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Maturational Changes in Binding Capacity of Fowl Sperm to the Epithelium of the Sperm Storage Tubules during Their Passage through the Male Reproductive Tract

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ABSTRACT : The objective of this study was to examine the binding potential of sperm to the epithelium of the sperm storage tubules (SST) *in vitro* and *in vivo* to assess the functional maturation of fowl sperm. Sperm from the testis, epididymis, as well as the proximal, middle and distal vas deferens were incubated *in vitro* with either the uterovaginal junction (UVJ)- or infundibular tissue containing SST at 39°C for 30 min. Aliquots of sperm were also artificially inseminated into the uteri of hens, and the UVJ and infundibulum were collected 24 h post artificial insemination (AI). After incubation and AI, tissues were washed to remove loosely adhered sperm and subjected to fluorescence staining with 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) for counting the number of bound sperm per 0.25 mm^2 of surface area. Sperm from the testis, epididymis, and the three segments of the vas deferens exhibited their differential ($p<0.05$) binding capacity, which increased gradually from the testicular to distal vas deferens sperm under both *in vitro* and *in vivo* conditions. Existing similar trend, sperm, regardless of their source had a lesser affinity to bind to the epithelium of the infundibular SST than to the UVJ-SST. These experimental results suggested that fowl sperm may undergo gradual changes in the process of functional maturation, whereby they gain the ability to bind to the epithelium of the SST during their passage through the male reproductive tract (MRT). (**Key Words :** Binding Capacity, Sperm Storage Tubules, Maturation, Male Reproductive Tract)

INTRODUCTION

Fowl sperm, unlike their mammalian counterparts, after natural mating or AI, are capable of surviving for a long period of time in the blind-ended SST located in the UVJ and infundibulum of the hen oviduct (Bakst, 1998). Study has revealed that sperm undergoing a process of storage maintain a direct contact with the epithelial cells of the SST, as evidenced by electron microscopy (Van Krey et al., 1967), and those remain free in the SST cannot survive longer (Zavaleta and Ogasawara, 1987). Sperm storage in the oviduct involves an intimate association of sperm with the epithelium of the storage tubules and a stabilization of sperm membrane integrity. Several authors have demonstrated that a strong adherence of sperm cells termed as “sperm binding” to the epithelium of the SST prolongs the life span (Ashizawa and Nishiyama, 1983) and maintains the fertilizing capacity of the resident sperm (Waberski et al., 2006). Therefore, the ability to bind to the epithelial cells of the SST is a very important sperm function with respect to their prolonged survival and fertile life in the oviduct. Although there is much early literature describing the mechanisms underlying the storage of fowl sperm in the hen oviduct, no attention has been concentrated on the acquisition of potential by the sperm in the MRT for the binding capacity.

The present study was therefore aimed at examining the capacity for binding of the testicular, epididymal, and vas deferens sperm to the epithelium of the SST under both *in vivo* and *in vitro* conditions as a means of assessing their functional maturation.

MATERIALS AND METHODS

Animals

A total of 24 mature White Leghorn and Silky fowl male chickens (*Gallus domesticus*) aged 37 to 40 wks with proven fertility were selected for conducting this

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experiment on the basis of their semen output and sperm quality. Single Comb White Leghorn females aged 43-45 wks, laying more than 5 eggs in a clutch with a 1-d pause between clutches for more than two wks prior to experiments, were used in this study. Experimental birds were caged individually under a photoperiod of 14 L:10 D. Male birds were fed on a commercial breeder's ration (11.72 MJ ME/kg and 190 g CP/kg) and female birds were fed on a laying ration (12.43 MJ ME/kg, 160 g CP/kg and 35 g/kg total calcium) (Feed Production Unit, Uehara Poultry Farm, Itoman, Okinawa, Japan) *ad libitum*. The present study was carried out in accordance with the regulations for the care and use of experimental animals prescribed by the Animal Care Committee of the University of the Ryukyus, Okinawa, Japan.

Collection and preparation of sperm

Uncontaminated semen devoid of transparent fluid was collected twice weekly by the massage method (Burrows and Quinn, 1935) from selected cockerels and pooled to obtain ejaculated sperm, which were used as the control. Immediately after collection, the pooled semen was diluted four times with minimum essential medium (MEM) (Eagle's Nissui, Pharmaceuticals Co., Tokyo, Japan), pH 7.4 (adjusted with 10% $NaHCO_3$) (added L-glutamine at 0.292 g/L and 10% new born calf serum to the pH adjusted MEM before use), washed two times by centrifugation at 600×g for 10 min, and the sperm were then resuspended in the MEM. Semen collection was discontinued for 3 d, and on the fourth day, the cockerels were sacrificed by cervical dislocation. After laparotomy, the male reproductive organ (MRO) was removed from the body cavity, and the testes, epididymides, and vas deferentia were detached from each other using scissors and forceps. The total length of the vas deferens was cut equally into the proximal, middle and distal portions.

Sperm were collected from the testes as well as the respective parts and portions of the MRT following the procedure described by Howarth (1983). Briefly, each testis was cut longitudinally using a surgical blade, and the sperm suspension exuded from the testis was collected. The epididymis and the proximal, middle and distal vas deferens were squeezed with broad pointed forceps to secure sperm. Sperm were then pooled according to their source of collection from the MRO and immediately diluted four times with MEM. Sperm number was counted with a haemocytometer (American Optical Co., New York, USA). The diluted sperm suspensions thus prepared from the samples obtained from the testis, epididymis, and three segments of the vas deferens, as well as the ejaculate were divided into series of aliquots for use in the sperm binding assay *in vitro* and *in vivo* conditions.

Artificial insemination of hens

Intrauterine AI of hens was made during plumping phase (between 6 and 7 h after the preceding oviposition) of the uterus. The expected time of egg arrival in the uterus was calculated from the record of oviposition of the preceding egg based on the standard time required for the process of an egg formation in the hen oviduct. The presence of an egg in the uterus was ascertained by palpation prior to insemination. A rubber inseminating catheter with blunt tip fitted with a disposable graduated syringe was used for intrauterine insemination of hens. Each hen was inseminated with 100×10^6 sperm in a 0.2 ml dose of diluted semen once in the whole period of the experiment.

Assessment of binding capacity of sperm

To carry out *in vitro* sperm binding assay, tissues of the UVJ- and infundibular sperm storage region (SSR) were collected from a total of 10 hens following the procedure described by Brillard and Bakst (1990). The sperm-epithelium interaction assay was used to assess *in vitro* binding capacity of sperm to the epithelium of the SST, according to the method described by Steel and Wishart (1996). Briefly, a small piece of tissue (1.0×1.0 cm) from either the UVJ- or infundibular SSR was incubated with sperm at a concentration of 1×10^7cells/ml taken from the testis, epididymis, and proximal, middle and distal vas deferens, as well as the ejaculate in 5 ml MEM at 39°C for 30 min in 5% CO_2 humidified atmosphere. On the other hand, to assess *in vivo* binding capacity of sperm to the epithelium of the SST, a total of 60 hens (10 hens/sperm aliquot) were artificially inseminated as described above with aliquots of sperm from those respective sources. The UVJ- and infundibular SSR were collected 24 h after AI.

Epithelial samples after *in vitro* incubation and AI were washed in Dulbecco's PBS (-) (PBS without added "calcium" and "magnesium" ion) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to remove the loosely adhered sperm. The washed epithelium was then incubated in 500 μl PBS containing fluorescent stain DAPI for 15 min at 39°C. Sperm that remained attached to the epithelium were observed under a fluorescence microscope (Nikon Eclipse 50*i*, Tokyo, Japan). The number of sperm bound to 0.25 mm^2 of surface area was counted at 400× magnification with a digital micro counter (Digital Sight DS-L2, Nikon Corporation, Tokyo, Japan) attached to the fluorescence microscope.

Statistical analyses

Values are presented as the mean of four independent experimental replicates. The variation among the values for each source of sperm is illustrated using SEM. Statistical analyses of the data were carried out by analysis of variance

Table 1. Number of sperm from the testis, epididymis, and proximal, middle and distal vas deferens, as well as the ejaculate bound to 0.25 mm^2 of surface area of the epithelium of the uterovaginal junction (UVJ) and infundibulum containing sperm storage tubules (SST) after incubation *in vitro* at 39°C for 30 min with 1×10^7 sperm/ml

Binding site (epithelium)	Source(s) of sperm					
	Testis	Epididymis	Vas deferens			Ejaculate
			Proximal	Middle	Distal	
UVJ-SST	21.0±0.8[a]	42.9±1.7[b]	55.3±1.2[c]	64.0±1.3[d]	73.8±1.1[e]	70.3±1.1[e]
Infundibular SST	13.1±0.6[a,]*	33.3±1.5[b]*	43.5±1.6[c]*	50.8±2.2[d]*	61.6±1.8[e]*	57.1±1.3[e]*

Values are expressed as mean±SEM (n = 4).
[a-e] Values with different superscripts in the same row for either UVJ- or infundibular epithelium are different (p<0.05) among sperm from different sources.
* Different (p<0.05) between the UVJ- and infundibular epithelium in terms of the number of bound sperm corresponding to their source either from the testis, epididymis, vas deferens or ejaculate.

(ANOVA) followed by the Tukey-Kramer test. All statistical analyses were performed with the Statistical Analysis System "R" software package (R Development core Team, 2008) using a generalized linear model (GLM). A probability of p<0.05 was considered to be statistically significant, unless stated otherwise.

RESULTS

When a given number of sperm from the testis, epididymis, three segments of the vas deferens, and ejaculate were incubated with a given piece of either the UVJ or infundibulum containing SST *in vitro*, as well as inseminated into the uteri of hens, they demonstrated differential (p<0.05) capacity to remain attached to the epithelial cells of the SST of either source (Table 1 and 2). The mean number of testicular sperm bound to the SST epithelium was very few. A marked (p<0.05) increase in the mean number of bound sperm was observed for the samples taken from the epididymis compared with the mean number of the testicular sperm bound to the epithelial cells of the sperm storage sites; this increase was followed by a gradual increase in the capacity for binding from the epididymal to the distal vas deferens samples. Ejaculated sperm exhibited binding capacity similar (p>0.05) to that of the distal vas deferens samples. Sperm, regardless of their origin in the MRO, in general, had higher (p<0.05; Table 1 and p>0.05; Table 2) binding affinity to bind to the epithelium of the UVJ-SST than to the infundibular SST.

DISCUSSION

The unique feature of avian reproduction is the presence of specialized SST in the female oviduct. In the present study, we observed that after intrauterine insemination or direct incubation of sperm with either the UVJ- or infundibular tissue containing SST, the mucosal epithelium of the sperm residence contained many sperm on its surface. However, after repeated rinsing of the mucosal tissue, a number of sperm remained attached to the epithelial cells of the SST, indicating a possible affinity of sperm to bind to the sperm storage epithelium. Our results have been in consistent with the observation of Ashizawa and Nishiyama (1983) who reported that sperm are held in the oviduct by binding to the mucosal epithelial cells. In mammals, it has also been shown that sperm after entering into the oviductal reservoir, their head come in direct contact with the epithelium, and through binding they maintain an intimate association with the isthmic mucosal epithelium during storage (Petrunkina et al., 2001).

Using sperm recovered from the testis, epididymis, and the three portions of the vas deferens, we demonstrated that sperm had the differential binding capacity, which has been shown to increase gradually from the testicular to distal vas deferens sperm. Our findings corresponded with those of Esponda and Bedford (1985) to the extent that they

Table 2. Number of sperm from the testis, epididymis, and proximal, middle and distal vas deferens as well as the ejaculate bound to 0.25 mm^2 of surface area of the epithelium of the uterovaginal junction (UVJ) and infundibulum containing sperm storage tubules (SST) 24 h after artificial insemination (AI) with 1×10^8 sperm into the uterus of each hen

Binding site (epithelium)	Source(s) of sperm					
	Testis	Epididymis	Vas deferens			Ejaculate
			Proximal	Middle	Distal	
UVJ-SST	15.2±0.6[a]	28.9±1.1[b]	43.7±1.4[c]	53.2±1.9[d]	62.3±1.8[e]	60.0±1.7[e]
Infundibular SST	12.9±0.6[a]	26.4±1.2[b]	39.0±1.3[c]	50.2±2.0[d]	59.1±1.5[e]	56.9±1.8[e]

Values are expressed as mean±SEM (n = 4).
[a-e] Values with different superscripts in the same row for either UVJ- or infundibular epithelium are different (p<0.05) among sperm from different sources.

demonstrated that plasma membranes of fowl sperm undergo biochemical maturation during the passage of sperm through the MRT, because sperm plasma membranes have been considered to be responsible for the recognition of storage sites in the oviduct and only the membrane intact sperm are capable of undergoing storage in the SST (Chalah et al., 1999). On the other hand, if the motility is directly correlated with binding capacity of sperm (Peterson et al., 1980), the minimal, medium and maximal motility of the testicular, epididymal and vas deferens sperm, respectively (Howarth, 1983; Ahammad et al., 2011) might have been another cause of the gradually increased capacity of sperm from the testis to distal vas deferens for binding to the epithelium of the SST. Furthermore, it is well known that avian sperm immediately after AI into the vagina undergo a process of selection, and only sperm with fertility potential are sequestered in the SST for storage (Bakst et al., 1994). Thus we inferred that the maturation of binding capacity of fowl sperm to the oviductal SST for prolonged storage may coincide with the acquisition of their fertilizing ability in the MRT. Consequently, the results presented in the current study are consistent with our previous study (Ahammad et al., 2011) in which we demonstrated that fowl sperm undergo a gradual process of functional maturation during their passage through the MRT to attain fertility potential.

A significantly higher number of bound sperm, regardless of their source to the epithelium of the UVJ-SST than to the infundibular SST, has been observed in our current study under both *in vitro* and *in vivo* conditions, indicating a possible difference in the receptivity to sperm ligands between the UVJ and infundibular epithelial cells, thereby inducing a preferential binding to the mucosa of the UVJ compared to the infundibular mucosa. Studies have shown that the UVJ-SST have higher capacity for holding sperm during storage than the infundibular SST, for which the UVJ- and infundibular SST are recognized as the primary and secondary residence of sperm, respectively (Bakst et al., 1994). Our results are in agreement with the findings of Howarth (1990), who demonstrated that AI into the uterus resulted in the access of a larger number of sperm in the UVJ-SST than in the infundibular SST. In contrast, early studies have indicated that a larger number of sperm after intrauterine insemination move to the infundibular SST than to the UVJ-SST (Zavaleta and Ogasawara, 1987; Bakst et al., 1994). In that case, the plausible explanation for the reduced number of bound sperm to the infundibular samples compared to the samples obtained from the UVJ in our *in vivo* experiment might be that a large number of resident sperm in the infundibulum, instead of establishing a binding relation with the wall of the infundibular SST, remain free and move towards the site of fertilization in order to increase the chances of sperm penetrating in the germinal disc region. Another reason might be that they deposited sperm deep into the uterus, whilst we inseminated hens distally to the uterus.

The mechanism by which sperm remain bound to the epithelial cells is not fully understood at the present time, but it is assumed that the binding of sperm to the mucosal cells of the oviductal sperm residence requires a specific receptor-ligand interaction. However, glycoproteins were identified both in the acrosomal region of the sperm (Robertson et al., 2000) and in the epithelium of the oviductal sperm-reservoir (P´erez et al., 2006). Studies provided evidence that fowl sperm acquire membrane-associated glycoproteins during their epididymal and vas deferens passage (Morris et al., 1987). Sperm surface glycoproteins have been shown to act as determinants of binding capacity of sperm and to be essential for the sperm to reside in the SST (Lake and Ravie, 1988). Thus it can be inferred that the differential binding capacity of sperm reported in this study might partly be attributable to the differences in the gradually increased expression of binding sites on the surface of sperm as they pass through the descending duct of the MRT.

The suppression of the ejaculated sperm binding capacity compared to that of the distal vas deferens sperm in our present study might be attributed to the harmful effect of seminal plasma at ejaculation (Douard et al., 2005). However, centrifugation is essential to discard seminal plasma, which causes damage to sperm cells that appears to be mediated by the release of reactive oxygen species, which in turn cause membrane damage by free oxygen radicals (Aitken and Clarkson, 1988).

As fowl sperm have been shown not to undergo changes in the morphology directly after spermiation (Jones and Lin, 1993), the results of the gradually increased binding capacity of the testicular, epididymal, and vas deferens sperm that were reported in the present study provide further evidence for the functional maturation of fowl sperm.

In conclusion, based on our *in vitro* and *in vivo* experimental evidence, we suggest that fowl sperm may undergo gradual changes in the process of functional maturation, whereby they gain the ability to bind to the SST epithelium during their passage through the MRT. However, unlike mammalian sperm, fowl sperm do not undergo a complete maturation in the epididymis, which is rudimentary in chickens. Rather, this process continues during the passage of sperm through the proximal, middle and distal vas deferens.

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