve specific receptors on the sperm and ZP [4], and aggregation of the sperm receptors during binding may elicit the acrosome reaction [5].

Mahi and Yanagimachi [6] found that canine spermatozoa can be capacitated in vitro and are able to penetrate the ZP within 7 h after initiation of incubation. We have recently demonstrated a significant increase in spontaneous acrosome reactions of canine spermatozoa under these in vitro conditions [7]. However, it is not known whether this timing has any relationship to the timing of acrosome reactions in vivo or whether the acrosome reactions are stimulated by canine ZP. It has been reported that bitches immunized with porcine ZP become infertile [8–10] and that antibodies raised against porcine ZP inhibit the penetration of canine ZP by canine spermatozoa [8, 9]. Although the technique of ZP immunization is promising for fertility control in the domestic dog, the mechanisms of action of this approach to immunocontraception will not be understood until more is known about canine sperm-ZP interaction. We have already found that the fluoresceinated lectin staining technique described by Cross et al. [11] is an efficient method for evaluating acrosome reactions of canine spermatozoa [7]. In the present study, we utilized these and other fluorescent labeling techniques to evaluate the relationship of canine sperm acrosomal status to ZP binding and to investigate whether canine spermatozoa can be induced to acrosome-react by homologous intact ZP and heat-solubilized ZP.

MATERIALS AND METHODS

Semen Collection and Sperm Preparation

Six normal beagle dogs 3–5 yr of age were used in these experiments. The dogs were housed in outdoor kennels with shelter provided. Commercial dry dog food (Kal Kan Mealtime, Kal Kan Foods, Inc., Vernon, CA) and water were available ad libitum. All animals were maintained in compliance with the Federal Animal Welfare Act and the NIH Guidelines for Care and Use of Laboratory Animals. Twelve ejaculates were studied (two ejaculates per dog) in each of the experiments using intact and solubilized ZP, respectively. The first fraction and the sperm-rich second fraction of ejaculated semen were collected by digital manipulation and were immediately transported to the laboratory in an insulated container. The concentration of sperm in the semen was determined by hemacytometer counts, and the percentage of motile sperm was estimated by observation of at least 100 cells with a phase-contrast microscope (BH-2, Olympus, Tokyo, Japan) and hemacytometer. Samples with good sperm motility (>90%) and adequate sperm concen-

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tration (>1 × 10^8 sperm/ml) were used in these experiments. The medium used for incubation of spermatozoa was canine capacitation medium (CCM) developed by Mahi and Yanagimachi [12]. This medium is a modified Krebs–Ringer bicarbonate-buffered saline containing 2 g/L BSA (Sigma Chemical Co., St. Louis, MO) and 50 mg/L gentamicin sulfate (Sigma). The semen was washed three times by centrifugation for 5 min at 300 × *g* in 5 ml of CCM. The final pellet was diluted in CCM to a concentration of 5 × 10^6 sperm/ml. Three-millimeter aliquots of the sperm suspensions were placed in 120 × 15-mm polystyrene test tubes (Fisher Scientific, Pittsburgh, PA), loosely capped, and incubated at 37°C with an atmosphere of 5% CO_2 in air. Spermatozoa were utilized for these experiments after 4 and 7 h of incubation.

Oocyte Collection and ZP Preparation

Canine ovaries were obtained from animals following ovariohysterectomy at a veterinary clinic and were put into cold Dunbar's PBS pH 7.4 [13] for transport to the laboratory. Some ovaries were immediately sliced with razor blades to release oocytes into cold Dunbar's PBS. Some oocytes were cryopreserved for use in the intact ZP binding experiments according to methods published previously [14]. Briefly, the oocytes were rinsed in cold Dulbecco's PBS (DPBS) [15] and transferred into cold DPBS. Dimethyl sulfoxide solution (DMSO; Sigma) was added to the DPBS four times at 5-min intervals to a final concentration of 2 M DMSO. Oocytes were loaded into 10-μl capillary tubes (Drummond Scientific, Broomall, PA) and stored at −80°C until used. Oocytes were thawed at room temperature and transferred to 1 ml of 75 mM sodium citrate buffer (pH 7.8) in a 5-ml test tube and were agitated for 2 min with a vortex mixer at high speed to remove the corona radiata cells [12]. The oocytes were then washed twice with CCM and incubated at 37°C in 5% CO_2 before the experiments. The other ovaries were stored frozen at −20°C until used for the solubilized ZP experiments.

Preparation of the solubilized ZP was carried out according to methods published previously [8]. Briefly, the ovaries that had been stored frozen were thawed in a 37°C water bath and sliced as described above. Ovarian debris were removed by filtration using 40-mesh stainless steel (Sigma) and nylon screens (200 μm; Spectrum Medical Industries, Inc., Los Angeles, CA) and cold Dunbar's PBS. Oocytes were then collected on a 52-μm nylon screen. ZP were counted with a hemacytometer, washed in Dunbar's PBS by centrifugation, and solubilized by heating at 70°C for 60 min. Residual particulate matter was removed by centrifugation in a microfuge for 2 min. The supernatant ZP solutions were dialyzed with a molecularporous membrane tubing (Spectrum Medical Industries) using CCM salts without sodium pyruvate, sodium lactate, glucose, BSA, and phenol red. The final ZP solution contained approximately 10 ZP/μl CCM. The solution was stored frozen at −20°C until used in the experiments.

Sperm-ZP Interaction

The experiments to determine the relationship of zona binding to the acrosome reaction were performed as described previously [14]. During the "pulse" phase of the experiment, 6 oocytes were placed in a 100-μl droplet of 5 × 10^6 sperm/ml CCM under silicon oil (Aldrich Chemical Co., Milwaukee, WI) for 1 min. The oocytes were washed to remove loosely bound sperm by pipetting in fresh CCM, and 3 of 6 oocytes were fixed by transfer to cold absolute ethanol in a multiwell glass slide (Polysciences, Inc., Warrington, PA). The remaining oocytes were incubated for an additional hour during the "chase" phase of the experiment in a 100-μl droplet of fresh CCM under oil at 37°C with 5% CO_2 in air.

To determine the effect of solubilized ZP on acrosome reactions of sperm, the ZP solution that had been stored frozen was thawed at room temperature. Sperm suspension (100 μl, 5 × 10^6 sperm/ml) was added to 100 μl of ZP solution (10 ZP/μl) in a polystyrene tube, and the mixture was incubated at 37°C with 5% CO_2 in air for 1 h. As a control, 100 μl of sperm suspension was added to 100 μl of CCM without solubilized ZP and incubated under the same conditions.

Evaluations of Sperm Viability and Acrosomal Status

The technique of indirect immunofluorescence using a polyclonal rabbit anti-human sperm antiserum [11] was modified for use in evaluating the acrosomal status of canine sperm bound to canine ZP. The methods for antibody production have been described previously [11]. ZP on multiwell glass slides were covered with an aliquot of antisperm antiserum diluted 1:10 with DPBS containing 50 mg/ml BSA (DPBS-BSA) and were incubated at 37°C for 2 h. After ZP were rinsed gently with DPBS-BSA, they were covered with an aliquot of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) diluted 1:10 with DPBS-BSA and were incubated at 37°C for 1 h. After ZP were rinsed, they were mounted using a medium consisting of 100 mg/ml of DABCO (1,4-diazabicyclo [2-2-2] octane; Sigma) in absolute ethanol. Samples were examined via fluorescence microscopy. Spermatozoa were scored as acrosome-reacted if there was little or no labeling of the anterior head region [11]. The equatorial region of acrosome-reacted sperm was often labeled. Spermatozoa with uniform or irregular labeling of the anterior head were scored as acrosome-intact. The sperm remaining in suspension after removal of the oocytes were assessed for viability by incubation with the supravital dye Hoechst 33258 (H258; Sigma) [11]; after sperm recovery on polycarbonate filters, the acrosomal status of the sperm was evaluated using *Pisum sativum* agglutinin conjugated with FITC (FITC-PSA; Vector Labs, Burlingame,

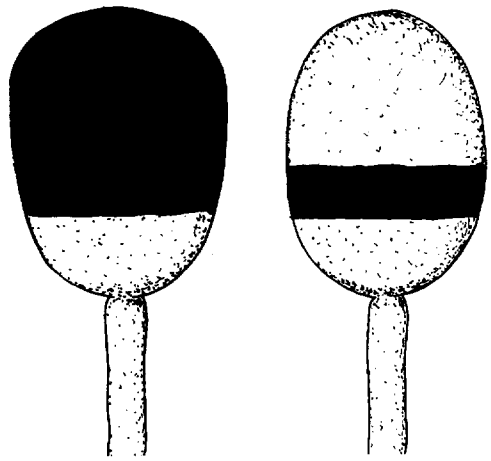

FIG. 1. Fluorescence patterns observed in intact and acrosome-reacted sperm. The acrosomal region of the acrosome intact sperm is heavily labeled. Acrosome-reacted sperm retain an equatorial band of label but there is little or no labeling of the anterior head region. The same staining pattern was observed when indirect immunofluorescence and FITC-PSA were used to assess acrosomal status. Photomicrographs of FITC-PSA-stained canine sperm have been published previously [7].

CA) [16]. The viability and acrosomal status of sperm in the solubilized ZP experiment were evaluated using H258 and FITC-PSA as previously reported [7].

The data were summarized as mean values ± SEM. Differences between means were tested using a paired *t*-test.

RESULTS

Comparison of Anti-Sperm Antiserum and PSA

To investigate the accuracy of assessing the acrosomal status of canine sperm with rabbit anti-sperm antiserum, the percentages of acrosome-reacted sperm in suspension after 4 h of incubation were compared using FITC-PSA and indirect immunofluorescence with the antisperm antiserum (Fig. 1). There was no significant difference between the mean percentage of viable, acrosome-reacted sperm (± SEM) detected with anti-serum and that detected with FITC-PSA (10.1 ± 1.5%, 9.7 ± 1.5%, respectively, n = 6).

Sperm Interaction with Intact ZP

The mean percentages of motile, viable, and acrosome-reacted sperm in the sperm suspension following removal of the oocytes in the pulse phase of the experiment are shown in Table 1. There were significant decreases in sperm motility and viability between 4 and 7 h of preincubation, and the percentage of spontaneous acrosome reactions increased between the 4-h and 7-h time points (Table 1).

The number and acrosomal status of sperm bound to the ZP are shown in Table 2. The total number of sperm bound to the ZP decreased after the chase period in comparison with the pulse, but only at the 4-h time point was the decrease statistically significant (Table 2). There were

TABLE 1. Percentages of motile, viable, and acrosome-reacted sperm in the suspension after the pulse phase of the experiments.[a]

Time of sperm preincubation (h)	Percent motile sperm	Percent total viable sperm	Percent viable, acrosome-reacted sperm[b]
4	71.2 ± 1.5	78.5 ± 1.5	11.4 ± 1.0
7	60.5 ± 1.5[c]	70.8 ± 1.5[d]	31.2 ± 1.0[c]

[a]Values are mean ± SEM for 12 experiments using 6 dogs.
[b]These percentages are based on the total viable sperm.
[c]Significantly different from 4 h ($p < 0.001$).
[d]Significantly different from 4 h ($p < 0.005$).

no significant differences between the percentages of acrosome-reacted sperm in suspension and the percentages of acrosome-reacted sperm on the surface of the ZP after the pulse at either the 4-h or 7-h time points (Tables 1 and 2). However, there were significant increases in the percentages of acrosome-reacted sperm on the ZP following the chase at both time points. The percentage of acrosome-reacted sperm on the ZP following the chase was higher at 7 h in comparison to 4 h (Table 2).

Sperm Interaction with Solubilized ZP

The percentages of motile and viable sperm declined between 4 and 7 h of incubation (Table 3), and neither motility nor viability was affected by solubilized ZP. The percentages of acrosome-reacted sperm increased significantly after incubation with solubilized ZP at both time points (Table 3).

DISCUSSION

Experiments with the mouse [17] and with the hamster [18] have indicated that acrosome-intact, but not acrosome-reacted sperm, are able to bind to the ZP. However, acrosome-intact and acrosome-reacted guinea pig spermatozoa are capable of binding to the ZP [19], and both acrosome-intact and acrosome-reacted human sperm appear to bind with equal efficiency to the ZP [20]. In the present study, acrosome-intact and acrosome-reacted canine sperm were observed on the surface of canine ZP after the 1-min coincubation (pulse) in proportions similar to those observed in the sperm suspension. These data suggest that both acrosome-intact and acrosome-reacted dog sperm are capable of binding to the ZP. Hartman and Hutchison [21] reported that capacitated hamster spermatozoa bound to the ZP immediately after insemination in vitro but dropped off by 5 min after insemination. Lee and Storey [22] observed that the number of mouse sperm bound to the ZP decreased over the time of incubation, whether or not loosely attached sperm were removed. However, significant sperm detachment does not appear to be a common occurrence following sperm-zona binding in humans [20] and nonhuman primates [14]. In the present study, the decrease in number of canine spermatozoa bound to the ZP at the end

TABLE 2. Total number of sperm bound to zona pellucida and the percentage of acrosome-reacted sperm on the zona pellucida after the pulse and chase phases of the experiments[a]

	Total number of sperm bound to zona pellucida		Percent acrosome-reacted sperm	
Time of sperm preincubation (h)	Pulse	Chase	Pulse	Chase
4	58 ± 9	36 ± 4[b]	15.7 ± 2.2	36.7 ± 1.6[c]
7	45 ± 9	28 ± 4	30.6 ± 2.2[d]	48.7 ± 1.6[cd]

[a]Values are mean ± SEM for 12 experiments using 6 dogs.
[b]Significantly different from pulse ($p < 0.05$).
[c]Significantly different from pulse ($p < 0.001$).
[d]Significantly different from 4 h ($p < 0.001$).

of the chase may have been caused by detachment of spermatozoa from the ZP during the chase.

The ZP or its components have been shown to induce sperm acrosome reactions in several species. Bleil and Wassarman [23] demonstrated that the mouse ZP glycoprotein, ZP3, induces the acrosome reaction. The 55-kDa glycoprotein component of pig ZP has acrosome reaction-inducing activity [24]. Homologous intact ZP can induce acrosome reactions in vitro in spermatozoa from humans [25], macaques [14], cattle [26], sheep [27], rats [28], hamsters [29], and mice [30–32]; and solubilized ZP has been shown to induce acrosome reactions in spermatozoa from humans [25], cattle [33, 34], pigs [24], rabbits [35], hamsters [29], and mice [31]. In the present study, there was an increase in the percentage of acrosome-reacted sperm bound to the ZP following incubation; a significant increase in the percentage of acrosome-reacted sperm was induced by incubation with solubilized ZP. Therefore, we conclude that canine ZP can induce the acrosome reaction of canine sperm in vitro.

Cherr et al. [29] reported that hamster ZP can induce acrosome reactions within 15 min after insemination. Although few human spermatozoa are induced to acrosome-react within 1 min of ZP binding, nonhuman primate spermatozoa acrosome-react in significant numbers within 1 min of co-incubation [14]. In the present study, there was no significant difference in the percentage of acrosome-reacted sperm on the surface of the ZP after the pulse in comparison with sperm in suspension. These data suggest that canine sperm, like sperm of rodents and humans, require a period of more than 1 min on the ZP before acrosome reactions are induced. It is possible that the rapidity of induction of acrosome reactions following macaque sperm-ZP binding is related to the synchony of capacitation that is induced in macaque sperm as a result of incubation with medium containing caffeine and dbcAMP [14]. It is interesting that canine spermatozoa, which demonstrate a significant increase in spontaneous acrosome reactions between 4- and 7-h capacitation in vitro, show no evidence of increased responsiveness to ZP during the same time period.

Mahi and Yanagimachi [6] found that canine sperm required capacitation before they were able to penetrate the ZP. Yamada et al. [36] found that canine sperm preincubated for 4 h can begin to penetrate the ZP within 1 h after insemination. Under the capacitation conditions used in these experiments, we have observed spontaneous acrosome reactions in canine spermatozoa after 4 h of incubation, with an additional significant increase after 7 h of incubation [7]. Similarly, in all treatments of the current study the percentage of acrosome-reacted sperm was higher at 7 h of preincubation than at 4 h, suggesting that more sperm became capacitated and were therefore able to respond to induction of the acrosome reaction as the incubation time increased. The sequence of events during fertilization and the conditions that promote sperm-ZP interaction in vivo are known to differ among species [1]. The events of sperm transport and fertilization in the bitch differ from those in many mammalian species in several respects, including the capability for prolonged sperm storage in the female tract and the fertilization of the germinal vesicle-stage oocyte [37]. Conservation of sperm function in the female tract may require specialized adaptations to control the rate of canine sperm capacitation in vivo. Nevertheless, the induction of the acrosome reaction in the fertilizing spermatozoa of do-

TABLE 3. Percentages of motile, viable, and acrosome-reacted sperm after incubation with solubilized zona pellucida for 1 h.[a]

	Percent motile sperm		Percent viable sperm		Percent viable, acrosome-reacted sperm[b]	
Time of sperm preincubation (h)	ZP solution	Control	ZP solution	Control	ZP solution	Control
4	65.7 ± 1.3	63.0 ± 1.1	77.3 ± 1.1	75.3 ± 1.3	22.5 ± 1.6[c]	13.3 ± 1.1
7	53.3 ± 2.0[d]	52.0 ± 1.9[d]	66.2 ± 1.8[d]	66.3 ± 2.3[d]	41.2 ± 1.9[cd]	29.8 ± 1.5[d]

[a]Values are mean ± SEM for 12 experiments using 6 dogs.
[b]These percentages are based on total viable sperm.
[c]Significantly different from control ($p < 0.001$).
[d]Significantly different from 4-h time point ($p < 0.001$)

mestic dogs appears to be accomplished by the same process as in other mammalian species.

REFERENCES

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), Physiology of Reproduction, vol 1. New York: Raven Press; 1988: 135.
2. Bedford JM. Ultrastructural changes in the sperm head during fertilization in the rabbit. Am J Anat 1968; 123:329–358.
3. Yanagimachi R, Usui N. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp Cell Res 1974; 89:161–174.
4. Bleil JD, Wassarman PM. Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell 1980; 20:873–882.
5. Leyton L, Saling P. Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. J Cell Biol 1989; 108:2163–2168.
6. Mahi CA, Yanagimachi R. Maturation and sperm penetration of canine ovarian oocytes *in vitro*. J Exp Zool 1976; 196:189–196.
7. Kawakami E, VandeVoort CA, Mahi-Brown CA, Tollner TL, Overstreet JW. Comparison of a fluoresceinated lectin stain with triple staining for evaluating acrosome reactions of dog sperm. J Exp Zool 1993; (in press).
8. Mahi-Brown CA, Huang TTF Jr, Yanagimachi R. Infertility in bitches induced by active immunization with porcine zonae pellucidae. J Exp Zool 1982; 222:89–95.
9. Mahi-Brown CA, Yanagimachi R, Hoffman JC, Huang TTF Jr. Fertility control in the bitch by active immunization with porcine zonae pellucidae: use of different adjuvants and patterns of estradiol and progesterone levels in estrous cycles. Biol Reprod 1985; 32:761–772.
10. Mahi-Brown CA, Yanagimachi R, Nelson ML, Yanagimachi H, Palumbo N. Ovarian histopathology of bitches immunized with porcine zonae pellucidae. Am J Reprod Immunol Microbiol 1988; 18:94–103.
11. Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 1986; 15:213–226.
12. Mahi CA, Yanagimachi R. Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. Gamete Res 1978; 1:101–109.
13. Dunbar BS, Wardrip NJ, Hedrick JL. Isolation, physicochemical properties, and macromolecular composition of zona pellucida from porcine oocytes. Biochemistry 1980; 19:356–365.
14. VandeVoort CA, Tollner TL, Overstreet JW. Sperm-zona pellucida interaction in cynomolgus and rhesus macaques. J Androl 1992; 13:428–432.
15. Dulbecco R, Vogt M. Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med 1954; 99:167–182.
16. Morales P, Cross NL. A new procedure for determining acrosomal status of very small numbers of human sperm. J Histochem Cytochem 1989; 37:1291–1292.
17. Saling PM, Storey BT. Mouse gamete interactions during fertilization *in vitro*. Chlortetracycline as a fluorescent probe for the mouse sperm acrosome reaction. J Cell Biol 1979; 83:544–555.
18. Philips DM, Shalgi RM. Surface properties of the zona pellucida. J Exp Zool 1980; 213:1–8.
19. Myles DG, Hyatt H, Primakoff P. Binding of both acrosome-intact and acrosome-reacted guinea pig sperm to the zona pellucida during *in vitro* fertilization. Dev Biol 1987; 121:559–567.
20. Morales P, Cross NL, Overstreet JW, Hanson FW. Acrosome intact and acrosome-reacted human sperm can initiate binding to the zona pellucida. Dev Biol 1989; 133:385–392.
21. Hartman JF, Hutchison CF. Nature of the prepenetration contact interactions between hamster gametes *in vitro*. J Reprod Fertil 1974; 36:49–57.
22. Lee MA, Storey BT. Endpoint of first stage of zona pellucida-induced acrosome reaction in mouse spermatozoa characterized by acrosomal H^+ and Ca^{2+} permeability. Gamete Res 1989; 24:303–326.
23. Bleil JD, Wassarman PM. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev Biol 1983; 95:317–324.
24. Berger T, Turner KO, Meizel S, Hedrick JL. Zona pellucida-induced acrosome reaction in boar sperm. Biol Reprod 1989; 40:525–530.
25. Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. Biol Reprod 1988; 38:235–244.
26. Crozet N. Ultrastructural aspects of *in vivo* fertilization in the cow. Gamete Res 1984; 10:241–251.
27. Crozet N, Dumont M. The site of the acrosome reaction during *in vivo* penetration of the sheep oocyte. Gamete Res 1984; 10:97–105.
28. Shalgi R, Phillips DM, Jones R. Status of the rat acrosome during sperm-zona pellucida interactions. Gamete Res 1989; 22:1–13.
29. Cherr GN, Lambert H, Meizel S, Katz D. *In vitro* studies of the golden hamster acrosome reaction: completion on the zona pellucida and induction by homologous soluble zonae pellucidae. Dev Biol 1986; 114:119–131.
30. Saling PM, Sowinski J, Storey BT. An ultra-structural study of epididymal mouse spermatozoa binding to zonae pillucidae *in vitro*: sequential relationship to the acrosome reaction. J Exp Zool 1979; 209:229–238.
31. Florman HM, Storey BT. Mouse gamete interactions: the zona pellucida is the site of the acrosome reaction leading to fertilization *in vivo*. Dev Biol 1982; 91:121–130.
32. Storey BT, Lee MA, Muller C, Ward CR, Wirtshafter DG. Binding of mouse spermatozoa to the zonae pellucidae of mouse eggs in cumulus: evidence that acrosomes remain substantially intact. Biol Reprod 1984; 31:1119–1128.
33. Florman HM, First NL. The regulation of acrosomal exocytosis. I. Sperm capacitation is required for the induction of acrosome reactions by the bovine zona pellucida *in vitro*. Dev Biol 1988; 128:453–463.
34. McNutt TL, Killian GJ. Influence of bovine follicular and oviduct fluids on sperm capacitation *in vitro*. J Androl 1991; 12:244–252.
35. O'Rand MG, Fisher SJ. Localization of zona pellucida binding sites on rabbit spermatozoa and induction of the acrosome reaction by solubilized zonae. Dev Biol 1987; 119:551–559.
36. Yamada S, Kawaji H, Nakazawa M, Naito K, Toyoda Y. Maturation, fertilization, and development of dog oocytes *in vitro*. Biol Reprod 1992; 46:853–858.
37. Mahi-Brown CA. Fertilization in dogs. In: Dunbar BS, O'Rand MG (eds.), A Comparative Overview of Mammalian Fertilization. New York: Plenum Press; 1991: 281–298.