Effect of semen storage on the number of spermatozoa in the perivitelline layer of laid turkey eggs

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A progressive decline in fertility over the course of egg production may be observed when turkey hens are inseminated weekly with semen stored for 24 h. *In vitro* storage of spermatozoa before insemination results in lower fertilization, possibly because fewer spermatozoa survive selection and storage in the hen's sperm storage tubules *in vivo*; alternatively, stored spermatozoa may be as capable of reaching the egg as are fresh spermatozoa, but unable to penetrate and fertilize the egg normally. The objective of this study was to determine whether this decline in fertility is a result of fewer spermatozoa reaching the egg after insemination with spermatozoa stored *in vitro*. Hens were inseminated weekly over the first 12 weeks of egg production with either fresh semen ($n = 30$ hens) or semen stored for 24 h ($n = 30$ hens). A total of 301 eggs was evaluated by determining the density distribution of spermatozoa embedded in the outer perivitelline layer. For the 12 weeks of egg production, the fertility of hens inseminated with fresh semen remained greater than 94%. Conversely, the percentage fertility of eggs from hens inseminated with stored semen in weeks 1–3 was greater than 94% but thereafter fertility averaged 86%. There was no difference in hatchability of fertile eggs between the two treatments over all weeks combined, and weekly throughout the study ($P > 0.05$). The mean number of spermatozoa in the perivitelline layer was higher ($P < 0.001$) when hens were inseminated with fresh (12.1 ± 1.3 spermatozoa per 5.5 mm^2 membrane) versus stored semen (2.5 ± 0.3 spermatozoa per 5.5 mm^2 membrane) over all weeks combined, and weekly throughout the study ($P < 0.05$). As a result of storage for 24 h, fewer spermatozoa are stored in the sperm storage tubules and, consequently, fewer spermatozoa are present at the site of fertilization, thus contributing to the depressed fertility.

Introduction

Significant progress has been made in understanding the physiological mechanisms that control sperm selection and storage in the oviduct of the turkey hen (Brillard and Bakst, 1990; Bakst, 1992, 1993; Brillard 1992; Bakst *et al.*, 1994). Many factors influence the efficiency of sperm storage and subsequent fertility, including the timing of insemination (Christensen and Johnston, 1977; McIntyre and Christensen, 1985), the duration of egg production (Brillard, 1993) and the age of the hens (Van Krey *et al.*, 1967). Artificial insemination (AI) before the onset of egg production maximizes the filling rate of the sperm storage tubules (SST) and results in higher fertility over the egg production period (McIntyre and Christensen, 1985). In addition, inseminations performed just before or after oviposition result in a decreased fertility

rate, possibly due to reduced numbers of spermatozoa in the SST (Christensen and Johnston, 1977). Fertility also declines over the egg production period after insemination with semen stored *in vitro* for 24 h (see review, Thurston, 1995). However, the effect of storage for 24 h *in vitro* on oviductal sperm storage and subsequent fertility is not fully understood.

Wishart (1987) developed a noninvasive bioassay that can provide an estimate of the duration of fertility of individual hens based on the density of spermatozoa embedded in the outer perivitelline layer of laid eggs. There is a significant positive correlation between the number of spermatozoa in the perivitelline layer and the number of spermatozoa residing in the SST at the time the egg is laid (Brillard and Antoine, 1990; Brillard and Bakst, 1990).

In this study, the perivitelline sperm counting procedure was used to determine whether the decline in fertility of hens inseminated with semen stored for 24 h is associated with fewer spermatozoa interacting with the ovum at the time of fertilization.

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Materials and Methods

Animals and semen collection

Commercial Large White breeder turkey tom and hen poults were purchased from a primary breeder and maintained under standard husbandry conditions during their brooding and growing periods. Toms and hens were photostimulated (03:00–17:00 h) at 26 and 28 weeks of age, respectively. Semen was first collected manually from toms at 28 weeks and, thereafter, at least once a week using two cloacal strokes (Bakst and Cecil, 1983). Semen from 5–10 toms was pooled and diluted 1:1 with turkey semen diluent (SemAid; Poultry Health Laboratories, Davis, CA) within 30 min of collection.

Semen storage and preparation for insemination

Diluted semen was divided into two aliquots, one part used for insemination within 90 min of collection (fresh), and the other part stored for 24 h and then used for insemination (stored). For storage, semen aliquots were kept in 10 ml Erlenmeyer flasks covered loosely with foil. These flasks were placed in beakers containing enough water to reach the upper level of semen in the flask and the beakers were placed on an orbital shaker (150 r.p.m.) in a refrigerator at 5°C (Sexton, 1988).

Hens were inseminated initially on days 14 and 16 after the onset of photostimulation and once a week thereafter for the next 12 weeks with 150×10^6 viable spermatozoa. The percentage of viable spermatozoa was determined using the modified ethidium bromide exclusion procedure (Bakst *et al.*, 1991). The insemination dose was adjusted accordingly to include a constant number of viable spermatozoa with each insemination. Two treatment groups consisting of 30 hens per group were established and inseminated with fresh or stored semen. Fifteen hens from each group were designated as egg donors for the evaluation of the number of spermatozoa in the perivitelline layer. Eggs from the remaining hens and those not used in the perivitelline bioassay were set once a week, candled at day 7–10 and then incubated to determine hatchability.

Quantification of spermatozoa in the perivitelline layer

Eggs laid by hens designated for determination of the perivitelline sperm counting procedure were collected four times a day and examined within 12 h of collection. After removing adherent albumen, a piece of perivitelline membrane about 15 mm $\times$ 25 mm was isolated and stained by the method of Wishart (1987) and modified using 5 μ g Hoeschst 33342 ml⁻¹ (Sigma, St Louis, MO; see Brillard and Bakst, 1990, for details). Spermatozoa in 50 randomly selected fields were counted at $\times 40$ magnification. The ovum was assumed fertile if > 2 spermatozoa per 5.5 mm² membrane were observed (Wishart, 1987).

Statistical analysis

Differences among the number of spermatozoa in the perivitelline layer, fertility and hatchability between hen

groups were examined using analysis of variance (ANOVA) using the least squares procedure and the general linear models procedure of the Statistical Analysis System (SAS, 1985). Percentage data were arcsine transformed before analysis. Data are displayed as arithmetic means for clarity of presentation.

Results

A total of 301 eggs was evaluated to determine the number of spermatozoa trapped in the perivitelline layer over the first 11 weeks of egg production. For both fresh and stored semen treatments, the average number of eggs evaluated was 14 per week per treatment and ranged from 4 to 22 eggs. Results obtained at 12 weeks were not included in the data set because fewer than four eggs were available from the stored semen treatment group.

The number of spermatozoa in the perivitelline layer was higher ($P < 0.05$) by week for fresh versus stored semen treatments throughout the 11 week evaluation period (Fig. 1) and over all weeks combined, 12.1 ± 1.3 spermatozoa per 5.5 mm² membrane for fresh and 2.5 ± 0.3 spermatozoa per 5.5 mm² membrane for stored treatments, respectively ($P < 0.001$). Although the number of spermatozoa in the perivitelline layer varied greatly within the fresh treatment group (range 0–83 spermatozoa per 5.5 mm² membrane), the mean number of spermatozoa in the perivitelline layer for stored semen (range 0–25 spermatozoa per 5.5 mm² membrane) never reached the mean number of spermatozoa observed in the fresh treatment group (Fig. 1).

Candling fertility remained high throughout the egg production period in hens inseminated with fresh semen (Table 1). There was no difference in the fertility of eggs from hens inseminated with fresh and stored spermatozoa for the first 3 weeks of egg production. Thereafter, fertility was lower for hens inseminated with stored semen (Table 1; $P < 0.001$). Estimated fertility (determined by counting perivitelline spermatozoa) was similar ($P > 0.05$) to true fertility (determined by candling) for the eggs from hens inseminated with fresh spermatozoa (94.8% estimated fertile versus 96.7% fertile, respectively). For hens inseminated with stored spermatozoa,

Fig. 1. Number of spermatozoa in the perivitelline layer (mean $\pm$ SEM) of eggs laid from turkey hens inseminated with fresh semen (—) or semen stored for 24 h (---).

Table 1. Percentage of turkey hen fertility ($\bar{x} \pm \text{SEM}$) after weekly inseminations of semen before (fresh) or after 24 h storage at 5°C*

Treatment	Egg production period (weeks)			
	1–3	4–6	7–9	10–12
Fresh	97.9 $\pm$ 0.5 ^a	95.7 $\pm$ 0.6 ^a	96.1 $\pm$ 0.6 ^a	97.0 $\pm$ 0.6 ^a
Stored	95.0 $\pm$ 0.7 ^a	85.6 $\pm$ 1.0 ^b	86.5 $\pm$ 1.1 ^b	87.4 $\pm$ 1.1 ^b

*Based on least square means of arcsine transformed data.

Means within columns with different subscripts are significantly different ($P < 0.001$).**Table 2.** Percentage hatchability ($\bar{x} \pm \text{SEM}$) of fertile eggs after weekly inseminations of turkey hens with spermatozoa before (fresh) or after 24 h storage at 5°C*

Treatment	Egg production period (weeks)			
	1–3	4–6	7–9	10–12
Fresh	88.5 $\pm$ 0.5	89.7 $\pm$ 1.1	80.0 $\pm$ 1.6	86.8 $\pm$ 1.5
Stored	93.2 $\pm$ 1.0	88.0 $\pm$ 1.7	80.7 $\pm$ 1.7	88.3 $\pm$ 1.4

*Based on least square means of arcsine transformed data. There were no significant differences between treatments with fresh and stored spermatozoa.

estimated fertility (71.5%) was lower than true fertility (88.5%, $P < 0.05$). Although the total number of hatched eggs was lower in the group treated with stored semen owing to decreased fertility, hatchability of fertile eggs between the fresh and stored treatments did not differ (Table 2).

Discussion

The mean number of spermatozoa in the perivitelline layer after insemination with stored spermatozoa was consistently lower than the number observed after insemination with fresh spermatozoa over the egg production period. Given that it has been clearly shown that there is a significant positive correlation between the number of spermatozoa in the perivitelline layer and the number residing in the SST (Brillard and Bakst, 1990; Brillard, 1992), our observations indicate that the depressed fertility observed in hens inseminated with spermatozoa stored for 24 h *in vitro* is due, in part, to fewer spermatozoa residing in the SST. The perivitelline layer bioassay (estimating the numbers of spermatozoa on laid eggs) has been used by several investigators as an indirect measure of efficiency of selection and storage *in vivo* in the SST in chickens (Brillard and Antoine, 1990; Alexander *et al.*, 1993; Wishart *et al.*, 1993; Wishart and Staines, 1995). Therefore, in the present study, the lower number of spermatozoa observed in the perivitelline layer of turkey eggs infers that selection and storage *in vivo* is compromised after sperm storage *in vitro*.

While hen fertility has been used as a final indicator of viability after storage for 24 h, the real problem is not the inability of spermatozoa to reach the ovum but an inability of spermatozoa to survive the oviductal selection and storage

process. Our results show a decline in fertility after the first 3 weeks of egg production in hens inseminated with stored semen. This decline has been demonstrated repeatedly when semen stored for 24 h is used (Sexton, 1988; Bakst *et al.*, 1991). Egg production and fertility decrease over the egg production period in hens even when fresh semen is used for insemination (Van Krey *et al.*, 1967; Brillard, 1993). Since hens are most efficient in producing fertile eggs at the beginning of egg production, the effect of using semen stored *in vitro* may not become apparent until several weeks into the production period.

In evaluating different flocks of naturally mated chickens using this bioassay, Wishart and Staines (1995) found that, in flocks with the highest fertility, the number of spermatozoa in the perivitelline layer was greater and there was a larger range in the numbers of spermatozoa on eggs evaluated (0–> 600) compared with a flock with lower fertility, in which most of the eggs evaluated had less than 20 spermatozoa. They reasoned that the bioassay is a better predictor of hen fertility than evaluation of day to day fertility, because it gives a better insight into the overall efficiency of hens to store spermatozoa and produce fertile eggs. Similar to the findings of Wishart and Staines (1995) of the relationship between flock fertility and numbers of spermatozoa trapped in the perivitelline layer, we found more and a much greater range in number of spermatozoa in the perivitelline layer of eggs from hens inseminated with fresh semen (range, 0–83 spermatozoa per 5.5 mm² membrane; average, 12 spermatozoa per 5.5 mm² membrane) compared with eggs from hens inseminated with spermatozoa stored for 24 h (range, 0–25 spermatozoa per 5.5 mm² membrane; average, 2.5 spermatozoa per 5.5 mm² membrane). Hens inseminated with fresh semen appear to have a greater

'reserve' of spermatozoa to ensure fertilization. In contrast, in hens inseminated with spermatozoa stored *in vitro*, there are only enough spermatozoa reaching the perivitelline layer to result in fertilization. Without any 'reserve spermatozoa', if fertilization is compromised in any way (i.e. by reduced sperm storage or lower sperm competence), hen fertility is lower.

There are several reasons why the efficiency of storage in the SST is lower after insemination with spermatozoa stored for 24 h. There is normally an intense selection pressure in the vagina. Considering that only about 1–2% of fresh, inseminated spermatozoa are stored in the SST (Brillard and Bakst, 1990), semen storage for 24 h may further reduce the ability of spermatozoa to survive this selection process. Reducing the number of competent spermatozoa inseminated even further (by *in vitro* storage) could result in an even smaller population of spermatozoa stored *in vivo* and capable of fertilization. This is evident in the present study, in which 80% fewer spermatozoa were observed on the perivitelline layer of eggs inseminated with spermatozoa stored for 24 h compared with fresh semen.

Storage of spermatozoa *in vitro* has been shown to reduce sperm viability (Bakst and Cecil, 1991). Although most semen evaluation tests do not reveal 'weak' spermatozoa after 24 h storage, hypo-osmotic stress tests have shown that the number of spermatozoa with poor plasmalemma integrity is greatly increased after storage (Bakst and Cecil, 1991; Donoghue *et al.*, 1995). Inseminating numbers of viable spermatozoa (after adjustment for sperm damage using the hypo-osmotic stress test) comparable to those in fresh semen does not prevent the fertility decline observed with stored semen (Bakst and Cecil, 1991). There is evidence that plasmalemma-associated proteins or glycoproteins are important components that allow spermatozoa to be stored in the SST (see reviews, Wishart and Steele, 1990; Bakst *et al.*, 1994). When neuraminidase was used to cleave neuraminic acids of plasmalemma-associated proteins in chicken spermatozoa, storage in the SST was reduced after intravaginal insemination but not intrauterine insemination (Froman and Thurston, 1984; Steele, 1992). Storage *in vitro* may alter the surface characteristics of the plasmalemma, or intrinsic proteins associated with the plasmalemma, of a greater proportion of spermatozoa, decreasing the number that survive selection through the vagina. Thus, selection and spermatozoa viability *in vivo* may contribute to lower numbers of spermatozoa available to fertilize eggs after *in vitro* holding.

Insemination before the onset of egg production fills the SST and it is thought that subsequent weekly inseminations replace spermatozoa that have been released from the SST (Bakst and Cecil, 1991; Bakst *et al.*, 1994). Since the reservoir of spermatozoa is initially lower with insemination of semen stored *in vitro* (based on a four times lower number of spermatozoa in the perivitelline layer of eggs from stored versus fresh semen inseminations), it is possible that the reduced fertility observed after the first 3 weeks of egg production for this treatment is due to the quantitative loss of spermatozoa in the SST and the lower efficiency of spermatozoa stored *in vitro* to replace them.

Our data on spermatozoa associated with the turkey perivitelline layer are similar to results observed with chickens (Wishart, 1987; Brillard and Antoine, 1990; Wishart *et al.*, 1993). Wishart (1987) reported that only 2.0 spermatozoa per 5.5 mm² were necessary to produce a fertile chicken egg.

Brillard and Antoine (1990) similarly found that 2.4 spermatozoa per 5.5 mm² were needed to predict 100% flock fertility. In the present study, 2 or more spermatozoa per 5.5 mm² were observed in the perivitelline layer of about 95% of all eggs from hens inseminated with fresh semen, which was similar to the overall candling fertility (96.7%). In contrast, after insemination with semen stored for 24 h, fertility estimates for eggs evaluated for perivitelline layer spermatozoa number was lower than that observed for candled eggs (71.5% versus 88.5%, respectively). Since fewer spermatozoa are observed in the perivitelline layer after storage of semen *in vitro* and the average number observed (2.5 spermatozoa per 5.5 mm²) is closer to Wishart's cut-off point for estimating fertility in chickens, it is possible that some eggs could be incorrectly considered infertile because so few spermatozoa are embedded in the perivitelline layer and are therefore missed when only a portion of the perivitelline layer is assessed. In contrast, the numbers of spermatozoa in the perivitelline layer for fresh semen averaged 2–40-fold higher; therefore, it is more likely that assessment of membrane resulted in estimating that these eggs are fertile.

Previous studies evaluating conditions affecting semen stored for 24 h have relied on fertility data that do not indicate the selection/storage ability of spermatozoa within the hen. The results of the present study show that about 80% fewer spermatozoa are embedded in the perivitelline layer after insemination with semen stored *in vitro* compared with fresh semen, resulting in lower fertility. The perivitelline layer bioassay was important for evaluating the effects of sperm storage conditions *in vitro* on subsequent selection and storage in the SST and fertility. Determining how spermatozoa are compromised *in vitro* and how this relates to reduced sperm storage in hens is essential for improving the use of stored semen in AI programmes.

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