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The Effects of Regular Fluid Secretion from the Uterus of Laying Hens on the Longevity and Fertilization Ability of Fowl Sperm in the Oviduct

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Abstract

The aim of this study was to examine whether the secreted fluid from the uterus influences the survival and fertilization capacity of fowl sperm in the hen oviduct. Hens with either regular uterine fluid secretion or irregular uterine fluid secretion were artificially inseminated through the transfer of sperm into the uterus. Twenty-four hours after artificial insemination, 3 hens with regular uterine fluid secretion and 3 hens with irregular uterine fluid secretion were killed, and the utero-vaginal junction and infundibular sperm storage tubules were observed for the presence of sperm. There was no difference ($P>0.05$) in the fill rate of either the utero-vaginal junction sperm storage tubules or the infundibular sperm storage tubules between hens with regular or irregular uterine fluid secretion. However, the sperm transferred into hens with regular uterine fluid secretion had a longer lifespan and fertilization ability than the counterpart group ($P<0.05$). In conclusion, these study results suggest that regular fluid secretion from the hen uterus may sustain the longevity and fertilization ability of fowl sperm in the oviduct.

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Introduction

After natural mating or artificial insemination (AI), fowl sperm are stored in the sperm storage tubules (SST) of the hen oviduct in a viable and fertile state for up to 3-4 wks (Fujii and Tamura, 1963; Ashizawa and Nishiyama, 1983; Brillard, 1993). Studies have shown that sperm stored in the utero-vaginal junction (UVJ) SST can survive longer than those stored in the infundibular SST, and hence, the UVJ-SST and infundibular SST are referred to as the primary and secondary SST, respectively (Brillard, 1993; Bakst *et al*., 1994). Considering the comparative location of the SST, the UVJ-SST are located near the uterus, which is capable of secreting a large volume of fluid for 19-20 hrs a day. It has been reported that the secretion of the uterine fluid (UF) continually bathes sperms during their long stay in the UVJ-SST and during their passage to the site of fertilization (Brillard *et al.*, 1987; Dupuy and Blesbois, 1996). The uterine secretions have been shown to contain a variety of organic and inorganic compounds that are known to supply nutrients and maintain optimal pH and osmotic pressure (El Jack and Lake, 1967; Lake, 1975; Slavesky and Leach, 1980), which provides sperms with a nutritive-protective aqueous milieu in the vicinity of the UVJ-SST. Moreover, AI into the hen uterus has been shown to allow sperms longer survival in the oviduct than other AI routes, indicating that the uterine environment is favorable for retaining the sperm's survival and fertility potential (Kempenich-pinto *et al*., 1970; Howarth, 1990). This effect is species specific. Thus, it seems reasonable to expect that the prolonged sperm survival in the UVJ-SST might be favored by UF secretion.

Examination of SST-oriented mechanisms underlying the prolonged fowl sperm survival in the UVJ-SST has been the subject of numerous studies since the discovery of the SST in the hen oviduct (Fujii and Tamura, 1963; Brillard, 1993; Das *et al*., 2010; Bakst, 2011). However, there are no studies examining whether regular sperm exposure to UF has biological significance in prolonging sperm survival and maintaining the fertilization ability of sperm in the UVJ-SST.

Therefore, this study was designed to examine the effects of the regular UF secretion on the sperm capacity in the oviduct for survival and fertilization. As it is well known that uterine fluid secretion is stimulated by the presence of an egg in the uterus and that the timing of UF secretion is directly associated with egg-laying patterns (Nakada and Koga, 1990), hens with either regular or irregular laying patterns were selected and subjected to AI for this study.

Materials and Methods

Birds

Mature White Leghorn, Japanese Shamo (Fighting cock) and Ukokkei (Silky fowl) male chickens (*Gallus gallus*) aged 43 to 45 wks with proven fertility were used as semen donors in this study. Fertilization capacity was selected as an

important sperm characteristic prior to pooling ejaculates collected from the semen donors. The fertility of individual males was tested at least two weeks prior to using the sperm for AI into the hen's vagina. Mature Single Comb White Leghorn female chickens aged 53-55 wks were used in this study. They were reared individually in cages (44 cm long x 40 cm wide x 36 cm high) under a photoperiod regimen of 14L:10D. Male birds were fed a commercial male breeder ration [(19% crude protein (CP) and 2800 ME (Kcal/Kg)], and female birds were fed a female breeder ration [(16% CP, 2900 ME (Kcal/Kg) and 3.5% Ca)] (Feed Production Unit, Uehara Poultry Farm, Itoman, Okinawa, Japan) *ad libitum*. This study was carried out in accordance with regulations for the care and use of experimental animals as prescribed by the Animal Care Committee of the University of the Ryukyus, Okinawa, Japan.

Semen collection and sperm preparation

Collection of uncontaminated ejaculated semen devoid of transparent fluid was performed twice weekly using the dorso-abdominal massage technique described by Burrows and Quinn (1935). To minimize breed and individual variations in sperm quality, the ejaculated semen was collected from the selected roosters and pooled. Immediately after collection, the pooled semen was diluted four times with Lake's solution, pH 7.1 (Lake, 1960). The diluted pooled semen was then washed twice and centrifuged at 600 × *g* for 10 min, and then was re-suspended in Lake's solution. The number of sperm cells was counted using a Neubauerhemocytometer (American Optical Co., New York, NY, USA). The average sperm concentration in the four-times-diluted sperm samples was 1.2×10^9 cells/mL. The final sperm concentration was adjusted to 5×10^8cells/mL by 2.4-fold further dilution of the sperm samples containing 1.2×10^9 cells/mL. The final diluted sperm suspensions were divided into a series of aliquots for use in AI.

AI of hens and fertility test

Laying hens with six to seven eggs in a sequence and a one-day pause between clutches were selected as hens with regular UF secretion, and laying hens with four or less eggs in a sequence and a pause of a variable number of days between clutches were selected as hens with irregular UF secretion. The selected hens were not exposed to male chickens nor subjected to AI in the two months preceding the experiment to ensure that no sperm remained in the hen oviduct. A total of 24 experimental hens (n = 12/UF secretory pattern group) were subjected to a single AI directly into the uterus with 1×10^8 sperm (Brillard *et al.*, 1987), with a 0.2 mL dose of diluted sperm per hen at eight hours following oviposition (two hours after the arrival of a membranous egg in the uterus). According to the standard time required

for egg formation in the hen oviduct, a membranous egg arrives in the uterus six hours after oviposition of the preceding egg (Warren and Scott, 1935). Prior to insemination, the presence of a membranous egg in the uterus was ascertained by palpation. A rubber inseminating catheter with a blunt tip fitted with a disposable graduated syringe was used for intrauterine AI of sperm into the hens' oviduct. Eggs were collected two times daily after the second day following AI and then incubated (37.8°C temperature, 60-65% relative humidity, <0.5% CO_2) in a forced-draft automatic incubator (Toyo Incubator TS15, Ashida Sangyo Co., Ltd., Okayama, Japan). Fertility [(number of fertilized eggs/total eggs set) × 100)] was determined by candling on 7th d of incubation. Eggs thought to be infertile were broken open, and the germinal disc region was examined microscopically for evidence of embryonic development.

Assessment of sperm survival in the oviduct

A day after AI, fertility was evaluated for a 3-wk period to assess the duration of the fertile period. The duration of fertility following fowl sperm AI into the hen oviduct is widely used to assess the length of sperm survival in the oviduct. The fertile period of the sperm was determined, according to the definition given by Brillard *et al*. (1989), as the number of days from the day after a single AI to the day before oviposition of the last fertile egg.

Observation of sperm in the SSTs

To determine the fill rate of the SST 24 h after AI, 3 hens with regular UF secretion and 3 hens with irregular UF secretion were killed by a rapid intravenous injection of a lethal dose of sodium pentobarbital (Nembutal; Dainippon Sumitomo Pharmaceutical, Osaka, Japan). This time interval was considered sufficient to ensure the maximal fill of the hen SST (Brillard, 1993). Preparation of the UVJ and infundibular mucosa for observation of SST was performed following the procedure described by Ito *et al.* (2011). Briefly, following laparotomy, the oviduct was removed from the body cavity, and the UVJ and infundibulum were excised longitudinally using fine scissors and forceps. The mucosal layer of each UVJ and infundibulum was scraped with a scalpel. The isolated individual mucosal folds containing SST from either the UVJ or infundibulum were incubated in phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 0.1% Triton X-100 (Sigma-Aldrich, Corp., St. Louis, Missouri, USA) at room temperature for 10 min, followed by washing with PBS. The specimens were then fixed overnight at -20°C with a 1:1 (v/v) mixture of acetone and methanol. After a final PBS wash, the fixed specimens were subjected to fluorescence staining with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min and observed

under a fluorescence microscope (Nikon Eclipse 50*i*, Tokyo, Japan). At least 100 SST were observed, and the number of sperm in the SST was counted to calculate the fill rate (%).

Statistical analyses

Data for the fill rate of the SST from three replicates and for the fertilization parameter from nine replicates were expressed as the mean ± SEM. Percentage data were subjected to arcsine transformation to satisfy the normality assumption prior to analysis of variance (ANOVA). All statistical analyses were performed using the Statistical Analysis System R software package (R Development Core Team, 2008). Data for the fertilization parameter were analyzed using the Chi-square test. Other data were analyzed by ANOVA using the general linear model (GLM) procedure and Student's t-test. A value of $P<0.05$ was considered statistically significant unless stated otherwise.

Results

When a given number of sperm were transferred into the uteruses of hens with either regular or irregular UF secretion, no difference ($P>0.05$; Table 1) was observed in the fill rate of either the UVJ or infundibular SST when comparing hens with regular UF secretion and hens with irregular UF secretion. As shown in Table 1, irrespective of the pattern of UF secretion, intrauterine AI of the hens resulted in a higher ($P<0.05$) fill rate of the UVJ-SST than the infundibular SST. Sperm transferred into the uterine of hens with regular UF secretion had longer survival and higher fertility ($P<0.05$; Table 2) than those transferred into the uterine of hens with irregular UF secretion.

Table 1. Fill rate (%) of the sperm storage tubules after artificial insemination of sperms into the uterus of hens with either regular or irregular pattern of secretion of fluid from the uterus

Pattern of uterine fluid secretion	Utero-vaginal sperm storage tubules	Infundibular sperm storage tubules
Regular	72.3 ± 3.6^{a}	23.5 ± 2.2^{b}
Irregular	69.4 ± 3.8^{a}	21.3 ± 2.9^{b}

Data are expressed as the mean ± SEM (n = 3). * Values differed between the utero-vaginal junction sperm storage tubules (SST) and infundibular SST sources as judged by two-factor ANOVA with GLM. Values within the same column did not differ between hens with regular uterine fluid secretion and hens with irregular uterine fluid secretion ($P>0.05$).

Table 2. Fertility (%) and duration of sperms survival (d) after artificial insemination of sperms into the uterus of hens with either regular or irregular pattern of secretion of fluid from the uterus

Pattern of uterine fluid secretion	Fertility of sperm (%)		Duration of sperm survival (d)
	First week	Second week	
Regular	72.3 ± 3.6 [a]	31.4 ± 2.2 [a]	27.4 ± 3.2 [a]
Irregular	59.4 ± 3.8 [b]	20.3 ± 1.6 [b]	16.2 ± 3.7 [b]

Data are expressed as the mean ± SEM (n = 9). [*] Values within the same column differed between hens with regular uterine fluid secretion and hens with irregular uterine fluid secretion ($P<0.05$).

Discussion

In this study, a significantly higher fill rate of the UVJ-SST compared to infundibular SST was observed 24 h after a single AI into the uteri of hens with either a regular or irregular UF secretion pattern. This finding indicated that after intrauterine AI, a large number of sperm cells migrated toward the primary residence site and entered into the UVJ-SST. The results of this study also revealed that the receptivity of the SSTs to sperm was not dependent on the regularity of UF secretion, as demonstrated by the similar fill rate of either the UVJ- or infundibular SST between hens with regular or irregular UF secretion. As reported by Brillard (1993), when sperm are introduced into the uterus, most of them find their way to the UVJ and enter into the UVJ-SST, while a few are directed to the infundibular SST. Recently, in a study on sperm maturation, we provided direct evidence that fowl sperm acquire the capacity to bind to the SST epithelium for storage and exhibit greater binding affinity to the UVJ-SST than to the infundibular SST (Ahammad *et al*., 2011).

Although it is clear that UF secretion plays an important role in the transport of sperm through the oviduct (Brillard, 1987), nothing is known about whether this fluid is associated with the length of survival and the maintenance of the fertilization capacity of the sperm. In avian species, the duration of fertility after a single AI into the oviduct is widely used to assess the length of sperm survival in the oviduct. In this study, to examine the effect of regular UF secretion on the survival of sperm in the oviduct, sperms were transferred into the uteri of hens with regular and irregular UF secretion. The results obtained revealed that the sperms introduced into the uteri of hens with regular UF secretion survived longer than those introduced into the uteri of hens with irregular UF secretion. It is well known that the more sperms are stored in the SSTs, the longer the duration of the fertile period for sperms in the oviduct (Brillard, 1993). However, this study revealed that following intrauterine AI, although the SST fill rate was not

significantly different between hens with regular and irregular UF secretory patterns, the sperms survival period was longer in the oviduct of hens with regular UF secretion compared with hens with an irregular UF secretion pattern. Thus, it seems likely that sperm survival in the oviduct is associated with the regularity of UF secretion.

Because fluid secretion from the uterus is directly associated with the production of shelled eggs, one may assume that egg production in hens may influence the longevity of sperm in the hen oviduct (Warren and Kilpatrick, 1929). However, according to a study by Lamoreux (1940), estrogen hormone, the key regulator of egg production in laying hens, is not involved in the length of sperm survival in the oviduct, which indicates that egg production in hens has no influence on sperms survival. Furthermore, a histological study by Bilgili *et al.* (1984) demonstrated that although the UVJ-SST contained an abundance of energy substrates for sperms metabolism, a large amount of the SST remained empty during the production of shell-less eggs by hens. Therefore, we can speculate that the secreted uterine fluid might be associated with the maintenance of sperms survivability in the oviduct. The prolonged survival of sperms in hens with regular UF secretion and the short lifespan of sperms in hens with irregular UF secretion observed in this study might be attributed to the fact that after entry into the UVJ-SST, sperms might remain exposed to the regular uterine secretions of hens with regular UF secretion and on an irregular basis in hens with irregular UF secretion. This study therefore suggests that regular UF secretion or the prolonged exposure of sperms to uterine secretions might be beneficial to sperms longevity in the oviduct.

As sperms remained fertile during their prolonged storage in the SST, this study also examined whether the secretion of fluid from the uterus sustains the fertilizing capacity of sperms in the oviduct. The results obtained revealed that sperms that were introduced into the uteri of hens with regular UF secretion exhibited significantly higher fertility than those introduced into hens with irregular UF secretion. This finding, coupled with the report indicating that the fertility of hens laying eggs in long clutches is higher than that of hens laying eggs with short clutches (Warren and Kilpatrick, 1929; Lamoreux, 1940), suggest that regular UF secretion might be beneficial to the maintenance of sperms fertilization potential. Moreover, sperms deposition into the uterus has been shown to significantly improve sperms fertility compared with other AI sites (Van Krey *et al.*, 1966; Ogasawara et al., 1966; Howarth, 1990). Thus, it can be inferred that following AI, a regular sperms exposure to UF is likely to be essential; the UF may help sperms retain their acrosomal membrane integrity (Blesbois and Brillard, 2007).

In conclusion, we suggest that regular fluid secretion from the uterus may have a pivotal role in the prolongation of functional sperms lifespan and maintenance of fertilizing capacity by providing an aqueous milieu in the oviduct.

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