Transfer of Sperm into a Chemically Defined Environment by Centrifugation Through 12% (wt/vol) Accudenz®[1]

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ABSTRACT Centrifugation is commonly used to wash sperm; however, most washing techniques do not put sperm in a chemically defined environment. Rather, washing by centrifugation, in effect, dilutes seminal plasma components. A 0.5-mL volume of 30% (wt/vol) Accudenz® was layered beneath 5 mL of 12% (wt/vol) Accudenz® in a 15-mL polypropylene centrifuge tube. Diluted semen from individual males (n = 10) was overlaid upon the 12% (wt/vol) Accudenz®. After centrifugation at 1,250 × *g* at 4 C for 25 min, washed sperm were present at the interface of the Accudenz® layers. Based upon hemacytometer counts, sperm recovery was 83% (CV = 12%). Neither sperm viability nor morphology was affected by washing. Efficacy of the washing procedure was evaluated by using extracellular glucose, glutamic acid, Ca^{+2}, and protein as markers. Washing eliminated 99% of the glutamic acid and glucose associated with sperm. Likewise, washing removed 98.5% of the extracellular Ca^{+2} associated with sperm. As evidenced by total protein analysis and SDS-PAGE, washing removed 98% of soluble seminal plasma proteins from sperm. In addition, washing did not affect sperm mobility or fertilizing ability. This procedure returns extended sperm to a physiological concentration in a chemically defined environment. By suspending washed sperm in distinct media, we induced differential sperm mobility. Therefore, this procedure is suitable for the study of the effect of specific substances upon sperm cell function.

(*Key words*: chicken, sperm, Accudenz®, motility, centrifugation)

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INTRODUCTION

Sperm motility is essential for maximal fertility in the fowl (Bakst *et al.*, 1994). Fowl sperm motility is sensitive to factors in seminal plasma and synthetic diluents. For instance, fowl sperm are motile if suspended in a calcium-free medium at 30 C. However, if the temperature is increased to 40 C, sperm become immotile (Thomson and Wishart, 1988). Motility is restored at 40 C by 2 m*M* calcium, 10% seminal plasma, or 10% peritoneal fluid collected at ovulation (Ashizawa *et al.*, 1989). Likewise, sperm respiration is strongly influenced by intracellular calcium concentrations (Ashizawa *et al.*, 1992). Of the substances that reactivate fowl sperm motility, calcium's effect on motility has received the most attention (Thomson and Wishart, 1991; Ashizawa *et al.*, 1994). In fact, calcium was identified as one of two substances in seminal plasma that stimulated motility at 40 C (Ashizawa and Wishart, 1987).

Proteins and serum replacements may also affect sperm activity. Bakst and Cecil (1992a,b) evaluated turkey sperm motility after storage in diluents containing BSA and two commercially available serum replacements using computer-assisted semen analysis. The presence of the BSA and serum replacements significantly increased sperm motility (Bakst and Cecil, 1992a, b). In addition, Mohan *et al.* (1995) reported that fowl seminal plasma contains a 75-kDa sperm motility inhibiting factor (SMIF). Furthermore, a number of enzymes (Lake, 1984) and a proteinase inhibitor (Lessley and Brown, 1978) have been reported in fowl seminal plasma; however, the precise biological roles of these proteins are unknown.

Fowl semen contains significant concentrations of proteins, glutamic acid, and inorganic ions (Freeman, 1984). Transparent fluid adds glucose to the seminal plasma (Freeman, 1984). Assessment of sperm function in relation to exogenous nutrients, motility agonists, and antagonists is necessary for analyzing how sperm react with their environment. Traditionally, centrifugation of sperm has been used as a method for washing sperm free of substances present in seminal plasma; however,

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Abbreviation Key: SMIF = sperm motility inhibiting factor.

this approach merely dilutes the components of the medium in which sperm are suspended. Because of the biochemical complexity of seminal plasma and the potential for interactions between compounds, it is desirable to study sperm in a chemically defined environment. In previous research (Froman and Thursam, 1994), sperm were washed through 12% (wt/vol) Accudenz® in order to remove an exogenous enzyme. We hypothesized that this simple technique could also be used to wash sperm free of seminal plasma components and thereby provide a means to study sperm in a chemically defined environment.

MATERIALS AND METHODS

Experimental Animals

New Hampshire roosters (n = 10) were used as semen donors. All roosters were housed in individual cages, consumed feed and water *ad libitum*, and were 26 to 42 wk of age when used for the following experiments.

Sperm Washing Procedure

A 30% (wt/vol) stock solution of Accudenz®[3] was prepared using 3 m*M* KCl containing 5 m*M* TES, pH 7.4, as the solvent. A 50 m*M* TES solution, pH 7.4, containing 130 m*M* NaCl (hereafter referred to as TES buffer), was used to prepare the 12% (wt/vol) Accudenz® and as a semen diluent. The TES buffer had an average osmolality of 315 mmol/kg. The portion of the TES buffer used to prepare the 12% (wt/vol) Accudenz® was diluted with deionized water to an osmolality of 275 mmol/kg. The 30% (wt/vol) Accudenz® stock was diluted with dilute TES buffer (275 mmol/kg) to prepare the 12% (wt/vol) Accudenz® (315 mmol/kg).

Each experiment was performed with sperm from individual males. Sperm concentration was determined fluorometrically according to Bilgili and Renden (1984). Ejaculates were diluted to 2×10^9 sperm per milliliter with TES buffer. Five milliliters of the 12% (wt/vol) Accudenz® was placed in a 15-mL polypropylene centrifuge tube. A 0.5-mL volume of 30% (wt/vol) Accudenz® was placed beneath the 12% (wt/vol) Accudenz® with a Pasteur pipet. The sperm suspension, not exceeding 2.0 mL, was overlaid on the 12% (wt/vol) Accudenz® solution and screw cap secured. Tubes were centrifuged at $1,250 \times g$ at 4 C for 25 min. After centrifugation, fluid above and below the washed sperm was removed by aspiration.

Evaluation of Fertility

Fertilizing ability of sperm from individual roosters (n = 10) was determined before and after washing. Sperm concentration in each ejaculate was determined as above. A sample of the ejaculate was diluted to 2×10^9 sperm per milliliter with 50 m*M* TES, pH 7.4, containing 120 m*M* NaCl, 10 m*M* glucose, and 2 m*M* $CaCl_2$, hereafter referred to as motility buffer. Each sperm suspension was used to inseminate 10 hens with 75×10^6 sperm per hen. The remaining neat semen was diluted with TES buffer and washed as described above. Washed sperm were diluted to 2×10^9 sperm per milliliter with motility buffer. Ten hens per male were inseminated as described above. Eggs were collected throughout a 10-d interval and set twice. Fertility was determined by breaking eggs open after 4 d of incubation and then examining the contents for embryonic development. Fertility was analyzed as described by Kirby and Froman (1991).

Evaluation of Washing Procedure

Sperm viability (n = 10 males, 1 ejaculate per male) was determined before and after washing by ethidium bromide exclusion (Bilgili and Renden, 1984). Recovery of washed sperm (n = 10 males, 1 ejaculate per male) was determined by calculating the number of sperm overlaid on the 12% (wt/vol) Accudenz® and the number of sperm recovered postwash. Sperm concentration of undiluted semen was determined using a hemacytometer. Then each ejaculate was diluted to 2×10^9 sperm per milliliter with TES buffer and washed as described above. The sperm suspension volume was recorded for washed sperm and sperm concentration was determined with a hemacytometer.

Glucose concentration (n = 10 males, 1 ejaculate per male) was determined with an assay kit.[4] Semen was diluted to 2×10^9 sperm per milliliter with motility buffer. A 0.5-mL sample of the sperm suspension was centrifuged at $12,000 \times g$ for 5 min and 20 μL of the supernatant was assayed for glucose. The residual sperm suspension was washed as described above. Washed sperm were resuspended to 2×10^9 sperm per milliliter with TES buffer and then centrifuged at $12,000 \times g$ for 5 min. The supernatant was assayed for glucose. Unknown glucose concentrations were determined using a standard curve (r = 0.99) with standards ranging from 25 to 0.1 m*M* glucose.

Calcium concentrations (n = 10 males, 1 ejaculate per male) were measured spectrophotometrically with the indicator Arsenazo III[5] according to Gratzer and Beaven (1977). A 5-m*M* Arsenazo III solution was passed three times through a column of Dowex 50W and then filtered prior to use. Neat semen was diluted to 2×10^9 sperm per milliliter with motility buffer. A 0.5-mL sample of the sperm suspension was centrifuged at $12,000 \times g$ for 5 min. A 0.4-mL volume of the supernatant was assayed by adding 0.1 mL of filtered Arsenazo III and then measuring the absorbance at 654 nm. The remaining sperm suspen-

[3]Accurate Chemical and Scientific Corp., Westbury, NY 11590.
[4]Catalog no. 115-A, Sigma Chemical Co., St. Louis, MO 63178-9916.
[5]A 9676, Sigma Chemical Co., St. Louis, MO 63178-9916.

sion was washed as described above. Washed sperm were resuspended to 2×10^9 sperm per milliliter with TES buffer and centrifuged at $12{,}000 \times g$ for 5 min. The supernatant was assayed for calcium. Unknown calcium concentrations were determined using a standard curve (r = 0.99) with standards ranging from 10 m*M* to 0.1 μM calcium.

Concentrations of glutamic acid (n = 5 males, 1 ejaculate per male) were determined by amino acid analysis.[6] Prior to analysis, a 0.5-mL volume of undiluted semen was centrifuged at $12{,}000 \times g$ for 5 min, and the supernatant was frozen at –20 C. The remaining neat semen was diluted to 2×10^9 sperm per milliliter with TES buffer and washed as described above. The washed sperm were microcentrifuged and the supernatant frozen as above. Protein concentrations (n = 10 males, 1 ejaculate per male) were determined using the Bradford protein assay.[7] A 0.25-mL sample of undiluted semen was centrifuged at $12{,}000 \times g$ for 5 min, and the supernatant was assayed for total protein. The remaining undiluted semen was diluted to 2×10^9 sperm per milliliter with TES buffer and washed as described above. Washed sperm were microcentrifuged and the supernatant was assayed for total protein as described above. Total protein was also evaluated before and after washing using SDS-PAGE (Laemmli, 1970). Electrophoresis was performed in a vertical slab gel containing 6 to 20% (wt/vol) acrylamide.

Evaluation of Sperm Mobility

Sperm mobility was measured using the procedure of Froman and McLean (1996). An ejaculate from each of 10 males was diluted to 5×10^8 sperm per milliliter with motility buffer. A 60-μL volume of sperm suspension was layered upon 0.6 mL of 6% (wt/vol) Accudenz® solution prewarmed to 41 C in a disposable microcuvette. The 6% (wt/vol) Accudenz® solution was prepared by diluting the 30% (wt/vol) Accudenz® stock solution with motility buffer diluted to 290 mmol/kg. The cuvette was incubated for 5 min in a 41 C water bath. Absorbance was measured at 550 nm 1 min after the incubated cuvette was loaded into a spectrophotometer. The remaining neat semen was diluted to 2×10^9 sperm per milliliter with TES buffer and washed as described above. The mobility of washed sperm was measured as above, but using each of the following media for sperm resuspension: 1) isotonic buffered saline containing 2 m*M* Ca^{+2}, 2) isotonic buffered saline containing 2 m*M* Ca^{+2} and 10 m*M* glucose, and 3) isotonic buffered saline containing 2 m*M* Ca^{+2} and 3 m*M* cyanide (CN^-). Each medium was also used to prepare 6% (wt/vol) Accudenz®.

Statistical Analysis

Single classification ANOVA was used to analyze the glucose, glutamic acid, calcium, and protein reduction data sets. Pre- and postwash viability was analyzed by *t*-test.

TABLE 1. Sperm recovery and sperm viability ($\bar{x}$ ± SEM) before and after washing sperm through 12% (wt/vol) Accudenz®

Roosters	Prewash viability	Postwash viability	Recovery
(n)		(%)	
10	99.7 ± 0.05	99.5 ± 0.05	83.8 ± 4.0

RESULTS

Semen diluted to 2×10^9 sperm per milliliter can be layered on top of 12% (wt/vol) Accudenz® without mixing layers. When centrifuged at $1{,}250 \times g$, sperm pass through the 12% (wt/vol) Accudenz® whereas the solution the sperm were suspended in does not mix with the Accudenz® solution. Furthermore, sperm do not penetrate the 30% (wt/vol) Accudenz® solution underlying the 12% (wt/vol) Accudenz®. Therefore, when this procedure is performed as described in the Materials and Methods section, sperm form a band approximately 1 cm deep at the interface of the 12 and 30% (wt/vol) Accudenz® solutions. Likewise, a thin white band is present at the interface of the medium in which the sperm were suspended and the 12% (wt/vol) Accudenz® solution. Transmission electron microscopy (TEM) of the material from this band revealed a few intact sperm, cellular debris, and spermiophages (data not shown). Conversely, sperm recovered from the interface of the 12 and 30% (wt/vol) Accudenz® solutions had normal morphology, as evidenced by TEM and phase contrast microscopy (data not shown). Likewise, these sperm were viable, as evidenced by ethidium bromide exclusion (Table 1). As shown in Table 1, recovery of washed sperm was 83% (CV = 12%). As shown in Table 2, washing removed 99.8, 99.2, and 98.5% of extracellular glutamic acid, glucose, and Ca^{+2}, respectively. Likewise, washing removed 98.3% of extracellular protein (Table 2). The efficacy of the washing procedure was corroborated by SDS-PAGE (Figure 1).

The postwash sperm mobility was 97% of prewash mobility when the resuspending medium contained glucose (data not shown). As shown in Table 3, the

TABLE 2. Reduction of marker compounds ($\bar{x}$ ± SEM) after washing sperm through 12% (wt/vol) Accudenz®

Roosters	Component	Reduction
(n)		(%)
9	Glucose	99.2 ± 0.14
5	Glutamic acid	99.8 ± 0.05
10	Calcium	98.5 ± 0.13
9	Protein	98.3 ± 0.23

[6]AAA Laboratory, Mercer Island, WA 98040.
[7]500-0006, Bio-Rad, Hercules, CA 94547.

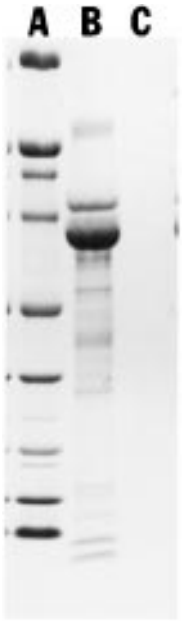

FIGURE 1. Polypeptides derived from chicken seminal plasma proteins following SDS-PAGE in a vertical slab gel containing a 6 to 20% (wt/vol) gradient of acrylamide. Polypeptides were stained with Coomassie blue. Lane A denotes molecular weight markers. Lane B shows proteins associated with unwashed sperm. After washing sperm through 12% (wt/vol) Accudenz®, the sperm suspension was centrifuged at 12,000 × *g* for 5 min and the supernatant used for electrophoresis. As demonstrated in Lane C, washing removed seminal plasma proteins.

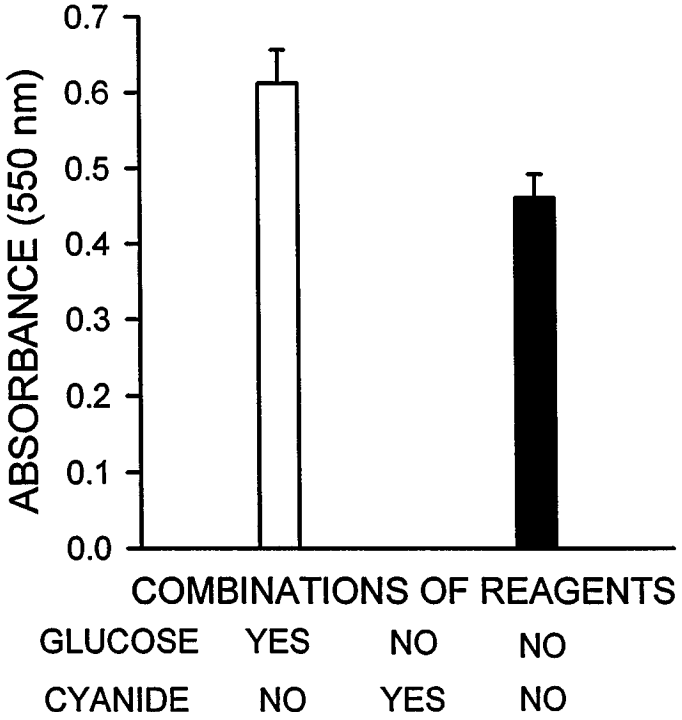

FIGURE 2. Sperm mobility as measured by a change in absorbance following an overlay of 6% (wt/vol) Accudenz® with sperm suspensions after centrifugation through 12% (wt/vol) Accudenz®. Each bar represents the mean and standard error of 10 replicate trials with semen from individual males. In each assay, pooled sperm were washed by centrifugation through 12% (wt/vol) Accudenz® prepared with buffered isotonic saline. This procedure washes sperm and returns them to a physiological concentration. Washed sperm were resuspended in each of three media. Each medium contained 2 m*M* Ca^{+2}. Media differed according to glucose and cyanide content. Combinations of reagents are shown along the X axis. Glucose and cyanide were used at 15 and 3 m*M*, respectively. Sperm were immotile in the glucose-free medium containing cyanide.

fertilizing ability of washed sperm did not differ from unwashed control sperm ($P \geq 0.05$; Table 3). Sperm mobility was 75% of controls when washed sperm were resuspended in glucose-free isotonic saline (Figure 2). In contrast, when washed sperm were resuspended in a glucose-free medium containing 3 m*M* cyanide, sperm were immotile (Figure 2).

DISCUSSION

Our primary objective was to determine whether we could obtain fully functional sperm in a chemically defined environment. In previous research, sperm were centrifuged through an Accudenz® solution in order to wash sperm free of an exogenous enzyme (Froman and Thursam, 1994). We hypothesized that this simple technique could also be used to wash sperm free of seminal plasma components so that the effect of specific compounds could be studied on sperm cell function. Attributes of washed sperm were evaluated *in vitro* and *in vivo*. The efficacy of washing was evaluated in terms of the extent sperm could be washed free of small and large molecular weight compounds.

Traditionally, centrifugation has been used as a method to wash sperm. However, this approach merely dilutes the components of the medium in which sperm are suspended. As alluded to above, Froman and Thursam (1994) prepared a sperm suspension containing exogenous neuraminidase, centrifuged the sperm through 12% (wt/vol) Accudenz®, and recovered washed sperm at a physiological concentration from the interface between the 12% and an underlying 30% (wt/vol) Accudenz® solution. Fertilizing ability was not affected and washed sperm were free of neuraminidase activity. Therefore, the washing procedure effectively removed an exogenous macromolecule from a population of sperm. However, we wanted to establish the efficacy of the procedure for compounds commonly found in extended semen.

TABLE 3. Fertilizing ability of sperm before and after washing through 12% (wt/vol) Accudenz®

Roosters	Treatment	Hens[1]	Eggs[2]	Fertility[3]
(n)		(n)		(%)
7	Nonwashed	70	393	91.3 ± 3.9
7	Washed	61	351	90.0 ± 3.3

[1]Each Single Comb White Leghorn hen was inseminated with a dose of 75 × 10^6 sperm in a volume of 75 μL.

[2]Collected over a 10-d interval after a single insemination.

[3]Each value is a mean ± SEM.

The efficacy of washing was tested using the following markers: glutamic acid, glucose, Ca^{+2}, and seminal plasma protein. Glutamic acid is the predominant free amino acid in fowl seminal plasma (Freeman, 1984). Due to the pK_a of the α-amino group, the α-carboxyl group and the side chain, this molecule is an organic anion at physiological pH. Glucose was used because it is present in semen (Freeman, 1984). Likewise, a hexose is commonly used as a nutrient in extenders. We chose the inorganic cation Ca^{+2} as the third marker because it is found in millimolar concentrations in seminal plasma and is a motility agonist at body temperature (Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987). Finally, we used seminal plasma proteins as macromolecular markers.

As evidenced by *in vitro* mobility at body temperature and the fertilizing ability of washed sperm *in vivo* (Table 2), the washing procedure did not impair sperm function. As shown in Table 2, the washing procedure reduced the presence of extracellular markers by an average of 99%. As evidenced by a CV of 0.7%, the efficacy of the washing procedure was independent of the chemical nature of the marker. In each case, the postwash concentration of the marker was negligible. Therefore, we concluded the washing procedure did indeed place sperm in a chemically defined environment.

Having shown it was possible to place functional sperm in a chemically defined environment, we conducted an experiment to demonstrate application of the method. We hypothesized that we could control sperm metabolism by controlling the extracellular environment. We chose sperm mobility as an endpoint. Fowl sperm metabolism is affected by glucose (Goldberg and Norman; 1961; Sexton, 1974; Wishart, 1982). Having considered that: 1) we could wash sperm free of glucose, 2) fowl sperm can utilize an endogenous substrate under aerobic conditions (Howarth, 1978, 1981), and 3) cyanide inhibits mitochondrial respiration, we formulated the following hypotheses. First, sperm would be motile in a glucose-free medium. Second, sperm would be immotile in a glucose-free medium containing cyanide. As shown in Figure 2, these hypothesized outcomes were observed. Therefore, profound differences in sperm function were experimentally induced by controlling the chemical composition of the spermatozoal environment. In conclusion, the procedure described herein represents a simple method to recover large numbers of viable fowl sperm in a chemically defined environment. We propose that it can be applied to the study of such phenomena as sperm metabolism, motility, and the acrosome reaction.

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