The surface of the plasma membrane of mammalian sperm is coated with various glycoproteins [13, 15, 25, 39, 41], and some of the glycoproteins on the surface of the sperm are thought to be related to sperm maturation in the epididymal duct [15, 25, 39] and sperm capacitation in the female reproductive tract [10, 31, 40]. Studies with fluorescein-isothiocyanate (FITC)-labeled lectins have shown that the glycoproteins secreted by the epithelial cells of the epididymal duct bind to the entire heads or acrosomal regions of the sperm during their transit through the epididymis [3, 6, 21, 27, 29] and that the ejaculated sperm are coated with glycoproteins produced by the accessory reproductive glands [5, 9]. Some of the glycoproteins on the surface of sperm have been suspected of acting as decapacitation factors [2, 10, 24, 31]. The secretory fluids in the lumen of the epididymal duct contain various enzymes [9, 29], and the concentrations and activities of the enzymes increase in the follicular phase of the ovarian cycle [38]. It has been found that some of the glycoproteins on the surface of the sperm of the rabbit [29] and hamster [38] are degraded and disappear as a result of the action of proteases, enzymes that hydrolyze proteins, and that the uterine fluid of the hamster contains proteases [38]. Sperm capacitation in the ewe [40], pig [10] and rabbit [31] is induced by removal of glycoproteins from the surface of the sperm plasma membrane after exposure to fluid in the female reproductive tract, and canine sperm capacitation has been also reported to be related to loss of glycoproteins from the acrosomal regions of sperm [13]. Sperm capacitation is initiated by an influx of Ca²⁺ into the sperm cytoplasm [11, 12, 32, 34]. The Ca²⁺ influx into the sperm induces abundant energy production by increasing cyclic AMP levels [32, 34]. Therefore, the disappearance of some glycoproteins on the sperm surface is important for the start of capacitation, but there have been few reports on the mechanism and process of canine sperm capacitation [18, 19].

In the present study, the protease concentration in the flush fluids of the uterine horns and oviducts of bitches was measured, and canine sperm were incubated in culture medium supplemented with protease to examine the relationships between the disappearance of glycoproteins on the heads of canine sperm as a result of the action of proteolytic enzymes, i.e., proteases, and the induction of sperm capacitation. After incubation the glycoproteins on the surface of the sperm were stained with 4 different FITC-lectins that bind to different saccharides, and the percentages of hyperactivated (HA−) sperm and acrosome-reacted (AR–) sperm were evaluated as indexes of sperm capacitation.

**MATERIALS AND METHODS**

**Animals**: Ten male beagle dogs (2–7 years old) with normal semen quality were used in this experiment. They were cared for in our university and housed in pens with ample runs. Commercial dry dog food was provided twice a day, and the dogs were given access to water *ad libitum*. All animals were maintained according to the guidelines of the Animal Care and Use Committee of the Nippon Veterinary
and Animal Science University.

Flushing of uteri and oviducts: Uteri and oviducts were obtained from 6 estrous, 5 diestrous, and 5 anestrous bitches (1–5 years old) by ovariohysterectomy for contraceptive purposes at the teaching hospital of our university and veterinary clinics in Tokyo. The stages of the estrous cycle were identified on the basis of vaginal bleeding and the observation of large antral follicles of the removed ovaries.

The uterine horns and oviducts on the right side were used to measure protease concentrations in flush fluids of the reproductive tract. A 20-gauge retaining needle connected to a 1-ml injection syringe was inserted into the lumen of the uterine horns and oviducts, and then the lumen of each portion was flushed with 1 ml of HEPES-EDTA (Sigma Chemical Co., Ltd., MO, U.S.A.) solution (25 mM HEPES and 1 mM EDTA, pH 7.3). The flush fluids were collected in test tubes.

Protease assay: The protease concentration of flush fluids from the uterine horns and oviducts of the bitches was measured with a protease assay kit (Quantikine Cleave, Pierce Biotechnology Co., Ltd., IL, U.S.A.). The assay method uses succinylated casein. In the presence of all kinds of proteases, the succinylated casein is cleaved at peptide bonds and produces an orange-yellow color. Briefly, 100 µl of succinylated casein solution was added to 48 wells (A) of a 96-well microplate, and 100 µl of assay buffer was added to the other 48 wells (B). A 50 µl volume of standard solution was added to 8 of the A wells and 8 of the B wells, and the other 40 A wells and 40 B wells were used to add 50 µl of the samples. The microplate was incubated for 20 min at room temperature, and 50 µl of working solution was added to each of the wells. After incubating the plate for 20 min at room temperature again, the amount of protease in the wells was measured with a spectrophotometer (UV-160A, Shimazu Co., Ltd., Tokyo, Japan) at an absorbance of 450 nm. The minimum detection limit by the protease assay kit was 0.05 µg/well.

Collection and evaluation of ejaculated sperm: The sperm-rich second fraction of ejaculated semen was collected from the 10 dogs by digital manipulation and immediately transported to our laboratory. The concentration of sperm in the semen was determined by hematocytometer plates (Fujihira Industry Co., Ltd., Tokyo, Japan) and a light microscope (BX60, Olympus Co., Ltd., Tokyo, Japan). A total of 500 sperm per slide were scored after each staining procedure.

Evaluations of HA-sperm and AR-sperm: The percentages of HA-sperm and AR-sperm were determined by counting the number of sperm with star-spin-like movement in a fixed position among 500 motile sperm [22]. The percentage of AR-sperm was determined by the triple-stain technique [36].

Statistical analysis: The data were analyzed by Student’s t test. Differences between means were analyzed for statistical significance by Student’s t test, and P values <0.05 were regarded as evidence of significance.

RESULTS

Protease concentrations in the flush fluids of the uterine horns and oviducts: The mean (± S.E.) protease concentrations in the flush fluids of the uterine horns and oviducts removed from the bitches are shown in Table 1. The mean protease concentration in the oviducts of the estrous bitches was significantly higher than in their uterine horns (P<0.01).

<table>
<thead>
<tr>
<th>Bitches</th>
<th>Uterine horns</th>
<th>Oviducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrous</td>
<td>1.07±0.25</td>
<td>5.95±1.02**</td>
</tr>
<tr>
<td>Diestrous</td>
<td>0.05&gt;</td>
<td>0.05&gt;</td>
</tr>
<tr>
<td>Anestrous</td>
<td>0.05&gt;</td>
<td>0.05&gt;</td>
</tr>
</tbody>
</table>

** P<0.01, in comparison with uterine horns.
The protease concentrations of the uterine horns and oviducts of the diestrous and anestrous bitches were below the minimum detectable protease concentration with the assay kit.

Changes in lectin-binding characteristics after incubating the sperm: Changes in the mean (± S.E.) percentages of FITC-lectin-binding sperm after 4 hr of incubation are shown in Table 2. Before incubation all of the sperm clearly stained with each of the FITC-lectins. The acrosomal regions stained with Con A, PNA, and WGA, and the entire heads were stained with PHA-E (Fig. 1). But the mean percentages of sperm binding each of the 4 FITC-lectins after incubation in MEM supplemented with 1 or 5 µg/ml protease and the control MEM were lower than before incubation. The mean percentages of the sperm binding each of the FITC-lectins in the MEM with 5 µg/ml protease were significantly lower than in the control MEM (P<0.05 and 0.01).

Percentages of motile sperm, HA-sperm, and AR-sperm after incubation: The mean (± S.E.) percentages of motile sperm, HA-sperm, and AR-sperm after 4 hr incubation are shown in Table 3. The percentages of motile sperm and HA-sperm in the MEM supplemented with protease were higher than the values in the control MEM, and the mean values in MEM with 5 µg/ml protease were significantly higher (P<0.01). But there was no significant difference in the mean percentages of AR-sperm between the sperm incubated in MEM with protease and in the control MEM.

Table 2. Mean (± S.E.) percentages of ejaculated sperm collected from 10 dogs that stained with 4 FITC-lectins after 4 hr incubation in MEM supplemented with 1 µg/ml or 5 µg/ml protease, and MEM without protease (control).

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Before incubation</th>
<th>After 4 hr of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1 µg protease</td>
</tr>
<tr>
<td>Con A</td>
<td>100 ± 0</td>
<td>64.3 ± 7.3</td>
</tr>
<tr>
<td>PHA-E</td>
<td>100 ± 0</td>
<td>47.9 ± 5.1</td>
</tr>
<tr>
<td>PNA</td>
<td>100 ± 0</td>
<td>60.9 ± 9.0</td>
</tr>
<tr>
<td>WGA</td>
<td>100 ± 0</td>
<td>49.9 ± 9.0</td>
</tr>
</tbody>
</table>

* P<0.05 and ** P<0.01, in comparison with control.

Fig. 1. Ejaculated canine sperm stained with FITC-*Canavalia ensiformis* agglutinin (a), FITC-*Phaseolus vulgaris* agglutinin (b), FITC-peanut agglutinin (c), and FITC-wheat germ agglutinin (d).
DISCUSSION

It has been reported that the acrosomal region or the entire head of sperm ejaculated by dogs is stained with FITC-labeled Con A-, PNA-, and WGA-lectins [3] and that the lectin-binding glycoproteins on the surface of canine sperm heads are gradually lost with the progression of sperm capacitation [4], the same as shown by the results of this study. The WGA-lectin-binding characteristics of the plasma membrane of swine sperm heads have been found to change with the process of sperm capacitation [15], and the lectin-binding characteristics of the surface of bovine ejaculated sperm change after exposure to the oviductal fluid of cows [28]. Some of the lectin-binding glycoproteins on the surface of the ejaculated sperm are thought to be decapacitation factors in the human [2] and mouse [11, 24]. It has been reported that uterine horn and oviduct fluids induce the disappearance of some glycoproteins on the sperm surface [10, 31, 40], and oviductal fluid induces an increase in HA-sperm [7, 8, 14, 17, 30, 33, 35].

The results of this study showed that protease is contained in the uterine horn and oviduct fluids of estrous bitches, and that the protease concentration in oviductal fluid is higher than in uterine fluid. The percentages of sperm binding to 4 different FITC-lectins in culture medium supplemented with protease were lower than in the control medium. These findings indicate that each of the 4 different glycoproteins to which the Con A-, PHA-E-, PNA-, and WGA-lectins bind disappear from the surface of the heads of ejaculated canine sperm as a result of protease action in the oviducts of estrous bitches.

The results of this study showed clearly higher percentages of motile sperm and HA-sperm in the medium with protease than in the control medium. The HA-movement of canine sperm is thought to be related to the disappearance of glycoproteins from the surface of the sperm membrane. Initiation of sperm capacitation requires an influx of Ca²⁺ from extracellular sources into the cytoplasm of the sperm [11, 12, 32, 34]. An increase in the intracellular Ca²⁺ concentration triggers abundant energy production in sperm by increasing cyclic AMP levels [32, 34], and HA-movement is induced as a result [1, 16, 34]. The influx of Ca²⁺ into cytoplasm of canine sperm has also been shown to induce an increase in cyclic AMP and active flagellar movement of the tail [37]. Therefore, the disappearance of some glycoproteins on the surface of canine sperm transported into the oviduct is presumably a result of exposure to the action of protease in the oviductal fluid of estrous bitches, and then HA-movement of sperm is induced by the Ca²⁺ influx into the sperm.

In the present study there was no difference in the percentage of AR-sperm among the sperm incubated in protease-supplemented medium and among the sperm incubated in the medium without protease. The authors have observed that the AR of canine sperm is mainly caused by attachment to the zona pellucida of oocytes [23]. The fertilizing ability of canine sperm must be maintained for a long period in the oviduct of the estrous bitch until the ovulated oocytes descend into the oviduct [20]. It has been found that canine spermatozoa in the oviduct of the estrous bitch attach to the oviduct epithelial cells and their viability is prolonged and the start of sperm capacitation is inhibited [20]. Therefore, the AR of canine sperm is not easily induced by the composition of the oviductal fluid.

REFERENCES


