Evaluation of a semen extender from a bioenergetic perspective

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ABSTRACT: Four premises for sperm preservation were previously outlined. The present work tested 2 of these. The first premise was that sperm mobility phenotype affects procedural efficacy. Random bred roosters were phenotyped with the sperm mobility assay. A normal frequency distribution was observed with 35% (SD = 16.4) mobile sperm. Test subjects had values >51% (high) or between 19 and 35% (below average). Phenotypes were confirmed by repeated measure analysis. Ejaculates were pooled by phenotype. Sperm were washed by centrifugation through 12% (wt/vol) Accudenz. Washed sperm were suspended in Beltsville Poultry Semen Extender (BPSE) at 2 × 10⁹ sperm/mL. Such sperm were stored at 10°C for 24 h. In the case of highly mobile sperm, an exponential decay was observed with a y-intercept of 72% and an asymptote of 53%. In contrast, postwash values for below-average males decreased linearly from a y-intercept of 31 to 17% after 24 h. A logistic decay was observed when sperm from high phenotype subpopulation males were extended with BPSE rather than washed before storage. Whereas y-intercepts were equivalent between experiments, end points were not, that is, 53 vs. 17% mobile sperm. This difference was attributed to the extent of cytotoxic edema. The second premise tested was that the sperm mobility assay can predict the status of sperm cell mitochondria in response to sperm manipulation. Highly mobile sperm were washed and then suspended in either saline or glucose-free extender. Solution pH and osmolality were equivalent. Extender osmolality was controlled by replacing glucose with mannitol. Sperm were stressed by incubation at 2 × 10⁹/mL at 20°C for 8 h. In each case, loss of sperm mobility approximated a logistic function. Whereas y-intercepts were equivalent, the time at which loss of function was half maximal was prolonged with the extender (P < 0.01). This difference was attributed to a diminution of the process whereby energy-deprived sperm were rendered immobile by cellular edema. An a posteriori analysis was limited to pretreatment data from males categorized a priori with the high phenotype. Phenotype was independent of time (P = 0.81) during the 14-wk interval in which experiments were performed. In summary, extender efficacy was affected by sperm mobility phenotype as well as the means by which the extender was used. To date, such effects have not been addressed in attempts to preserve chicken sperm in vitro.

Key words: bioenergetics, chicken, semen extender, semen preservation, sperm, sperm mobility


INTRODUCTION

Sperm mobility was identified as a new quantitative trait of chickens by Froman and Feltmann (1998). Mobile sperm penetrated 6% (wt/vol) Accudenz (Accurate Chemical and Scientific Corp., Westbury, NY) from an overlaid sperm suspension. Such sperm moved against resistance imposed by a physical interface. Thereafter, male fertility was shown to be a function of sperm mobility phenotype (Froman et al., 1999), and phenotype determined the outcome of competitive fertilization (Birkhead et al., 1999). Next, computer-assisted sperm motion analysis demonstrated that immobile sperm were typically motile at body temperature but had a straight line velocity less than 30 μm/s (Froman et al., 2003). Phenotype was predicted by the proportion of such sperm within an ejaculate. Subsequent experiments demonstrated that mitochondrial Ca²⁺ cycling enables sperm motion (Froman and Feltmann, 2005). Additional experiments demonstrat-
ed that immobile sperm contain moribund mitochondria as evidenced by electron microscopy, \( O_2 \) consumption, and sperm egress rate from the hen’s sperm storage tubules (Bowling et al., 2003; Froman and Kirby, 2005; Froman et al., 2006). These discoveries warranted viewing artificial semen storage from a bioenergetic perspective (Froman and Feltmann, 2010). In this regard, it is noteworthy that the poultry industry has yet to implement any consistent form of artificial semen storage. It is also noteworthy that chicken semen quality can be defined as the number of mobile sperm produced per male per day (Froman and Rhoads, 2013).

Most recently, the author has demonstrated how the sperm mobility assay can be used to predict mathematical relationships that characterize fowl sperm function (Froman, 2014). Four premises for sperm preservation were derived from this effort. The present work was conducted to test 2 of these premises. First, how is procedural efficacy affected by sperm mobility phenotype? Second, can the sperm mobility assay be used to detect the effects of in vitro sperm cell manipulation?

**MATERIALS AND METHODS**

**Semen Donors**

Roosters in a random bred flock of New Hampshire chickens \((n = 454)\) were phenotyped at 27 wk of age with the sperm mobility assay as outlined by Froman (2014). Data were assigned to frequencies in increments of 10 percentage units. The Kolmogorov–Smirnov test for goodness of fit was used to determine whether observed frequencies approximated a normal distribution (Sokal and Rohlf, 1969a). Estimates of the mean and SD were used to calculate the normal probability density function (Sokal and Rohlf, 1969d). Those roosters with values >1 SD above the mean and those with scores between the mean and 1 SD below the mean were phenotyped again at 29 wk of age. Forty roosters were selected from each group based on phenotype, sperm concentration, and semen volume. Sperm mobility data were evaluated by nested ANOVA (Sokal and Rohlf, 1969c). Roosters reserved as semen donors were maintained on a 14:10 photoperiod and fed ad libitum in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching, third edition (FASS, 2010). Experiments were conducted with the approval of the Oregon State University Institutional Animal Care and Use Committee.

**Reagents**

Accudenz was purchased from Accurate Chemical and Scientific Corp., Westbury, NY. All other solutes were purchased from Sigma Chemical Co., St. Louis, MO. All solutions were prepared with sterile-filtered, cell culture-tested water purchased from Sigma Chemical Co. Stock solutions of 30% (wt/vol) Accudenz were prepared with 3 mM KCl and 5 mM \( N \)-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES). Stock solution pH was adjusted to 7.4 with 5 M KOH. Isotonic TES-buffered saline was prepared with 50 mM TES in 125 mM NaCl. The pH was adjusted to 7.4 with 5 M NaOH. Stock solutions of 12% (wt/vol) Accudenz were prepared with 54% (vol/vol) TES-buffered saline, 40% (vol/vol) 30% (wt/vol) Accudenz, and 6% (vol/vol) water. Beltsville Poultry Semen Extender (BPSE), pH 7.4, was prepared as a modification of that described by Sexton and Fewlass (1978). Specifically, the extender contained 55 mM anhydrous dibasic potassium phosphate, 46 mM sodium glutamate, 28 mM glucose, 32 mM sodium acetate, 10 mM TES, 2 mM potassium citrate, 5 mM monobasic potassium phosphate, and 2 mM magnesium chloride. Osmometer standards (100, 290, and 1,000 mmol/kg) were purchased from Wescor, Inc., Logan, UT. The osmolality of each experimental solution was measured with a Model 5500 Vapor Pressure Osmometer (Wescor, Inc.).

**Experiment 1**

Solutions to be used were warmed to room temperature. Ejaculates were pooled by phenotype until a volume of 5 mL was obtained. Sperm concentration was measured with a spectrophotometer as outlined by Froman and Rhoads (2012). Sperm mobility was measured as described by Froman (2014) at an osmolality of 290 mmol/kg. Next, sperm were diluted to \( 2 \times 10^9 \) sperm/mL with TES-buffered saline. A 1-mL volume of 30% (wt/vol) Accudenz was layered beneath a 30-mL volume of 12% (wt/vol) Accudenz in a 50-mL polypylene centrifuge tube. A 6-mL volume of sperm suspension was overlaid on the 12% (wt/vol) Accudenz, and the tube was centrifuged at 20°C for 30 min at 1,250 \( \times \) g. Supernatants were removed by aspiration, and washed sperm was recovered from the interface between Accudenz solutions. Washed sperm were diluted to \( 2 \times 10^9 \) sperm/mL in BPSE. Seven 300-μL volumes of the sperm suspension were pipetted into microcentrifuge tubes within a floating rack, and the rack was placed in a 20°C water bath. As postwash sperm mobility was measured, the water bath was cooled at a rate of –0.4°C/min to 10°C. Sperm mobility was measured again after 1, 2, 4, 8, 12, and 24 h after the onset of cooling. This procedure was repeated until 5 replicates per phenotype were obtained. Each data set was plotted as a function of time. Parameters of \( y(x) = \alpha + \beta e^{-\lambda(x)} \) and \( y(x) = \alpha + \beta(x) \) were estimated with the PROC NLIN procedure.
and PROC GLM features of SAS, respectively (SAS Inst. Inc., Cary, NC). Finally, a 2-way ANOVA (Sokal and Rohlf, 1969e) was used to analyze pooled pre- and postwash data (n = 10 observations).

**Experiment 2**

Ejaculates from high sperm mobility roosters were pooled and sperm mobility was measured as outlined above. Next, semen was diluted to 2 × 10⁹ sperm/mL with BPSE. Seven 300-μL volumes of the sperm suspension were pipetted into microcentrifuge tubes as outlined above. As the mobility of extended semen was measured, the water bath was cooled at a rate of −0.4°C/min to 10°C. Sperm mobility was measured again after 1, 2, 4, 8, 12, and 24 h after the onset of cooling. This procedure was repeated until 5 replicate data sets were obtained. Data were plotted as a function of time. Parameters of the logistic function $y(x) = (\gamma)/[1 + e^{\beta(\tau - x)}]$ were estimated with the PROC NLIN feature of SAS.

**Experiment 3**

Ejaculates from high sperm mobility roosters were pooled, prewash sperm mobility was measured, sperm were washed by centrifugation through 12% (wt/vol) Accudenz, and postwash sperm mobility was measured as outlined above. Washed sperm were diluted to 2 × 10⁹ sperm/mL in either TES-buffered saline or BPSE in which mannitol was substituted for glucose. In each case, eight 300-μL volumes of sperm suspension were pipetted into microcentrifuge tubes within a floating rack as outlined above. Next, the rack was incubated in a 20°C water bath for 8 h. Sperm mobility was measured hourly after immersion of the rack within the water bath. This procedure was repeated until 5 replicate data sets were obtained for each type of sperm suspension. Each data set was plotted as a function of time. Parameters of the logistic function $y(x) = (\gamma)/[1 + e^{\beta(\tau - x)}]$ were estimated for each data set with the PROC NLIN feature of SAS. Thereafter, the principle of conditional error was used to determine if estimates of $\tau$ were estimates of a common parameter. An extra sums of squares $F$-test was performed as follows:

$$f_{r,(n-p)} = (\text{SSH}/r)[\text{SSE}/(n-p)],$$

in which SSH was the difference between the conditioned sum of squared residual errors and the sum of squared residual errors obtained unconditionally (SSE), $r$ was the number of independent parametric statements implied by the condition ($\tau_1 = \tau_2$), $n$ was the number of observations, and $p$ was the number of parameters within the observational model.

**RESULTS AND DISCUSSION**

As shown in Fig. 1, sperm mobility phenotype approximated a normal distribution for the base population of roosters. The population mean was 35% (SD = 16.4) mobile sperm. Semen donors were selected from 2 subpopulations: roosters with either below-average or high sperm mobility. These phenotypes were confirmed by nested ANOVA.

**A Posteriori Analysis**

The mobility of freshly ejaculated sperm from the high phenotype observed in Exp. 1, 2, and 3 was plotted as a function of rooster age. Parameters of $y(x) = \alpha + \beta(x)$ were estimated with the PROC GLM feature of SAS, respectively. The principle of conditional error outlined above was used to test the hypothesis that the predicted slope was equivalent to 0.
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below-average sperm. This difference was attributed to mitochondrial well-being at the onset of storage (Froman and Kirby, 2005), which in turn is affected by the interaction between sperm cell glycolytic ability (Froman et al., 2011) and duration of sperm passage through the excurrent ducts of the testis (Froman and Rhoads, 2013). In any event, the experimental outcome shown in Fig. 2 demonstrated that semen donor choice affected extender efficacy.

Experiment 2 tested the efficacy of BPSE with diluted semen as opposed to washed sperm from the high phenotype. Two distinct responses to storage were observed as shown in Fig. 2 and 3. Whereas the y-intercepts were consistent between experiments, a logistic decay was observed with a retention of only 17% mobile sperm after 24 h of storage. There are 2 interrelated explanations for these experimental outcomes. On one hand, the effective concentration of glucose differed between experiments. Whereas the glucose concentration in BPSE was 28 mM, the glucose concentration, on average, for washed and extended sperm was 18 vs. 12 mM at the start of Exp. 1 and 2, respectively. Therefore, glucose may have become limiting in the case of extended sperm. This explanation is consistent with the importance of the glycolytic pathway to fowl sperm survival (Sexton, 1974; Wishart, 1982, 1984; Froman et al., 2011). That said, this explanation does not account for the different patterns observed. Had the data set in Exp. 2 decayed exponentially from a y-intercept of 70% to an asymptote of 17% due to inadequate glucose, then exponential decay would have been observed in each experiment. However, this was not the case. Consequently, the best explanation for the different experimental outcomes may be the fact that centrifugation of sperm through 12% (wt/vol) Accudenz put sperm in a chemically defined environment that minimized cytotoxic edema (Liang et al., 2007). The ultrastructure of immobile sperm described by Froman and Kirby (2005), that is, swollen mitochondria with disorganized cristae, is consistent with cytotoxic edema, which occurs when energy-dependent cation efflux fails, ion influx follows, and water uptake is enabled by increased cellular osmolality. Consequently, the extent to which an anion is permeant or impermeant becomes critical in conditions promote cellular edema. This possibility was tested in Exp. 3.

Washed sperm were suspended in either TES-buffered saline or BPSE in which mannitol replaced glucose. Therefore, neither solution contained exogenous energy. In addition, sperm were incubated at 20°C rather than 10°C to increase metabolic rate. These 2 conditions used in combination constituted a stress test. The parameter τ within the logistic model predicts the time at which a half-maximal response is observed. These estimates were 2.9 and 4.9 h for TES-buffered saline and glucose-free BPSE, respectively. The F-statistic was 9.33 when the principle of conditional error was used to test whether these values were estimates of a common parameter. This hypothesis was rejected because F(0.01; 1, 27) was 7.68 (Sokal and Rohlf, 1969b). In summary, Exp. 3 demonstrated that loss of sperm function can be predicted in a stochastic manner for sperm in a chemically defined environment. Moreover, this experiment demonstrated an ameliorative effect of BPSE independent of exogenous energy. This effect is attributed to the fact that BPSE contains phosphate and glutamate as its principal anions rather than chloride.

Figure 2. Decrease in the mobility of washed sperm during storage at 10°C. Each symbol represents a mean ± SD. Open squares denote observations made with sperm categorized beforehand with below-average sperm mobility. In contrast, open circles denote observations made with sperm categorized by high sperm mobility. The solid line in each plot denotes a predicted function. In the case of highly mobile sperm, a gradual exponential decay was observed. In contrast, sperm mobility decreased in a linear manner in the case of the below-average sperm mobility. In each case, data sets were based on 5 replicate semen pools.

Figure 3. Decrease in the mobility of sperm diluted with Beltsville Poultry Semen Extender before storage at 10°C. Each symbol represents a mean ± SD. Open circles denote observations made with roosters categorized beforehand with high sperm mobility. Data approximated a logistic function. The solid line denotes the predicted function. This data set was based on 5 replicate semen pools.
Experiment 3 also served to test the premise that the sperm mobility assay can predict the status of sperm cell mitochondria in response to sperm manipulation. In this case, the sperm cell environment affected mitochondrial well-being as evidenced by the inability of sperm to respond to extracellular Ca\(^{2+}\) under physiological conditions (Froman, 2014). This conclusion is valid because fowl sperm propulsion, as measured by either the sperm mobility assay or computer-assisted sperm motion analysis, is absolutely dependent on mitochondrial Ca\(^{2+}\) cycling. This phenomenon serves to activate phospholipase A2 and thereby provide endogenous long-chain fatty acids for β-oxidation (Froman, 2003, 2013; Froman and Feltmann, 2005, 2010; Froman et al., 2006). In other words, when sperm mitochondria are functional, they then respond to the experimental conditions used. In contrast, this response was gradually lost as the stress test progressed (Fig. 4). The importance of this outcome can be illustrated with the following argument: when mitochondrial Ca\(^{2+}\) cycling occurs, mobile sperm will then be observed. Therefore, when immobile sperm are observed, it is logical to conclude that mitochondrial Ca\(^{2+}\) cycling has failed. In review, the experimental outcomes shown in Fig. 2, 3, and 4 served to reinforce the author’s assertion that the sperm mobility assay is a bioassay for sperm mitochondria.

The a posteriori analysis of sperm mobility was performed to reinforce the importance of sperm mobility phenotype, which was assigned in a preliminary experiment (Fig. 1). This categorization was neither contrived nor arbitrary because it was statistical and independent of time (\(P = 0.8119\)) over the course of subsequent experiments (Fig. 5). Moreover, phenotype connotes biological significance. This principle has been established by noncompetitive and competitive fertility trials (Froman et al., 1997, 1999, 2003; Birkhead et al., 1999; Pizzari et al., 2008). In short, sperm mobility phenotype matters. Based on the author’s experimental outcomes to date, one’s choice of semen donor, as defined by sperm mobility phenotype, may be more important to successful artificial sperm preservation than one’s choice of extender or storage conditions.

**LITERATURE CITED**


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