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Quantification of Spermatozoa in the Sperm-Storage Tubules of Turkey Hens and the Relation to Sperm Numbers in the Perivitelline Layer of Eggs

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ABSTRACT

This study was conducted to determine the number of spermatozoa residing in the oviduct sperm-storage tubules (SST) and the relationship between these numbers and the number of spermatozoa embedded in the perivitelline layer of oviductal eggs after a single insemination of 200 × 10^6 spermatozoa. The SST of hens inseminated within one week before the expected onset of egg production were filled faster (4 h vs. 2 days) and possessed more spermatozoa (4.1 vs. 2.0 × 10^6) than the SST of hens inseminated after the onset of egg production. Furthermore, for hens in egg production, significantly fewer spermatozoa were recovered from the SST if the hen was inseminated within 2 h before or after oviposition than if inseminated more than 2 h before or after the oviposition. There was a strong positive correlation between the number of spermatozoa in the SST and the number of spermatozoa embedded in the perivitelline layer of the oviductal eggs ($r = 0.85$, $p < 0.01$). These data show that the population of spermatozoa actually accepted by the SST is quite small relative to the number of spermatozoa inseminated and that maximum sperm-storage is achieved when the hen is inseminated just prior to the onset of egg production. It is suggested that the sperm-storage capacity of the oviduct and the quality of the semen sample can be estimated on the basis of numbers of spermatozoa embedded in the egg perivitelline layer.

INTRODUCTION

Female birds have the capacity to store spermatozoa in their oviduct for varying amounts of time, depending on the species. In the domestic turkey (*Meleagris gallopavo*), fertilized eggs have been recorded 10–15 weeks after a single insemination (Christensen and Bagley, 1989). The anatomical unit in the oviduct responsible for prolonged sperm storage is the sperm-storage tubule (SST) (for reviews, see Zavaleta and Ogasawara, 1987, and Bakst, 1987). In the turkey, the SSTs are located in a 2- to 3-cm-wide band in the anterior portion of the vagina, a region referred to as the uterovaginal junction (UVJ). Spermatozoa enter the SSTs, which are discrete tubules confluent with the UVJ luminal epithelium, and over a period of several weeks are gradually released to ascend to the infundibulum, the site of fertilization. It is this mechanism of sperm storage and slow release that assures a succession of fertilized eggs in the absence of repeated copulations or artificial inseminations.

The turkey industry takes full advantage of the sperm-storage capacity of the hen through the extensive use of artificial insemination (AI). There is adequate information regarding semen collection, semen dilution and storage, and insemination techniques, yet we know little about the fate of spermatozoa in the oviduct immediately after AI. Howarth (1971) reported that within 30 min of insemination, 84% of the spermatozoa was excreted. Thus, sperm loss is considerable. Depending on the number of weeks the hens have been in egg production, hens could be inseminated weekly with 120–300 million spermatozoa.

There is evidence of a sperm-selection process at the level of the SST (Ogasawara et al., 1966; Bakst, 1989). It has long been known that debris and dead spermatozoa fail to enter the SST. More recently, Lake and Ravie (1988) and Froman and Engel (1989) demonstrated that chicken hen fertility was lowered and the SST capacity to store spermatozoa was reduced following AI with neuraminidase-treated spermatozoa. The fertilizing capacity of similarly treated spermatozoa was not affected if they were introduced surgically into the magnum, a segment anterior to the UVJ. The existence of a sperm-selection mechanism would imply that only a certain subpopulation of spermatozoa within the semen sample is retained by the oviductal SST.

The precise sperm population in the UVJ SST is not known but has been estimated to be about 7.5 × 10^6 spermatozoa (Bakst, 1987). It was our objective to study sperm population dynamics within the oviduct of the domestic breeder turkey. More specifically, we determined the relationship between the number of sperm residing within the SST to number of sperm embedded in the perivitelline layer of oviductal eggs.

MATERIALS AND METHODS

Hen Groups and AI

A total of 85 commercial strain turkey hens were used in this study. Hens were caged individually in an environ-

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ment-controlled house and provided feed and water ad libitum. At 30 wk of age, the photoperiod was increased from 6 h to 14 h of light (lights-on: 0300–1700 h).

Hens were divided into three groups. Group 1 (n = 32) consisted of hens inseminated before the onset of egg production (12–21 days after the onset of photostimulation) and did not have an egg mass in the oviduct when euthanized. Group 2 (n = 45) consisted of hens inseminated after the onset of egg production (minimum of 3 eggs prior to insemination) and did not exceed 34 wk of age. Group 2 hens were inseminated at least 2 h before or after oviposition. Group 3 hens (n = 8) differed from Group 2 hens only by being inseminated less than 2 h before or after oviposition.

For each AI, semen was manually collected from a minimum of 15 males, pooled, and diluted 1:1 with Beltsville Poultry Semen Extender (Continental Plastics, Delavan, WI). The sperm concentration was determined with a hemocytometer. Within 1 h of semen collection, hens were inseminated once with 200×10^6 spermatozoa.

Sperm Recovery from the Oviduct

Hens were euthanized with sodium pentabarbitol. To recover spermatozoa from the lumina of the infundibulum and the uterus and vagina (the uterus and vagina were handled as one segment), the oviduct was carefully removed and the infundibulum was clamped with hemostats at the base of the fimbriated region of the infundibulum and about 1 cm distal to the infundibulum-magnum junction. The uterus and vagina were removed and the proximal end of the uterine pouch and the distal end of the vagina were clamped. The vagina was stripped of connective tissue until straight. Each clamped segment was injected with 3.5 ml of Hanks' Balanced Salt Solution (HBSS) plus 0.05% Triton X-100. The segment was gently massaged for 30 s to disperse the wash solution throughout the mucosal folds. One end of the segment was then placed in a preweighed beaker, the hemostat was removed, and the wash fluid was collected and weighed to determine the volume of fluid recovered. The number of spermatozoa recovered was determined by hemocytometer counts (10 replicate counts per washing). Sperm heads, which are characteristically uniformly dense, slightly arced, filiform-shaped, and about 0.6 μm in diameter, were readily discernible from cell debris by phase-contrast microscopy.

To determine the total number of spermatozoa residing in the SSTs, the uterus and vagina were cut longitudinally and their mucosae were exposed. Starting in the vagina about 8 cm from the distal boundary of the uterus, the surface folds and lamina propria were separated from the muscularis mucosae by gently scraping with a scalpel. The sheet of mucosa was spread on a glass surface and the precise location of the SSTs was determined by microscopy (Bakst, 1987). The mucosa containing the SSTs was isolated, minced in HBSS, and dissociated with a Polytron (Brinkman Instruments, Westbury NY) operated at 7000 rpm for one 5-s burst. Preliminary work showed that this speed and duration are optimal and resulted in the recovery pf 86.2% ± 10.2% (10 samples) of the spermatozoa added to HBSS. Spermatozoa in the dispersed SST-containing mucosa were counted with a hemocytometer (10 replicate counts per sample).

Quantification of Spermatozoa in the Perivitelline Layer

Thirty hens in Group 2 had either a shell-less, soft-shell, or a hard-shell egg in the uterus when euthanized. To facilitate the determination of hen fertility, all uterine eggs were incubated in a commercial incubator for 16–20 h, and then the blastoderm was examined for signs of cleavage divisions. After removing adherent albumen, a piece of perivitelline layer about 15 × 25 mm was isolated (preliminary data indicated that sperm was randomly distributed within the perivitelline layer) and stained by the method of Wishart (1987), with one modification. The stain used in this study was Hoechst 33342 (Sigma, St. Louis, MO) at a concentration of 5 μg/ml of diluent (Lake and Ravie, 1979).

Statistical Analyses

Comparisons between groups for sperm numbers recovered from washed or dispersed oviductal segments were performed with a factorial analysis of variance model. When necessary, data were subjected to logarithmic transformation prior to the analysis of variance. Maximum sperm storage in the SST was determined using the following linear model: spermatozoa in SST = intercept + slope (days) in which days from 0.16 (for 4 h) up to 28 days were entered into the model in a stepwise manner. Maximum storage included the days that gave the best fit (largest r^2 value) with a positive slope.

RESULTS

Inseminations before or after Onset of Egg Production

Group 1 had significantly more spermatozoa residing in the SST 4 h, and 5 and 7 days after insemination than Group 2 (Tables 1, 2). Successive linear adjustments indicated that maximum sperm storage in the SST was at 4 h after AI in Group 1 (3.2×10^6 spermatozoa) and 2 days after AI in Group 2 (1.6×10^6 spermatozoa). In Groups 1 and 2, the number of spermatozoa recovered from uterine and vaginal washes was in excess of 100,000 between 4 h and 2 days and dropped precipitously thereafter. In contrast, spermatozoa recovered from the infundibulum region never exceeded a few thousand in both groups.

Inseminations near Time of Oviposition (±2 h)

A significant ($p < 0.01$) group effect was observed for the numbers of spermatozoa in the SST of hens in Group 2 vs. Group 3 ($F = 6.74$; $p < 0.02$). Seventy-one percent fewer spermatozoa were recovered from Group 3 hens than from Group 2 hens. Due to the small number of hens within

TABLE 1. Spermatozoa ($\times 10^3$) recovered from washing the uterus and vagina (combined) and infundibulum and from dispersed mucosa containing SST.*

Segment/Group	Interval after insemination								
	4 h	1 day	2 days	3 days	4 days	5 days	7 days	14 days	28 days
Uterus/Vagina									
Group 1	847 ± 341	100 ± 18	223 ± 138	74 ± 26	23 ± 6	42 ± 17	30 ± 12	—	—
Group 2	1195 ± 690	232 ± 167	132 ± 60	25 ± 8	20 ± 13	11 ± 8	8 ± 5	0	0
Group 3	91	21 ± 21	0	31	0	0	—	—	—
SST									
Group 1	3212 ± 911[a]	1865 ± 1055[a]	1261 ± 249[a]	1559 ± 440[a]	2132 ± 1245[a]	1807 ± 442[a]	1527 ± 106[a]	—	—
Group 2	1139 ± 305[b]	1249 ± 195[a]	1629 ± 244[a]	1189 ± 309[a]	797 ± 217[a]	484 ± 138[b]	678 ± 194[b]	328 ± 71	271 ± 120
Group 3	228	256 ± 256	415	418	67	305 ± 93	—	—	—
Infundibulum									
Group 1	7 ± 3	18 ± 2	4 ± 1	0	0	1 ± 0.5	4 ± 3	—	—
Group 2	1 ± 0.4	4 ± 1	3 ± 1	1 ± 0.4	0	12 ± 5	0	3 ± 1	0
Group 3	0	0	28	0	0	6 ± 4	—	—	—
Hen number									
Group 1	5	5	4	5	4	4	5	0	0
Group 2	5	5	5	5	5	5	5	5	5
Group 3	1	2	1	1	1	2	0	0	0

*Hens were inseminated one time with 200 × 10^6 spermatozoa before the onset of egg production (Group 1) and either more (Group 2) or less (Group 3) than 2 h before or after oviposition.

[ab]Between Groups 1 and 2, means (±SEM) with different superscripts within the same interval after insemination differ significantly ($p < 0.05$). Between Groups 1 and 2, no significant differences were observed in the number of sperm recovered from washing uterus and vagina.

TABLE 2. Time and group effects on sperm numbers recovered from washings of the uterus and vagina (combined) and from dispersed SST in turkey hens inseminated before (Group 1) or after (Group 2) the onset of egg production.*

Source of variation	Degrees of freedom	*F* value	Pr > F
Uterus/vagina (wash)			
Time	8	4.78	<0.01
Group	1	0.03	0.86
Time × Group	8	0.33	0.92
SST (dispersed)			
Time	8	2.85	<0.01
Group	1	7.55	<0.01
Time × Group	8	1.79	0.12

*Hens were inseminated one time with 200 × 10^6 spermatozoa.

each time period following AI, statistical comparisons involving Group 3 were not conducted. However, Table 1 shows a tendency for fewer spermatozoa in the uterus/vagina washings in Group 3 vs. Group 2 up to Day 2 after AI.

Sperm Numbers in SST and Perivitelline Layer

Due to the small numbers of eggs recovered in the oviducts of hens in Groups 1 and 3 (3 eggs combined), data on the number of spermatozoa in the perivitelline layer in these groups were not used. In Group 2 there was a significant relationship between the number of spermatozoa in the SST and the number of spermatozoa in the perivitelline layer of oviductal eggs recovered from Day 2 to Day 28 after insemination. The linear regression equation describing this relationship is $Y = 0.015X - 2034$ ($r^2 = 0.72$) where Y equals the number of spermatozoa embedded in the perivitelline layer and X equals the number of spermatozoa residing in the SST.

DISCUSSION

Spermatozoa fill the SST faster and in greater numbers if hens are inseminated just prior to the onset of egg production rather than after the onset of egg production. Adjusting the number of spermatozoa recovered from the SST to an 86.2% recovery rate (see *Materials and Methods*), an estimated 4.1 × 10^6 spermatozoa reached and entered the SST within 4 h of insemination in prelaying hens (Group 1). In contrast, after the onset of egg production, 2 days are required to attain a maximum population of 2.0 × 10^6 spermatozoa in the SST (Group 2). Considering that each hen was inseminated with 200 × 10^6 spermatozoa, it is obvious that AI is an inefficient process resulting in a dramatic loss of spermatozoa. Similar observations have been reported previously by Allen and Grigg (1957) and Howarth (1971). Alternatively, maybe such a large insemination dose is necessary so that an adequate number of a more fecund subpopulation of spermatozoa can populate the SST and thereby assure long-term fertility. The latter appears to be true for those nondomesticated birds that copulate frequently before the onset of egg production (first ovulation). Such frequent copulations may allow a population of viable spermatozoa to be selected by and accrue within the SST. Under some as yet unknown stimulation associated with follicular maturation and/or ovulation, spermatozoa are then released from the SST to populate the site of fertilization (Bakst and Bird, 1987).

With both domesticated and nondomesticated birds there is general agreement that spermatozoa from the second male takes precedence over the first male in paternity. This has been demonstrated in chickens (Warren and Gish, 1943; Compton et al., 1978; Van Krey et al., 1981), turkeys (Payne and Kahrs, 1961; Christensen, 1981), Mallards (Cheng et al., 1983), and ringed turtle-doves (Sims et al., 1987). In domesticated species, this has been attributed to stratification of the sperm in the SST (Van Krey et al., 1981). In several studies, females were inseminated initially with semen from one strain and inseminated again 4–6 h later with semen from a phenotypically different strain, and the subsequent phenotypes of the progeny were monitored. The results obtained with this tandem insemination procedure are compatible with our observations made with prelaying hens but are difficult to reconcile with our observations regarding the rate of filling of the SST from hens inseminated after the onset of egg production. We, as did Prasad (1967) using histological techniques, noted that filling the SST required 2 days after insemination of hens already in egg production. Verma and Cherms (1965), also using histological techniques, indicated that it required 5 days to attain maximal SST filling. However, they only collected tissues at varying periods within 90 min of AI and again 5 days after AI. Regardless if it required 2 or 5 days to fill the SST, after a tandem insemination sequence with a 4-h interval, there would be a mixing of the spermatozoa in the UVJ and vagina and, thereafter, limited stratification of the spermatozoa in the SST based on the order of insemination. Interestingly, Christensen (1981) noted that second-male precedence in poult paternity was less evident in hens in the last half of their egg production cycle following tandem inseminations. We are currently attempting to resolve this paradox between delayed filling of the SST in hens in egg production and the second-male precedence in paternity.

Differences in the numbers of spermatozoa recovered from the SST of hens inseminated before (Group 1) or after (Group 2) the onset of egg production support previous observations that hen fertility is improved due to increased retention of spermatozoa by the SST if the hen is inseminated prior to the onset of egg production (see Bakst, 1989, for review). How the onset of egg production affects the fate of spermatozoa in the oviduct is not known. Events associated with egg transport and oviposition may reduce the sperm transport capacity of the UVJ (Brillard et al., 1987). Possibly the oviduct's luminal environment during egg production is not conducive to sperm transport or there are fewer functional SST after the onset of egg production. Answers to this question may lead to improvements in AI technology and subsequently a longer, more productive breeding season.

The number of spermatozoa recovered from SST of hens inseminated within 2 h before or after oviposition (Group 3) was about one-third the number of spermatozoa recovered from SST of hens inseminated more than 2 h before or after oviposition (Group 2). These observations provide an explanation for the observed decrease in fertility associated with AI around the time of oviposition and ovulation (Christensen and Johnston, 1975, 1977). Similarly, the insemination of chickens 3 h before or after oviposition has a detrimental effect on fertility (Brillard et al., 1987) postulated that the oviductal contractions associated with oviposition somehow interfer with the transport and storage of spermatozoa in the oviduct.

Wishart (1987) first developed a technique to determine the density of spermatozoa embedded in the perivitelline layer of laid eggs. He found this number to be correlated with the duration of hen fertility. In this study, there was a strong positive correlation between the numbers of spermatozoa present in the perivitelline layer and the number of spermatozoa recovered from the SST. Brillard (unpublished) also observed a strong positive correlation ($0.56 < r < 0.87$; $p < 0.01$) in chickens between the number of spermatozoa present in the perivitelline layer and the estimated number of spermatozoa in the SST derived from histological sections. Taken together, these observations indicate that this procedure is a reliable biological assay for the evaluation of semen quality and oviductal sperm storage capacity at the time of insemination. Since there are no semen evaluation procedures that can predict the fertilizing capacity of a semen sample and no noninvasive techniques to estimate the number of spermatozoa residing in the SST, quantification of spermatozoa embedded in the perivitelline layer of eggs will provide estimates both of the duration of fertility and the number of spermatozoa from a single insemination that actually were selected by the SST for storage.

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