

## Lectin-Binding Characteristics and Capacitation of Canine Epididymal Spermatozoa

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**ABSTRACT.** Cross sections of the testes and the caput, corpus and cauda epididymides removed from 12 dogs were stamped on glass slides, and the sperm on the slides were stained with 6 different FITC-lectins (Con A, DBA, PNA, PSA, SBA, and WGA) to examine the characteristics of the surface glycoproteins (GPs) on canine epididymal sperm. The corpus epididymal sperm were washed three times by centrifugation, and their lectin-binding characteristics were investigated. The washed sperm from the corpus and cauda epididymides were incubated for 24 hr, and the fertilizing capacity of the sperm was evaluated by calculating the percentages of actively motile sperm (%MO), hyperactivated sperm (%HA), and acrosome-reacted sperm (%AR), and the number of canine zona-pellucida (ZP)-binding sperm. The testicular sperm did not stain with SBA lectin, but the SBA lectin fluorescence was observed on the surface of the entire heads of the caput epididymal sperm. Although all of the entire heads or acrosomal regions of the corpus epididymal sperm stained with all 6 FITC-lectins, the heads and acrosomal regions of the cauda epididymal sperm did not stain with DBA or SBA lectins. Washing the sperm from the corpus epididymis resulted in loss of the fluorescence of the FITC-DBA and -SBA lectins. The mean %MO, %HA, %AR, and ZP-binding number of the cauda epididymal sperm after 24 hr of incubation were higher than the values for the corpus epididymal sperm. All of the mean values for the washed sperm from the corpus and cauda epididymides were higher than the values for the unwashed sperm from the corpus and cauda, and with the exception of %AR, the values from the washed sperm from the corpus epididymis were significantly higher ( $P < 0.05, 0.01$ ). The results indicate that DBA- and SBA-lectin-binding GPs on the surface of canine epididymal sperm are associated with the fertilizing capacity and may be decapacitation factors.

**KEY WORDS:** canine, capacitation, epididymis, lectin, sperm.

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It is well-known that the mammalian sperm plasma membrane surface is coated with various glycoproteins (GPs) [8, 9, 20, 21, 25]. Although some GPs are already present on the surface of immature sperm in the seminiferous tubules of the testis [18, 19], the surface of the sperm becomes covered with a wide variety of GPs secreted by the epididymal epithelium during sperm transit through the epididymis [5, 6, 17, 23]. Sperm surface GPs are thought to induce sperm maturation and fertilizing capacity [10, 20] in the epididymal duct, and techniques utilizing fluorescein-isothiocyanate (FITC)-labeled lectins have revealed that the sperm surface GPs are biochemically modified during sperm transit through the epididymis and that there are species-dependent differences in sperm surface GPs [1, 5, 6, 17, 20, 23]. Some of the GPs on the surface of epididymal sperm have been suspected of acting as decapacitation factors (DFs) [16, 21], and while sperm fertilizing capacity is induced by removing DFs from the sperm surface [7, 16], there have been few reports on the lectin-binding characteristic of the GP on the surface of canine epididymal sperm [1]. In the present study, the characteristics of binding of 6 FITC-lectins to the surface of canine sperm collected from the testes and the caput, corpus and cauda epididymides were investigated to examine modifications in the surface GPs of canine epididymal sperm. The lectin-binding characteristics of corpus epididymal sperm washed with culture medium by centrifugation were also examined. The fertilizing capacity of the corpus and cauda epididymal sperm washed and incubated for 24 hr was assessed by calculating the percentages

of actively motile sperm, hyperactivated sperm, and acrosome-reacted sperm, and the number of canine zona-pellucida (ZP)-binding sperm.

### MATERIALS AND METHODS

*Collection of testicular and epididymal sperm:* Canine testes and epididymides were obtained from 12 dogs, 1–3 yr of age, during orchidectomy for contraceptive purpose at veterinary clinics in Tokyo, and the surgical specimens were placed in Ringer's solution for transport to our laboratory. Cross sections of the testes and the caput, corpus and cauda epididymides were made with a scalpel to release sperm into 38°C culture medium, i.e., Eagle's MEM without BSA, and the debris was removed by filtration through a 40-mesh stainless steel and 100- $\mu$ m nylon screen. Some of the corpus epididymal sperm collected with washed three times by centrifugation for 5 min at 400  $\times$  g, and then the sperm were smeared on glass slides for staining with FITC-lectins.

*Fluoresceinated lectin staining:* Six different FITC-labeled lectins (Honen Inc., Tokyo), *Canavalia ensiformis* agglutinin (Con A), *Dolichos biflorus* agglutinin (DBA), peanut agglutinin (PNA), *Pisum sativum* agglutinin (PSA) soybean agglutinin (SBA) and wheat germ agglutinin (WGA) were used to examine the modifications of the surface GPs of canine testicular and epididymal sperm. FITC-lectin staining of the surface was carried out according to the method of Lee and Damjanov (1985) [19]. 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/ml phosphate-buffered saline solution) in the dark for 30 min. After a brief rinse with phosphate-buffered saline solution, the slides were covered with glycerol and examined under a fluorescence microscope (BX60, Olympus Inc., Japan).

**Sperm incubation:** Approximately half of the sperm collected from the corpus and cauda epididymides were washed three times by centrifugation for 5 min at 400 × g, and the sperm suspensions were diluted in Eagle's MEM at 38°C to a concentration of 5 × 10<sup>6</sup> sperm/ml. Three-milliliter aliquots of the sperm suspensions were placed in 120 × 15-mm glass test-tubes, which were loosely capped, and incubated for 24 hr at 38°C under an atmosphere of 5% CO<sub>2</sub> in air. Unwashed sperm were also incubated by the method described above, as controls.

**Evaluations of motile sperm, hyperactivated sperm, and acrosome-reacted sperm:** The percentages of actively motile sperm (%MO), hyperactivated sperm (%HA), and live and acrosome-reacted sperm (%AR) were determined 24 hr after the start of incubation. The %MO was evaluated by using a warm-plate. The %HA and %AR were estimated by counting the number of sperm with star-spin-like movement in a fixed position among all motile sperm [12] and by the triple-stain technique [27], respectively.

**Oocyte collection and ZP-binding assay:** Ovaries collected from proestrous or estrous bitches were sliced with a razor blade to release the oocytes into Eagle's MEM. The oocytes were then transferred to 1 ml of 75 mM sodium citrate buffer solution (pH 7.8) in a test-tube and agitated for 2 min with a vortex mixer to remove the cumulus cell layer [14]. After the oocytes were washed by pipetting in fresh culture medium, and cryopreserved at -20°C until used for the ZP-binding assay.

THE ZP-binding assay was performed by the method described previously [11]. Briefly, after the oocytes were thawed at room temperature, three oocytes were placed in each of three 100-µl droplets of sperm suspension that had been incubated for 24 hr under paraffin oil in 35-mm plastic tissue culture dishes. After incubating the oocytes and sperm were incubated together for 1 hr, the oocytes were washed by pipetting to remove loosely bound sperm and transferred to fresh medium in other culture dishes. The number of sperm strongly bound to the ZP (number of ZP binding sperm) was counted under a phase-contrast microscope (× 400).

**Statistical analysis:** The data have been summarized as mean values ± standard error (S.E.). Differences between means were statistically analyzed by Student's *t* test.

## RESULTS

**FITC-lectin-binding characteristics of the surface of testicular and epididymal sperm:** The FITC-lectin-binding characteristics of the surface of the plasma membrane of canine sperm collected from the testes and the caput, corpus and cauda epididymides are shown in Table 1. Although the

Table 1. FITC-lectin-binding characteristics of canine testicular and epididymal sperm

Lectins	Testicular sperm	Epididymal sperm		
		Caput	Corpus	Cauda
ConA	a (-)	a (-)	a (+)	a (+)
DBA	h (+)	h (+)	h (+)	h (-)
PNA	h (+)	h (+)	h (+)	h (+)
PSA	a (+)	a (+)	a (+)	a (+)
SBA	h (-)	h (+)	h (+)	h (-)
WGA	a (+)	a (+)	a (+)	a (+)

a: Acrosomal regions of sperm. h: Entire heads of sperm. (+): Stained with FITC-lectins. (-): Not stained with FITC-lectins.

Table 2. FITC-lectin-binding characteristics of sperm collected from canine corpus epididymides before and after washing by centrifugation

Lectins	Before washing	After washing
ConA	a (+)	a (+)
DBA	h (+)	h (-)
PNA	h (+)	h (+)
PSA	a (+)	a (+)
SBA	h (+)	h (-)
WGA	a (+)	a (+)

a: Acrosomal regions of sperm. h: Entire heads of sperm. (+): Stained with FITC-lectins. (-): Not stained with FITC-lectins.

testicular sperm did not stain with Con A and SBA lectins at all, the SBA lectin fluorescence was observed on the surface of the entire heads of the caput epididymal sperm, and the acrosomal regions or the entire heads of the corpus epididymal sperm stained with all 6 FITC-lectins, but no DBA or SBA lectin fluorescence at all was seen on the surface of the heads of the cauda epididymal sperm. FITC-DBA lectin stained the cytoplasmic droplet attached to the mid-piece of all of the testicular and epididymal sperm.

The FITC-lectin-binding characteristics of the corpus epididymal sperm washed by centrifugation are shown in Table 2. The acrosomal regions or the entire heads of the washed sperm from the corpus epididymides stained with Con A, PNA, PSA and WGA lectins, the same as observed in the unwashed sperm from the cauda epididymis. Nevertheless, the fluorescence of the DBA and SBA lectins on the surface of the entire heads of the corpus epididymal sperm disappeared after washing by centrifugation (Fig. 1).

**Fertilizing capacity of corpus epididymal sperm incubated after washing:** The mean %MO, %HA, %AR and number of sperm bound to the ZP among the washed sperm from the corpus and cauda epididymides after 24 hr of incubation are shown in Figs. 2, 3, 4 and 5. All of the mean values for the cauda epididymal sperm were higher than the values for the corpus epididymal sperm, and all of the mean values for the washed sperm from the corpus and cauda epididymides were higher than the values for the unwashed sperm. It was especially noteworthy that the mean %MO, %HA and number of ZP binding sperm in the washed sperm

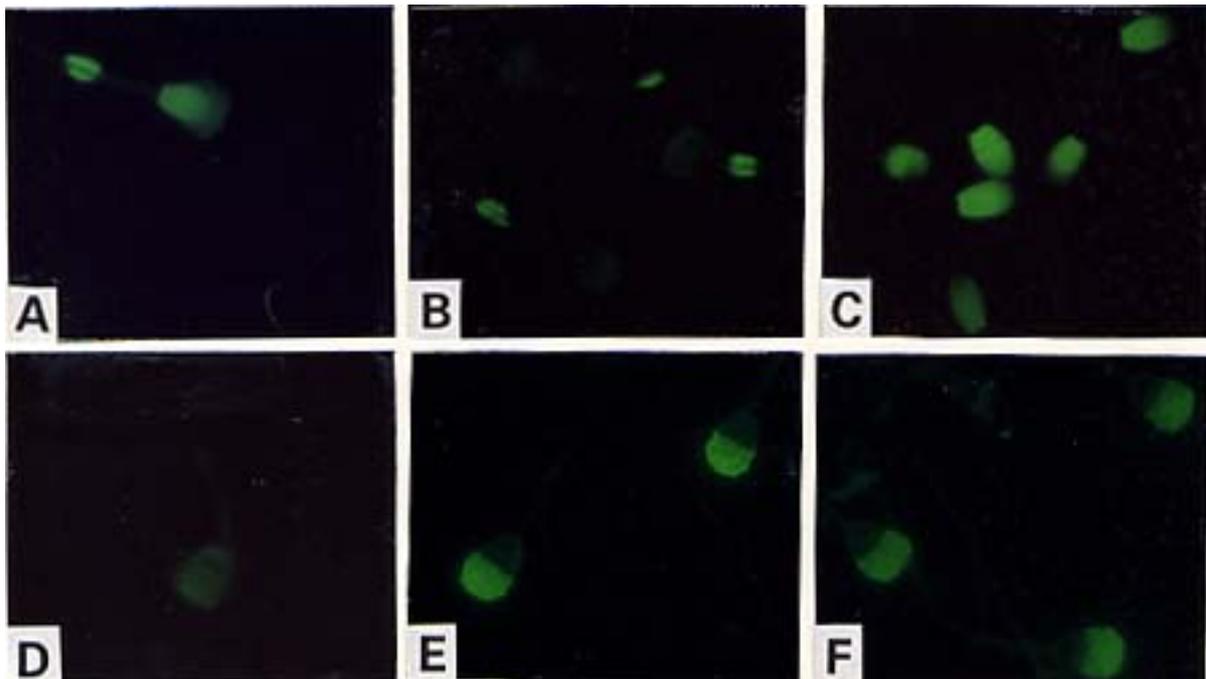


Fig. 1. Canine corpus epididymal sperm stained with FITC-DBA, -SBA, and -WGA lectins, before and after washing by centrifugation ( $\times 1,000$ ). A: Sperm stained with DBA lectin, before washing. The entire head and cytoplasmic droplet of the sperm are stained. B: Sperm stained with DBA lectin, after washing. The fluorescence over the entire head of the sperm has been lost but not on the cytoplasmic droplet. C: Sperm stained with SBA lectin, before washing. The entire head of the sperm has stained. D: Sperm stained with SBA lectin, after washing. The fluorescence of the entire head of the sperm has been lost. E: Sperm stained with WGA lectin, before washing. The acrosomal region of the the sperm has been stained. F: Sperm stained with WGA lectin, after washing. The fluorescence of the acrosomal region of the sperm is still visible.

were significantly higher than in the unwashed sperm from the corpus epididymides ( $P < 0.05, 0.01$ ).

## DISCUSSION

GPs that coat the sperm surface are acquired or lost during sperm transit through the epididymis, and the characteristics of the GPs on the surface of the plasma membrane of mammalian sperm vary with the species [6, 9, 20, 23]. Some GPs coating the sperm surface are derived from epididymal fluid [5, 15, 17, 23]. Although the testicular sperm of humans [19] and goats [18] do not stain with FITC-DBA lectin, this study showed that the entire heads of the canine testicular sperm fluoresced with DBA lectin, demonstrating that the surface of canine testicular sperm is coated with a GP composed of methyl-2-acetamide-2-deoxy-D-galactose, which is a DBA lectin-binding saccharide [19]. The heads of the human [19] and the goat [18] testicular sperm stain with FITC-SBA lectin. In the present study the canine testicular sperm did not stain with either the FITC-SBA or -Con A lectin, but fluorescence was observed on the entire heads of the caput epididymal sperm and the acrosomal regions of the corpus epididymal sperm by FITC-SBA lectin and -Con A lectin, respectively. These findings

indicate that GPs composed of N-acetyl-D-galactosamine, which is an SBA-lectin-binding saccharide [19], and  $\alpha$ -mannose, which is a Con A-lectin-binding saccharide [20], may be secreted by the epithelium of the canine caput epididymis and corpus epididymis, respectively, and may coat the sperm surface in the epididymal duct.

In the rat [28], a PNA-positive GP is secreted by the caput epididymal epithelium and is associated with sperm maturation during epididymal transit. In the pig [9], PNA lectin tightly binds to the surface of the plasma membrane of cauda epididymal sperm, and sperm maturation is also induced by PNA-positive GP. In the bull [22] and the ram [20], a WGA-lectin-binding GP, which is composed of N-acetyl-D-glucosamine, appears to be associated with acquisition of fertilizing capacity by the sperm during epididymal transit. The present study, however, did not demonstrate that PNA- and WGA-lectin-binding GPs induce canine sperm maturation and/or fertilizing capacity. FITC-PSA lectin has been reported to stain only the acrosomal regions of canine epididymal [1] and ejaculated [8, 13] sperm. The results of this study showed that FITC-PSA lectin also binds to the acrosomal cap of canine sperm collected from the testes and the caput, corpus and cauda epididymides, and the lectin-binding characteristics of canine sperm did not

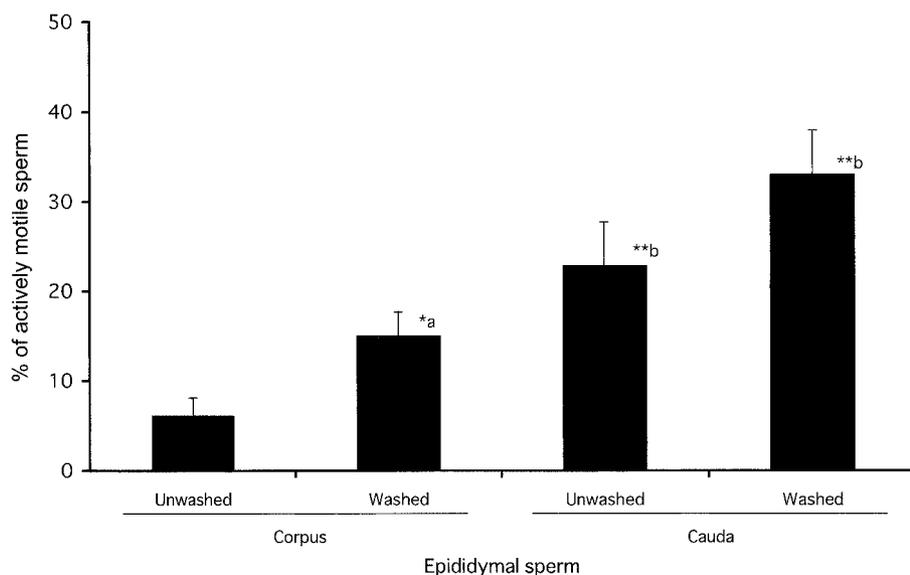


Fig. 2. The mean ( $\pm$  S.E.) percentages of actively motile sperm collected from the corpus and cauda epididymides of 12 dogs and incubated for 24 hr after washing by centrifugation. \* a:  $P < 0.05$ , in comparison with unwashed corpus epididymal sperm. \*\* b:  $P < 0.01$ , respectively, in comparison with corpus epididymal sperm.

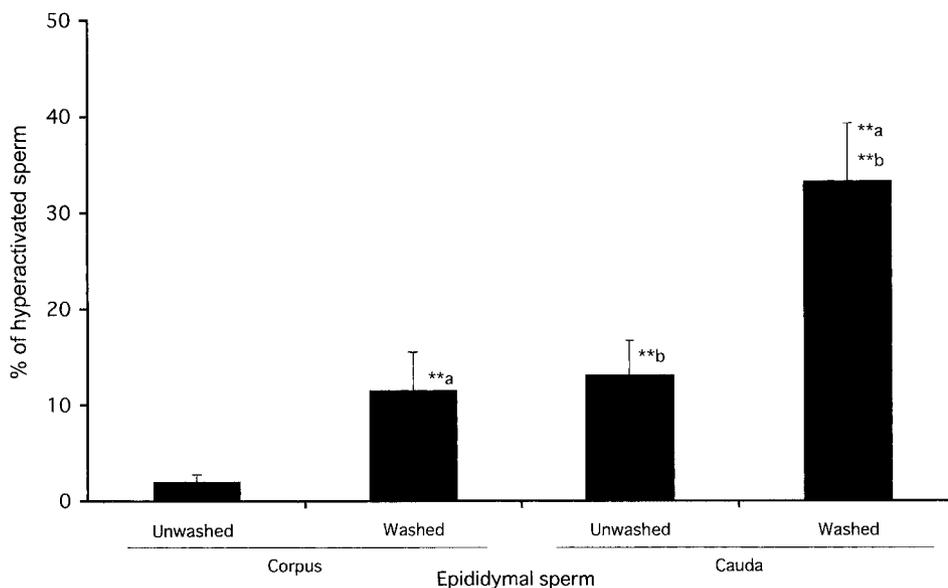


Fig. 3. The mean ( $\pm$  S. E.) percentages of hyperactivated sperm collected from the corpus and cauda epididymides of 12 dogs and incubated for 24 hr after washing by centrifugation. \*\* a:  $P < 0.01$ , in comparison with unwashed sperm. \*\* b:  $P < 0.01$ , respectively, in comparison with corpus epididymal sperm.

change during epididymal transit. Therefore, the PSA-lectin-binding GP may be unrelated to maturation and the fertilizing capacity of canine epididymal sperm.

FITC-DBA and -SBA lectin fluorescence was observed on the entire surface of the head of canine corpus epididymal sperm, but not of cauda epididymal sperm. The

canine corpus epididymal sperm washed by centrifugation no longer stained with the DBA and SBA lectins. These findings indicate that both DBA- and SBA-lectin-binding GPs, which are composed of methyl-2-acetamide-2-deoxy-D-galactose and N-acetyl-D-galactosamine, respectively, are removed from the surface of the head of canine sperm

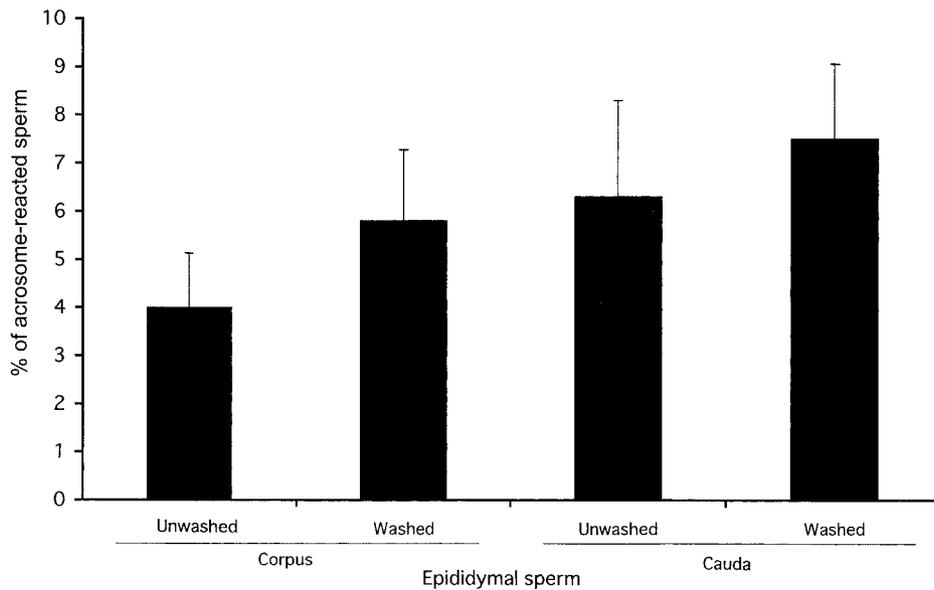


Fig. 4. The mean ( $\pm$  S. E.) percentages of acrosome-reacted sperm collected from the corpus and cauda epididymides of 12 dogs and incubated for 24 hr after washing by centrifugation.

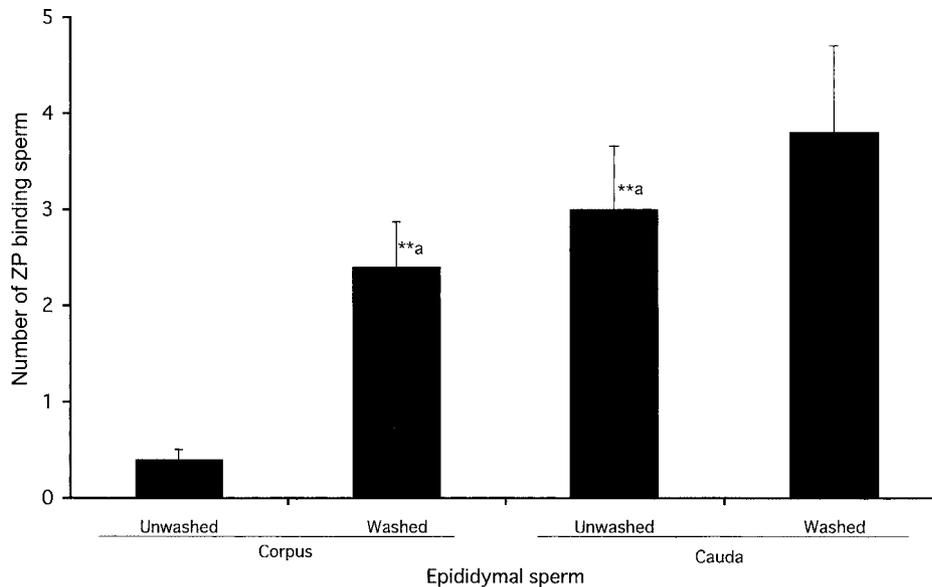


Fig. 5. The mean ( $\pm$  S. E.) percentages of zona-pellucida-binding sperm collected from the corpus and cauda epididymides of 12 dogs and then incubated for 24 hr after washing by centrifugation. \*\* a:  $P < 0.01$ , in comparison with unwashed corpus epididymal sperm.

during sperm transit from the corpus epididymides to the cauda epididymides and by washing the corpus epididymal sperm. In the ram [29] and the pig [4, 6], GP content on the sperm surface decreases during sperm transit through the epididymis, and the decrease is thought to be associated with sperm maturation [6, 29]. The GPs coating the surface of boar epididymal sperm are gradually lost due to the

hydrolytic effects of enzymes secreted by the epididymal epithelium [6]. Canine epididymal fluid contains various types of glycosidases, e.g., N-acetyl- $\beta$ -glucosaminidase,  $\beta$ -galactosidase and  $\alpha$ -mannosidase [26]. Thus, DBA- and SBA-lectin-binding GPs on canine sperm surface are therefore presumably removed by the effects of the enzymes in the epididymal fluid during sperm transport through the cor-

pus epididymides and the cauda epididymides. SBA lectin binds to ram sperm collected from the caput and corpus epididymides, but not to the cauda epididymal sperm, the same as shown by the results for canine epididymal sperm in this study [20]. It has been reported that canine immature epididymal sperm acquire fertilizing capacity after co-incubation with epididymal epithelial cells in the dog [24], but the relationship between the loss of DBA- and SBA-lectin-binding GPs on the canine cauda epididymal sperm and sperm maturation was not elucidated by our study.

The epididymal fluid of mammalian species contains DFs, which cover the sperm surface and inhibit sperm capacitation [2, 3]. Some of the specific proteins secreted by mouse epididymal epithelium are DFs, and removal of the proteins from the surface of mouse sperm induces sperm capacitation [16]. It has been found that DFs can be removed from the surface of mouse sperm by washing [7]. In the present study, after 24 hr of incubation the mean %MO, %HA and number of ZP-binding sperm in the washed sample from the canine corpus epididymides were much higher than for unwashed sperm from the corpus epididymides. Therefore, canine sperm maturation or loss of DFs from the sperm surface appears to occur during sperm surface appears to occur during sperm transit through the corpus epididymides and the cauda epididymides. After 24 hr of incubation the mean %MO, %HA and number of ZP-binding sperm in the washed sample from the canine corpus epididymides were much higher than for unwashed sperm from the corpus epididymides. The results for canine corpus epididymal sperm are presumably associated with the modification of DBA- and SBA-lectin-binding GPs on the corpus epididymal sperm surface. Although the FITC-DBA or -SBA lectins bound to the entire heads of unwashed canine sperm collected from the corpus epididymides, no fluorescence of either FITC-DBA and -SBA lectins was observed on the heads of the unwashed cauda epididymal sperm or the washed corpus epididymal sperm. Such a change in the lectin-binding characteristics of canine epididymal sperm suggests that loss of DBA- and SBA-lectin-binding GPs on the surface of epididymal sperm induces active movement and acquisition of fertilizing capacity. Therefore, the DBA- and SBA-lectin-binding GPs coating canine epididymal sperm may be the DFs of canine caput and corpus epididymal sperm. Enzymes that hydrolyze DBA- and SBA-lectin-binding GPs are presumably present in canine cauda epididymal fluid.

The authors have reported that the AR of canine sperm is efficiently induced by homologous ZP [14]. Therefore, the low %AR of both the corpus and cauda epididymal sperm after 24 hr incubation without homologous ZP in this study is understandable. The mean %MO, %HA, %AR and number of ZP-binding sperm in washed samples from the canine cauda epididymides were all higher than the values for unwashed sperm from the cauda epididymides. These results suggest that various DF-like GPs are present on the surface of the canine epididymal sperm and that some DF-like GPs remain on the plasma membrane surface of the

cauda epididymal sperm.

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