Induction of Dog Sperm Capacitation by Oviductal Fluid

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ABSTRACT. Four estrous beagles were inseminated with 1×10^8 sperm into both the right and left uterine horns, and the uterine horn and oviduct on one side were removed under anesthesia after 7 hr and 24 hr, respectively. The lumen of the uterine horns and oviducts was flushed with canine capacitation medium (CCM), and movement of the sperm in CCM was assessed by phase-contrast microscopy. In a second experiment, ejaculated sperm obtained from 5 normal beagles was incubated in CCM supplemented with oviductal flush fluid (OF-CCM) at 38°C with 5% CO₂ in air. Motility of sperm, and percentages of hyperactivated sperm (%HA) and acrosome-reacted sperm (%AR) among freely swimming (FS) sperm were investigated until 24 hr after the start of incubation. After 7 hr of incubation the sperm was co-incubated with canine oocytes in OF-CCM for 2 hr, and the number of zona pellucida-binding (zona-binding) sperm was then counted. The %HA among the sperm in the oviductal flush fluid both 7 hr (mean $\pm$ S.E.; $15.0 \pm 2.4\%$) and 24 hr ($77.5 \pm 5.2\%$) after intrauterine insemination were significantly higher than in the uterine flush fluid ($P < 0.05$, 0.01 , respectively). The motility and %HA among FS-sperm in OF-CCM were higher than in the control medium without oviductal fluid. However, there was no difference in the %AR between OF-CCM and control medium. The number of zona-binding sperm in OF-CCM (8 ± 1) was significantly greater than in control medium (5 ± 1) ($P < 0.05$). These results suggest that oviductal fluid in the estrous bitch maintains sperm motility and induces sperm capacitation. — **KEY WORDS:** canine, oviductal fluid, sperm capacitation.

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The estrous period in the bitch is longer than in most animals and lasts an average of 10 days [9, 39]. Ovulation occurs 2 or 3 days after the onset of estrus in most bitches [29, 38]. The eggs are ovulated as primary oocytes and are incapable of being fertilized until at least 48 hr later when they become secondary oocytes in the oviduct [15, 40]. In spite of this long delay, a single mating can be fertile even when it occurs on the first day of estrus. Therefore, it appears that the fertile life of canine sperm must be maintained for at least 5 days in the female reproductive tract.

There is evidence that the oviduct has the function to store sperm [27] and to maintain sperm viability [7, 16, 37] until the time of ovulation and fertilization. The ewe [5], pig [35], rabbit [4], hamster [18] and mouse [26] have been found to have high rates of freely swimming (FS) sperm exhibiting star-spin-like active movement or vigorous flagellar movement called “hyperactivation” in their oviducts. In the bull [25], sperm motility and viability were maintained for a longer period and more effectively induced by addition of oviductal fluid to the sperm medium *in vitro*. However, observations of canine sperm interactions with homologous oviductal fluid have not been reported. In the present study, the motility and capacitation of dog sperm in the flush fluid of the reproductive tracts of estrous bitches after intrauterine insemination and in medium supplemented with oviductal fluid obtained from estrous bitches were examined to search for the relation between maintenance of motility of dog sperm for a long period and oviductal fluid.

MATERIALS AND METHODS

Animals: Five male beagles with normal semen quality

and four beagles in the estrous period, aged 2–5 years, were used in this experiment. The optimum time for intrauterine insemination in the estrous bitches was determined by daily examination of receptivity for a male dog after vaginal bleeding.

Four anestrous bitches ovariectomized at the teaching hospital of our university were used for the zona pellucida-binding assay.

Semen collection and evaluation: The sperm-rich second fraction of ejaculated semen [14] was collected by digital manipulation and immediately transported to the laboratory. The concentration of sperm in the semen was determined by hemacytometer counts. The percentage of motile sperm was estimated by using a sperm motility examination plate (Fujihira Industry Inc., Tokyo), and semen samples with good sperm motility (>90%) were used.

Intrauterine insemination and flushing of uteri and oviducts: Intrauterine insemination was performed on four estrous bitches on the 4th or 5th day after the start of acceptance, as described in the report by Tsutsui *et al.* [41]. All of the bitches were laparotomized under halothane inhalation anesthesia. A 20-gauge retaining needle connected to a 1 ml/ injection syringe was inserted into the middle portion of the right and left uterine horns, and semen adjusted to a concentration of 1×10^8 sperm/0.3 ml/ seminal plasma collected from one of four dogs was injected into each uterine horn. The uterine horns and oviducts on the right and left side were removed 7 and 24 hr, respectively, after the intra-uterine insemination. The flush fluid was collected in a watch glass on a warm plate, and the movement of sperm in the flush fluid was immediately examined under a phase-contrast microscope (HFX-IIA, Nikon Inc., Japan). The percentage of motile sperm among

the free swimming (FS)-sperm and the percentage of sperm exhibiting active tail movement (%VT) among the sperm attached to oviductal epithelial cells (OEC) collected by the oviductal flushing were determined.

Sperm incubation and evaluation of hyperactivated and acrosome-reacted sperm: The spermatozoa collected from 5 dogs were washed twice by centrifugation at 300 g for 5 min in 5 ml of canine capacitation medium (CCM) as described by Mahi and Yanagimachi [24]. The final pellet was diluted in CCM supplemented with oviductal fluid (OF-CCM) to a concentration of 5×10^6 sperm/ml. The OF-CCM contained a final concentration of 20% CCM which was used to flush the oviducts. The oviducts were removed from estrous bitches under general anesthesia and then flushed each with 1.0 ml of CCM. Sperm incubation was performed in 120×15 mm glass test tubes, and the tubes were loosely capped and cultured at 38°C in an atmosphere of 5% CO₂ in air. The percentages of PM, HA, and acrosome-reacted (AR) sperm among the FS-sperm were examined 1, 2, 4, 7, and 24 hr after the start of incubation. The percentages of hyperactivated sperm (%HA) and acrosome-reacted sperm (%AR) were determined by counting the sperm with star-spin-like movement [19] in all of motile sperm and by the triple stain technique [36], respectively. The %VT among the sperm attached to the OEC collected by the oviductal flushing was investigated. This experiment was replicated three times for each dog.

Oocyte collection and zona pellucida-binding assay: the ovaries collected by ovariectomy from the 4 anestrous bitches were sliced with razor blades to release oocytes into CCM [20]. The oocytes were transferred to 1 ml of 75 mM sodium citrate buffer (pH 7.8) in a 5-ml test tube and agitated for 2 min with a vortex mixer to remove the corona radiata cells [20]. The oocytes were then washed by pipetting in fresh CCM and cryopreserved with CCM

containing 2 M dimethyl sulfoxide at -20°C until used for the zona pellucida-binding assay (zona-binding assay).

The extinct oocytes were later thawed at room temperature and washed twice with fresh CCM. Five oocytes were placed in a 100- μ l droplet of 5×10^6 sperm/ml CCM under paraffin oil (Nacalai Tesque, Inc., Japan) in 35-mm plastic tissue culture dishes. The sperm were co-cultured with the oocytes 7 hr after the start of incubation in OF-CCM and control CCM without oviductal fluid. The oocytes co-cultured for 2 hr were washed to remove loosely bound sperm by pipetting in fresh CCM and then transferred into other culture dishes without sperm. The number of sperms strongly bound to zona pellucida were counted under a phase-contrast microscope.

Statistical analysis: The data are summarized as mean values $\pm$ S.E.M. Differences between means were analyzed statistically using Student's *t* test.

RESULTS

Capacitation of sperm collected from uteri and oviducts: The mean %HA of FS-sperm in the oviductal flush fluid was significantly higher than in the uterine flush fluid both 7 and 24 hr after intrauterine insemination ($P < 0.05$ and 0.01 , respectively) (Fig. 1). The motility among the sperm in the oviductal flush fluid ($55.3 \pm 2.9\%$) was higher than in the uterine flush fluid ($38.0 \pm 5.7\%$) 24 hr after the insemination, and the %HA at 24 hr ($77.5 \pm 5.2\%$) was significantly higher than at 7 hr ($15.0 \pm 2.4\%$) ($P < 0.01$). Although the %VT ($81.5 \pm 4.4\%$) of the sperm attached to the OEC in the oviductal flush fluid decreased slightly 24 hr after intrauterine insemination, it was significantly higher than the motility of the FS-sperm ($P < 0.01$).

Capacitation of sperm incubated with oviductal fluid: The motility of FS-sperm in OF-CCM was maintained at significantly higher levels than in the control medium

Fig. 1. Mean ($\pm$ S.E.) percentages of motile sperm in the flush fluid of the oviducts and uterine horns obtained from estrous bitches at 7 hr and 24 hr incubation after flushing. * $P < 0.05$, ** $P < 0.01$, in comparison with uterine flush fluid. This experiment was replicated three times for each dog.

Fig. 2. Changes in the mean ($\pm$ S.D.) percentages of motile sperm collected from 5 dogs in CCM containing oviductal fluid obtained from estrous bitches during the injection. $**P<0.01$, in comparison with freely swimming sperm. This experiment was replicated three times for each dog.

Fig. 3. Changes in the mean ($\pm$ S.E.) percentages of hyperactivated sperm collected from 5 dogs in CCM containing oviductal fluid obtained from estrous bitches during the incubation. $**P<0.01$, in comparison with control CCM. This experiment was replicated three times for each dog.

without oviductal fluid throughout this experiment ($P<0.01$) (Fig. 2). However, the motility ($20 \pm 2\%$) of FS-sperm in OF-CCM was significantly lower than the %VT ($67 \pm 3\%$) of sperm attached to the OEC after 24 hr of incubation (Fig. 2).

The %HA of FS-sperm in OF-CCM was higher than in control CCM throughout this experiment. The values in OF-CCM after both 4 ($59 \pm 5\%$) and 7 hr ($74 \pm 3\%$) of incubation were 1.5 fold those in the control medium (Fig. 3). There was little difference between the %AR of FS-sperm in OF-CCM and in control medium during 1–7 hr of incubation (Fig. 4).

The number of sperm attached to the zona pellucida in the sperm incubated in OF-CCM for 7 hr was significantly more than in the control medium (Fig. 5).

DISCUSSION

In the bull [7, 30], horse [37], pig [16], rabbit [27], hamster [32] and mouse [34], it has been observed that sperm transported to the oviduct through the uterine horn attach to the OEC to maintain motility and viability. The OEC is known to secrete substances that support and maintain sperm motility and viability [1, 17, 21]. Glycosaminoglycans, a group of glycoproteins, in the oviductal fluid are candidates for factors that maintain sperm motility and induce capacitation [2, 12, 22, 28, 33]. High percentages of FS-sperm in the oviduct were found to exhibit active HA-like movement, a capacitation phenomenon, in the human [13], sheep [5], pig [35], rabbit [4], hamster [18], and mouse [26]. As described in the above reports, the %HA of the sperm collected from the oviduct in this

Fig. 4. Changes in the mean ($\pm$ S.E.) percentages of acrosome-reacted sperm collected from 5 dogs in CCM containing fluid obtained from estrous bitches during the incubation. This experiment was replicated three times for each dog.

Fig. 5. The mean ($\pm$ S.E.) number of zona pellucida-binding sperm for 2 hr co-incubation after 7 hr preincubation in CCM containing oviductal fluid obtained from estrous bitches during the incubation. * $P < 0.05$, in comparison with control CCM. Sperm were collected from 5 dogs and this experiment was replicated three times for each dog.

study was also higher than those collected from the uterus. It has been reported that a large number of sperm were found in the uterine glands of copulated bitches and that sperm motility was maintained for ten days after mating [6]. However, HA-like movement of sperm has not been found. Therefore, we concluded that the volume of sperm capacitation-related materials contained in the uterine secretion is less than in the oviductal secretion. It has been reported that dog sperm were able to be capacitated before and after 7 hr of incubation [23] and that the fertile life of sperm in the reproductive tract of the bitch is at least 6 days

[3]. In the present study, it is assumed that the number of capacitated sperm and %HA gradually increased in the oviduct of the bitch after 7 hr of artificial insemination.

We found that addition of canine oviductal flush fluid to the homologous sperm medium results in longer maintenance of sperm motility and higher percentages of capacitated sperm, which has been similarly described in the reports on other animals [22, 25, 28]. Induction of sperm capacitation by canine oviductal fluid was demonstrated not only by the results of the assessments of HA and AR but also by the results of the zona-binding assay as well. The zona-binding assay is a method that enables a more precise assessment of sperm fertility [8, 10, 11, 42].

Dog sperm attached to the OEC had longer lasting flagellar movement of the tail than FS-sperm, and similar results have been reported for other animals [7, 31]. It is believed that OEC-binding sperm are able to maintain their motility and viability for more days than FS-sperm because they receive a direct supply of energy sources from the OEC and because of suppression of active movement, such as HA. Stallion and dog sperm must retain their viability longer because of the very long estrous period in the mare [37] and bitch [9, 39]. It has been claimed that attaching to the OEC by stallion sperm results in longer motility in the oviduct [37]. The results of the present study suggest that dog sperm transported into the oviduct lumen are capable of waiting for ovulation and maturation of the ovulated oocytes because of their sustained motility and fertility resulting from attachment to the OEC and the effects of the oviductal fluid. As a next step, it will be necessary to analyze which components of canine oviductal fluid are related to the capacitation of dog sperm.

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