FULL PAPER *Theriogenology*

Disappearance of the PHA-E Lectin Binding Site on the Surface of Ejaculated Sperm and Sperm Capacitation in the Dog

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ABSTRACT. Ejaculated semen and cross sections of the cauda epididymides collected from 3 normal dogs were smeared or stamped on glass slides, and the sperm on the slides were stained with 7 different FITC-lectins (Con A, DBA, GS-1, PHA-E, PSA, UEA-1, WGA) to examine the relation between sperm-binding glycoprotein derived from the canine prostate and sperm capacitation. The only lectin that stained the ejaculated sperm but not the cauda epididymal sperm was PHA-E. The sperm ejaculated by 5 other dogs were incub ated for 4 hr in fluid flushed from the uterine horns or oviducts of estrous bitches, and then the percentages of actively motile sperm and hyperactivated sperm (HA-sperm) were determined. The percentages of PHA-E-labeled sperm and sperm labeled with fluoresceinated Ca indicator to assess the influx of Ca into the sperm were also evaluated. The mean percentages of actively motile sperm, HA-sperm, and Ca-labeled sperm after 4 hr of incubation in the uterine flush fluid and oviductal flush fluid were significantly higher tha n in control medium ($P<0.05$, 0.01), but the mean percentages of PHA-E-labeled sperm were lower (both $P<0.01$). The percentages of PHA-E-unlabeled sperm correlated with the percentages of both HA-sperm and Ca-labeled sperm ($r^2=0.787$ and 0.812, respectively). The results indicate that loss of the glycoprotein secreted by the canine prostate on the sperm surface induces the influx of Ca into the sperm, and then hyperactivation of the sperm.

KEY WORDS: canine, capacitation, lectin, prostate, sperm.

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It has been reported that the surface of the plasma membrane of mammalian sperm becomes coated with various glycoproteins secreted by the epididymal epithelium during sperm transit through the epididymis [9, 11], and some of the glycoproteins on the sperm surface are thought to induce sperm maturation in the epididymal duct [10, 17, 29]. It is known that the glycoproteins produced by the prostates and seminal vesicles are contained in the semen [2, 5, 24] and that some of them act as decapacitation factors [2, 24]. The decapacitation factors on the surface of the sperm are lost as a result of the action of proteolytic enzymes in the uterus and oviduct [5, 6, 19], and sperm capacitation is then promoted by some components of oviductal fluid, e.g., glycosaminoglycans [11, 15, 22]. Sperm capacitation is induced by an influx of Ca into the cytoplasm of the sperm [23, 25, 27]. Techniques utilizing many different fluorescein-isothiocyanate (FITC)-labeled lectins have revealed the characteristics of the glycoproteins coating the surface of the sperm in many species [9, 16–18, 29], but there have been few reports on the relation between sperm-binding glycoproteins and sperm capacitation in the dog [3, 7]. In the present study, canine epididymal sperm and ejaculated sperm were stained with 7 different FITC-lectins to examine for the presence of sperm-binding glycoproteins derived from canine prostate in the seminal fluid and to assess the relation between the glycoproteins and sperm capacitation. Changes in the binding characteristics of the FITC-lectins to the surface of the canine sperm were observed after incubating the sperm in medium that had been flushed through the uterine horns or oviducts of estrous bitches. The influx of Ca into the cytoplasm of canine sperm was assessed to investigate the induction of sperm capacitation.

MATERIALS AND METHODS

Animals: Eight male beagle dogs (2–6 years old) having 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 water was available *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.

Collection of ejaculated sperm and evaluation of semen quality: The sperm-rich second fraction of ejaculated semen was collected from all 8 dogs by digital manipulation and immediately transported to our laboratory. The concentration of sperm in the semen was determined by hemocytometer counts, and the percentage of actively motile sperm on glass slides was estimated by examining 500 sperm with a warm-plate (Fujihira Industry Co., Ltd., Tokyo) and a light microscope (VBS-FT-2, Nikon Co., Ltd., Tokyo). The ejaculated sperm of the 3 dogs from which collection of epididymal sperm was planned were smeared on glass slides to examine the lectin-binding characteristics of the sperm.

Collection of cauda epididymal sperm: Epididymides were obtained from 3 of the 8 dogs by orchio-epididymectomy. Cross sections of the cauda epididymides were stamped on the glass slides to stain the sperm with lectins.

The cauda epididymides were sliced with a scalpel to release sperm into 38°C Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co.. Ltd., Tokyo) without

bovine serum albumin, and the debris was removed by filtration through a 40-mesh stainless steel and 100-μm nylon screen. The cauda epididymal sperm collected were smeared on the glass slides for staining with lectins.

Fluoresceinated-lectin staining of epididymal and ejaculated sperm: Seven different fluorescein-isothiocyanate (FITC)-labeled lectins (Honen Co. Ltd., Tokyo), *Canavalia ensiformis* agglutinin (Con A), *Dolichos biflorus* agglutinin (DBA), *Griffonia simplicifolia* agglutinin (GS-1), *Phaseolus vulgaris* agglutinin (PHA-E), *Pisum sativum* agglutinin (PSA), *Ulex europaeus* agglutinin (UEA-1), and wheat germ agglutinin (WGA), were utilized to examine the modifications of the glycoproteins on the surface of the cauda epididymal and ejaculated sperm. FITC-lectin staining of the surface was carried out according to the method of Lee and Damjanov [16]. Briefly, the spermatozoa smeared on the glass slides were fixed with acetone at 4°C for 10 min, and the slides were immersed in FITC-lectin solutions (50 μg/m*l* phosphate-buffered saline solution; PBS) in the dark for 30 min. After a brief rinse with PBS, the slides were covered with glycerol and examined under a fluorescence microscope (BX60, Olympus Co., Ltd., Tokyo). Five hundred sperm per slide were scored after each staining procedure.

Flushing of uteri and oviducts: Uteri and oviducts were obtained from 5 estrous bitches after ovariohysterectomy for contraception at the veterinary teaching hospital of our university. The stages of the estrous cycle were judged from the appearance of the ovaries and vaginal bleeding. The connective tissue, blood vessels, and fat surrounding the uteri and oviducts were removed. A 20-gauge retaining needle connected to a 5 m*l* injection syringe was then inserted into the lumen of each uterine horn and oviduct, and the lumen was flushed with 3 m*l* of Eagle's MEM. The flushed fluid was collected in a test tube to incubate the ejaculated sperm in the fluid.

Sperm incubation: The ejaculated sperm of the 5 dogs in which collection of epididymal sperm was not planned were used for incubation in the uterine flush fluid and oviductal flush fluid. The sperm were washed twice by centrifugation at 300 × g for 5 min in 5 m*l* of Eagle's MEM warmed to 38°C, and the final sperm pellet was diluted to a concentration of 1×10^7 sperm/m*l* in Eagle's MEM (control), the uterine flush MEM and the oviductal flush MEM. The sperm were incubated in loosely capped 120 × 15 mm glass test tubes for 4 hr at 38°C in an atmosphere of 5% CO_2 in air.

Evaluation of hyperactivated sperm and acrosome-reacted sperm: The percentages of hyperactivated sperm (HA-sperm) and live and acrosome-reacted sperm (AR-sperm) were determined 4 hr after the start of incubation. The percentages of HA-sperm and AR-sperm were estimated by counting sperm with star-spin like movement in a fixed position among 500 motile sperm [13] and by the triple-stain technique [26], respectively.

Evaluation of influx of Ca into sperm cytoplasm: The influx of Ca into the sperm cytoplasm was assessed by staining with a fluoresceinated Ca indicator (Calcium Crimson, Molecular Probes Co., Ltd., Oregon, U.S.A.). Briefly, 30 μ*l* of 20% Ca indicator fluid in dimethyl sulfoxide was added to 1 m*l* of each sperm suspension in glass test tubes after incubation for 4 hr, and the test tubes were gently stirred with a shaker (EYELA MMS, Tokyo Rikakikai, Co., Ltd., Tokyo) for 30 min. The sperm suspension was centrifuged at 300 × g for 5 min, and after transferring the sperm pellet to 1 m*l* of fresh Eagle's MEM, it was shaken for 30 min to remove the Ca indicator on the sperm surface. The sperm were then dropped onto glass slides and mounted in dimethyl sulfoxide, and the percentage of sperm with fluoresceinated heads (Ca labeled sperm) was estimated by examining 500 sperm with a fluorescence microscope.

Statistical analysis: The data are summarized as mean values ± standard error (S.E.). Differences between means were statistically analyzed by Student's *t* test. Values of $P<0.05$ were regarded as significant. Correlations between the data were examined by Spearmann's test.

RESULTS

Lectin-binding characteristics of epididymal and ejaculated sperm: The FITC-lectin-binding characteristics of the surface of the plasma membrane of the cauda epididymal and ejaculated sperm are shown in Table 1. There were no differences in Con A, DBA, GS-1, PSA, UEA-1, or WGA lectin-binding characteristics between the cauda epididymal sperm and the ejaculated sperm. Nevertheless there was a difference in PHA-E lectin-binding: no PHA-E lectin fluorescence at all was seen on the surface of the cauda epididymal sperm, but the entire heads and mid-pieces of all of the ejaculated sperm were stained with FITC-PHA-E lectin (Fig. 1).

Change in PHA-E lectin-binding characteristics after incubation of ejaculated sperm: The mean percentages of actively motile sperm, HA-sperm, AR-sperm, fluoresceinated PHA-E lectin-labeled sperm, and Ca-labeled sperm (Fig. 2) after 4 hr of incubation in the uterine flush MEM and oviductal flush MEM are shown in Table 2. The mean percentages of actively motile sperm and HA-sperm incubated in the uterine flush MEM and oviductal flush MEM were significantly higher than in the control MEM ($P<0.05$, 0.01), and the percentage of HA-sperm in the oviductal

Table 1. Binding characteristics of cauda epididymal and ejaculated sperm from 3 dogs with seven different FITC-labeled lectins

Lectins	Cauda epididymal sperm	Ejaculated sperm
Con A	Acrosomal regions (+)	Acrosomal regions (+)
DBA	(–)	(–)
GS-1	(–)	(–)
PHA-E	(–)	Entire heads and mid-pieces (+)
PSA	Acrosomal regions (+)	Acrosomal regions (+)
UEA-1	(–)	(–)
WGA	Acrosomal regions (+)	Acrosomal regions (+)

(+): stained with FITC-lectins and (–): not stained with FITC-lectins.

Fig. 1. Ejaculated canine sperm stained with FITC-PHA-E lectin after incubation for 4 hr (× 400). The entire head and mid-piece of the sperm for stained with FITC-PHA-E lectin.

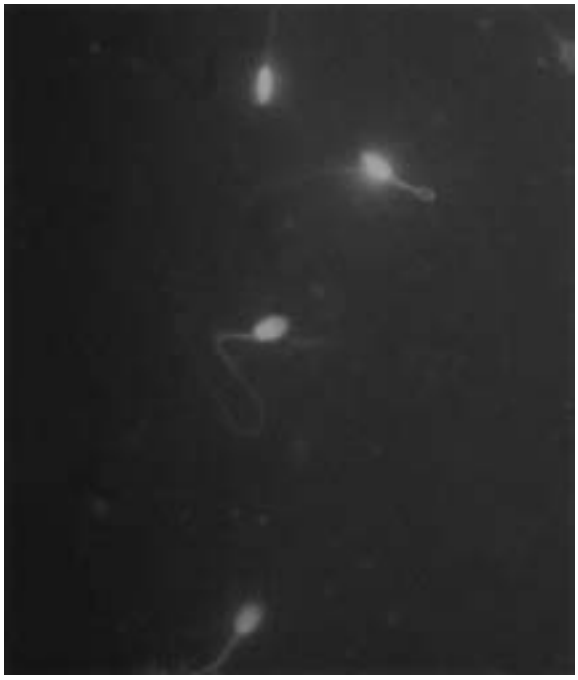

Fig. 2. Ejaculated canine sperm stained with fluoresceinated Ca indicator after incubation for 4 hr (× 400). The entire head of the sperm stained with fluoresceinated Ca indicator.

flush MEM was significantly higher than in the uterine flush MEM ($P<0.01$), but there were no significant differences between the percentages of AR-sperm in the uterine flush MEM, oviductal flush MEM, and in control MEM.

Although the mean percentages of PHA-E-labeled sperm incubated in the uterine flush MEM and oviductal flush MEM were both significantly lower than in the control MEM ($P<0.01$), the percentages of Ca-labeled sperm incubated in the uterine flush MEM and oviductal flush MEM were significantly higher than in the control MEM ($P<0.01$).

The percentages of PHA-E-unlabeled sperm were positively correlated with both the percentages of HA-sperm ($r^2=0.787$) (Fig. 3) and of Ca-labeled sperm ($r^2=0.812$) (Fig. 4).

DISCUSSION

Some of the glycoproteins coating the surface of the plasma membrane of mammalian sperm are derived from the fluid secreted by the epithelium of the epididymis [17, 29] and accessory glands [2, 5]. It is well-known that the glycoprotein-binding sites on the sperm surface change during sperm transit through the male [4, 30] and female [19, 31] reproductive tracts. There have been several reports on the Con A, PSA, and WGA lectin-binding characteristics of the surface of canine epididymal and ejaculated sperm [3, 7], and the results of this study agree with their findings. The changes in lectin-binding sites on the sperm surface are thought to be associated with sperm maturation [9,29] and sperm capacitation [7, 18]. The ejaculated semen of many species contains decapacitation factors, that cover the sperm surface and inhibit sperm capacitation [6, 21], and some of the specific glycoproteins secreted by the accessory glands act as decapacitation factors [28]. The presence of PHA-E lectin-binding glycoprotein on the surface of the entire head and mid-piece of canine ejaculated sperm, but not on epid-

Table 2. Changes in the mean (± S.E.) percentages of motile sperm, hyperactivated (HA–) sperm, acrosome-reacted (AR–) sperm, PHA-E lectin-labeled sperm, and fluoresceinated Ca indicator in sperm ejaculated by 5 dogs and incubated for 4 hr in control MEM, uterine flush MEM and oviductal flush MEM

Incubation period (hr)	Motile sperm (%)	HA-sperm (%)	AR-sperm (%)	PHA-E-labeled sperm (%)	Ca-labeled sperm (%)
0 (Control MEM)	88.0 ± 3.0	0	0	100.0 ± 0.0	0
4 (Control MEM)	57.0 ± 4.4	18.2 ± 2.0	7.2 ± 1.2	71.8 ± 3.0	24.6 ± 5.5
4 (Uterine flush)	69.0 ± 2.2*[a)]	24.2 ± 1.0*[a)]	9.0 ± 1.4	50.4 ± 1.9**[a)]	51.8 ± 2.4**[a)]
4 (Oviductal flush)	72.0 ± 1.8*[a)]	62.2 ± 2.7**[ab)]	10.4 ± 1.4	22.6 ± 1.8**[ab)]	73.0 ± 2.7**[ab)]

*: $P<0.05$ and **: $P<0.01$.

a) in comparison with control MEM after 4 hr of incubation.

b) in comparison with uterine flush MEM.

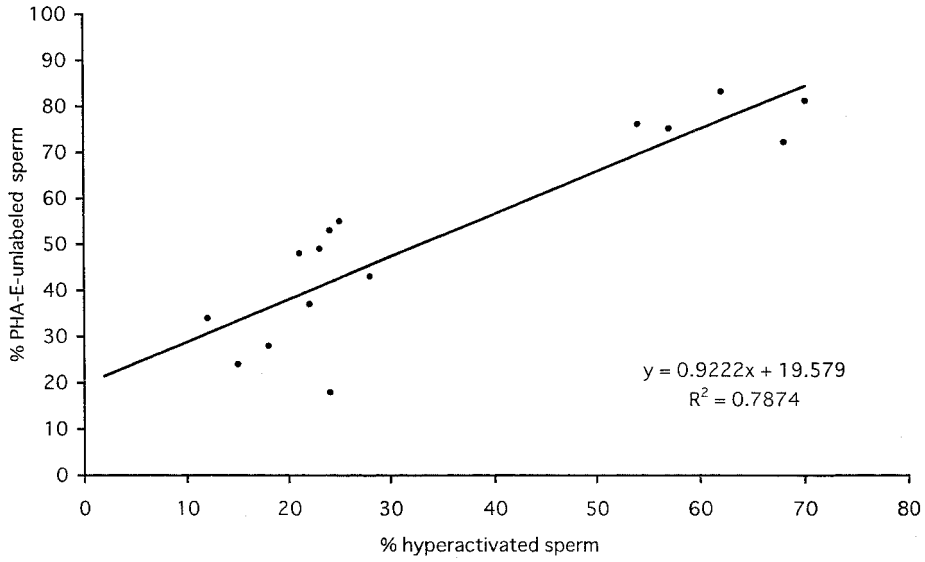

Fig. 3. Correlation between the percentages of PHA-E-unlabeled sperm and the percentages of HA-sperm (r^2=0.787).

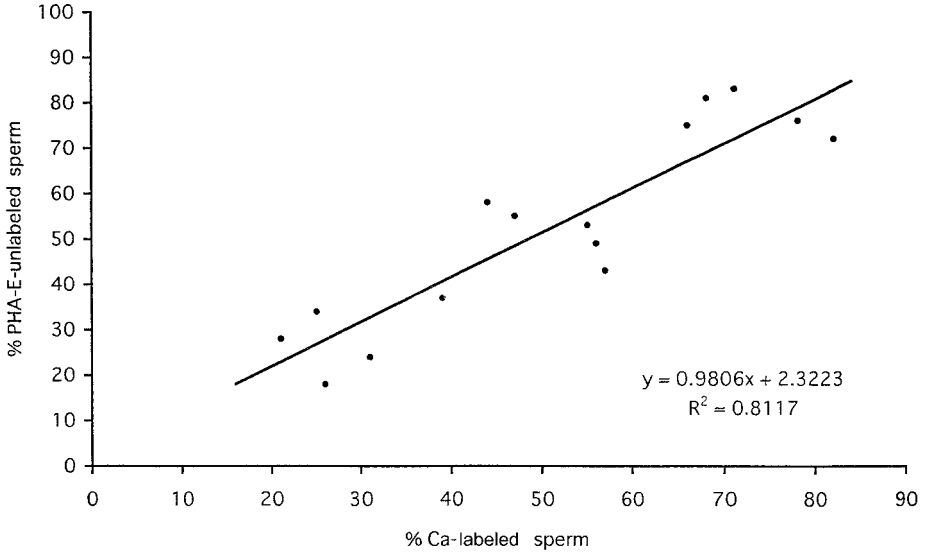

Fig. 4. Correlation between the percentages of PHA-E-unlabeled sperm and the percentages of Ca-labeled sperm (r^2=0.812).

idymal sperm, was demonstrated in this study. Since PHA-E lectin specifically binds to a monosaccharide, N-acetylgalactosamine [16], it was concluded that a glycoprotein containing N-acetylgalactosamine is present in ejaculated canine semen and that the glycoprotein secreted by the canine prostate binds to the surface of the entire head and mid-piece of the sperm. It has been reported that the surface of the ejaculated sperm of humans [16], rabbits [20], and hamsters [20] is coated by a glycoprotein containing N-acetylgalactosamine. PHA-E lectin binds to the plasma membrane of ejaculated human sperm [16], and a glycoprotein containing N-acetylgalactosamine, which is a PHA-E lectin-binding saccharide, has been assumed to be a decapacitation factor for human sperm [2].

In the present study, the mean percentage of HA-sperm in canine sperm incubated in the oviductal flush MEM was higher than in the control MEM, and the mean percentage of PHA-E lectin-labeled sperm was lower. The percentage of HA-sperm correlated with the percentage of PHA-E-unlabeled sperm. These results suggest that HA-sperm move-

ment-inducing substances are present in the oviductal fluid of estrous bitches, the same as in other species [12, 15, 22], and that loss of PHA-E lectin-binding glycoprotein on the surface of canine sperm is associated with the start of sperm capacitation. The authors [14] previously reported that glycosaminoglycans, a class of mucopolysaccharide, in the oviductal fluid of the estrous bitches induce HA-movement of canine sperm, the same as in the cow [1, 8]. Glycosaminoglycans have been shown to cause sperm capacitation as a result of inducing functional changes in sperm cell membrane, absorption of Ca into the sperm and activation of cyclic AMP in the sperm [1, 8]. The results of the present study indicate that a PHA-E lectin-binding protein which is thought to be secreted by the canine prostate is one of the decapacitation factors for canine sperm and that loss of the PHA-E-binding protein on the surface of canine sperm in the oviducts causes induction of sperm capacitation by certain substances, e.g., glycosaminoglycans, in the oviductal fluid of the estrous bitch.

It has been found that the glycoproteins on the surface of sperm are removed after exposure to fluids of the female reproductive tract of estrous animals and that their removal induces sperm capacitation [5, 6, 21, 31]. Proteases (enzymes that hydrolyze proteins) in the fluids of the uterus and oviduct have been reported to hydrolyze sperm surface glycoproteins [5, 19], and it will be necessary to examine the reproductive tract fluid of estrous bitches for the presence of proteases. Sperm capacitation phenomena, hyperactivation, and the acrosome reaction, require an influx of Ca from extracellular sources into the cytoplasm of the sperm [23, 25, 27], and the flagellar movement of the tail of canine sperm requires an increase in the intracellular Ca concentration [27]. Since the percentage of fluoresceinated Ca-labeled sperm after incubation correlated with the percentage of PHA-E-unlabeled sperm in the present study, it is concluded that the loss of N-acetylgalactosamine-containing glycoprotein secreted by the canine prostate from the sperm surface causes the influx of Ca into cytoplasm of the sperm, thereby inducing hyperactivated sperm movement.

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