Induction of Bovine Sperm Capacitation by TEST-Yolk Semen Extender[1]

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ABSTRACT

Ejaculated bull semen was diluted 1: 10 in the TEST-yolk buffer, cooled slowly to 4°C, and stored for up to 48 h. Aliquots were taken at 0, 4, 8, 16, 24, and 48 h and washed once or three times in bovine serum albumin-saline and the sperm pellets resuspended in this saline. Fertilization of zona-free hamster oocytes was used to assess sperm capacitation. Motility differed between samples washed once or three times (53.7 vs. 21.7%). Motility was highest at 4 h storage but did not differ between 16, 24, or 48 h of storage. More sperm without intact acrosomes were found at 4 h than at 0 h, but the percentage did not change further until after 24 h. Penetration of oocytes was not different between sperm washed once or three times (28.5 vs. 26.9%). No penetration occurred at 0 h, and highest penetration rates occurred at 4 and 8 h of storage (32.1 and 33.4%). Penetration rates at 16, 24, and 48 h were not different (25.3, 25.2, 22.5%). In conclusion, storage of bull sperm in TEST-yolk buffer for 4 to 48 h resulted in capacitation. Even though capacitation was induced by 4 h, at least 71% of the sperm population had not undergone an acrosome reaction by 48 h of storage. This may explain why penetrability was maintained over this period.

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INTRODUCTION

Mammalian spermatozoa undergo a series of biochemical and biophysical changes, collectively referred to as "capacitation", before they can fertilize ova. This confers on the spermatozoa the ability to undergo the acrosome reaction and subsequently fuse with egg oolemma (2). One of the major problems of in vitro fertilization in large domestic animals is failure to achieve in vitro capacitation of sperm. Since capacitation was first reported (1, 12), a number of techniques have been used to evaluate the capacitation status of sperm. Systems developed have been based on bioassay (13, 43), fluorescent labeling of sperm surface antigens (10, 25), lectin-induced sperm agglutination (37), and radiolabeling of sperm surface proteins (32).

The discovery that heterologous sperm could penetrate the zona-free hamster oocyte (ZFHO) (42, 44) made it possible to use an in vitro penetration assay as a test for capacitation and fertilizing potential. The ZFHO permit entry of sperm from a wide variety of species, provided the sperm are capacitated and acrosome reacted (43). Capacitation is assumed to have taken place in the sperm penetration assay if the spermatozoa undergo nuclear decondensation following entry into the ooplasm (43). Lorton and First (30) demonstrated that bull sperm capacitated in vitro could penetrate the ZFHO. Since then a number of systems have been used to capacitate bull sperm in vitro with varying degrees of success (6, 7, 8, 18, 19, 22, 41).

Likewise, storage of human sperm at 4 and 5°C for at least 18 h in TEST-yolk extender preserved sperm motility (24, 46) and increased sperm penetration into ZFHO (5, 11, 26). Bovine semen extended with TEST-yolk extender and stored 24 to 48 h at 4°C can fertilize bovine oocytes (20), signifying that capacitation had occurred during storage.

The objectives of the present study were to

determine if TEST-yolk semen extender can 1) maintain bovine sperm motility for at least 48 h at 4°C; 2) maintain bovine acrosome integrity for at least 48 h at 4°C; and 3) induce capacitation of bovine sperm.

MATERIALS AND METHODS

TEST-Yolk Buffer Preparation

The buffer was prepared as described by Graham et al. (17). Briefly, Tes (Sigma Chemical Co., St. Louis, MO) 22.35 g and Tris (Sigma) 5.91 g were dissolved in 300 and 150 ml of twice glass-distilled water, respectively. Tes was titrated against Tris to pH 7.00. Chicken egg yolk (20%, vol/vol) was added, the mixture centrifuged (12,000 × *g*; 10 min), and the supernatant fluid used for semen extension. The final pH and osmotic pressure of the TEST-yolk buffer were 7.0 and 320 mOsmol/kg. The TEST-yolk buffer was stored for up to 2 mo at –20°C until used.

Semen Collection and Preparation of Sperm Suspensions

Bovine ejaculated semen was collected biweekly by artificial vagina from two fertile, normal Holstein bulls. Semen was transported to the laboratory in a 37°C insulated container within 5 to 10 min of collection. Motility was subjectively scored microscopically and the sperm concentration was determined by hemocytometer counts. Semen (1 ml) was diluted 1:10 in 37°C TEST-yolk buffer in a 15 ml plastic conical centrifuge (Corning #25310). The tube was placed inside a 180 ml glass beaker (Pyrex) containing 120 ml of water (37°C). The water filled beaker was then placed inside a 4°C room and cooled to 4°C in 110 to 130 min. The time when 4°C was reached was designated time 0. The semen was stored at 4°C for up to 48 h.

At 0, 4, 8, 16, 24, and 48 h, two 1-ml aliquots of extended semen were pipetted into two 15-ml plastic conical centrifuge tubes and washed in 5 ml of pH 7.2, .15 *M* NaCl containing .1% bovine serum albumin, Fraction V (BSA-saline) (37°C). One aliquot was washed once (1×) and the other three times (3×) by centrifuging it for 5 min at 1000 × *g* and discarding the last supernatant. The final resuspension of both aliquots was in BSA-saline (37°C) to a concentration of 2×10^6 sperm/ml.

Measurements of Motility and Acrosome Reaction

Samples (10 µl) of the final sperm suspension were evaluated microscopically for progressive motility on a warm slide (37°C) using phase at 400×. Three observations were made each time. All estimates were made by the same observer.

Slides for acrosome assessment were prepared by smearing 10 µl of sperm on a glass slide and drying them for 48 h. To examine acrosome status, the procedure of Bryan and Akruk (9) as modified by Lenz et al. (29) was used. This procedure employs a sequential naphthol yellow S and erythrosin B stain. Sperm so prepared were examined using phase microscopy and 200 sperm per slide were classified as having intact acrosomes or as being acrosome reacted. Intact acrosomes were bright cherry red with a densely stained apical ridge, whereas reacted acrosomes were uncolored or dull grey. This procedure can not identify a "true acrosome reaction" from a "false acrosome reaction". However, it can distinguish sperm with intact acrosomes from those without acrosomes.

Measurement of Sperm Capacitation with Zona-Free Hamster Oocytes

Zona-free hamster oocytes were obtained by standard methods for superovulation, ovum recovery, and removal of zona pellucida (44). Female golden hamsters were superovulated with 40 IU pregnant mare serum gonadotropin (Sigma) followed 55 h later by 40 IU human chorionic gonadotropin (Sigma). Hamsters were sacrificed 18 to 20 h post-hCG injection, and ovaries and oviducts were transferred to a 60 × 15-mm culture dish containing 5 ml, pH 8.5, Ca^{++}-free Tyrode's medium (CFT). The oviducts were opened and the cumulus mass removed. The ova were freed from the surrounding cumulus cells by treating them for 7 to 10 min at 37°C with .1% (wt/vol) hyaluronidase (Sigma) in CFT then treated for 2 or 3 min at 37°C with .03% (wt/vol) trypsin (Sigma) in CFT to remove the zona pellucida.

Droplets (50 µl) of sperm suspended in BSA-saline, pH 7.2, were placed in a 60- ×

15-mm culture dish beneath a layer of liquid paraffin. The ZFHO were added to the droplets (10 oocytes per drop) and incubated for 3 h in a 37°C, 5% CO_2 humid environment.

Evaluation of the oocytes for penetration was at completion of coincubation. Oocytes were transferred from fertilization droplets to a glass microscope slide and overlaid with a #1 glass coverslip supported at each corner with dots of vaseline-paraffin mixture (1:1). The coverslip was gently pressed down until oocytes expanded under pressure to allow better observation of pronuclei. Oocytes were considered penetrated if a swollen sperm head or two or more pronuclei were visible within the oocyte vitellus.

Capacitation Controls

The effect of time and BSA-saline on bovine sperm motility and their ability to penetrate ZFHO (capacitation) was determined as follows: 1) Undiluted semen (2 ml) was incubated for 4 h at 37°C in a culture dish (10 × 35 mm). After 4 h of incubation, a .5-ml aliquot of semen was taken and washed 3× in 5 ml of BSA-saline, pH 7.2. 2) Undiluted semen (.5 ml) plus 1.5 ml BSA-saline were incubated for 4 h at 37°C in a culture dish. After 4 h, it was centrifuged and washed 3× in 5 ml of BSA-saline. 3) Undiluted semen (.5 ml) was washed 3× in BSA-saline (5 ml each time). After the third wash, the sperm pellet was resuspended in 2 ml BSA- saline and incubated for 4 h at 4 or 37°C. 4) Undiluted semen (.5 ml) was diluted 1:10 in BSA-saline and cooled slowly to 4°C and stored for 4 h. After 4 h, it was centrifuged and washed 1× in 5 ml of BSA-saline.

Experimental Design

Two 1-ml aliquots were taken from the TEST-yolk extended semen at 4°C at 0, 4, 8, 16, 24, and 48 h and washed in 5 ml of 37°C, pH 7.2 BSA-saline. One aliquot was washed 1× and the other 3× (1000 × *g*; 5 min). Each sperm pellet was resuspended in pH 7.2 BSA-saline (37°C) at 2 × 10^6 sperm/ml and analyzed for motility, acrosome integrity, and capacitation (ability to penetrate ZFHO).

Statistical Analysis

Analysis of variance was performed on motility, acrosome reaction, and penetration data employing split, split-plot design (35). The analysis of variance was based on the model:

$$Y_{ijkl} = \mu + R_i + B_j + e_{i(j)} + T_k + (BT)_{jk} + e_{ijk} + W_l + (BW)_{jl} + (BTW)_{jkl} + e_{ijkl}$$

where:

μ = overall mean,
R_i = replicate effect,
B_j = bull effect,
T_k = time effect,
W_l = wash effect,
$e_{i(j)}$ = whole plot error (Error 1),
e_{ijk} = split plot error (Error 2),
e_{ijkl} = split-split plot error (Error 3),
$(BT)_{jk}$ = bull × time interaction,
$(BW)_{jl}$ = bull × wash interaction, and
$(BTW)_{jkl}$ = bull × time × wash interaction.

Means from analysis that were different at $P<.05$ were tested using Duncan's multiple range test (16). Ejaculates (or days) were considered as replicates and were treated as random variables; treatments were considered fixed. Means transformed to arcsins were tested by the honestly significant difference test (35).

TABLE 1. Effect of sperm storage time in TEST-yolk at 4°C and one or three bovine serum albumin (BSA)-saline washes on bull sperm motility.

	% Motility		
Storage	Before washing in BSA-saline	One wash	Three washes
(h)			
0	75.0	60.6	22.5
4	68.1	60.6	31.3^{a}
8	65.0	56.3	20.6
16	63.1	51.9^{a}	17.5
24	60.0	48.1^{a}	20.0
48	58.1^{a}	44.4^{b}	18.1
Average	64.9^{A}	53.7^{B}	21.7^{C}

^{a,b,c}Superscripts denote differences within column ($P<.05$).

^{A,B,C}Superscripts denote differences between columns ($P<.05$).

TABLE 2. Analyses of variance for the effect of bull, wash procedure, and length of incubation time on sperm motility in TEST-yolk buffer at 4°C.

Factor	df	SS	MS	F-Value
Replicate	3	242.5	80.8	1.36
Bull	1	250.3	250.3	4.22
Error 1	3	177.9	59.3	
Time	5	2355.5	471.1	26.84**
Bull × time	5	332.6	66.5	3.83
Error 2	30	526.6	17.6	
Wash	1	24,544.0	24,544.0	1183.01**
Bull × wash	1	.3	.3	.01
Bull × time × wash	10	521.4	52.2	1.51*
Error 3	36	746.9	20.7	
Total	95			
	Avg.	Within-cell SD		
Bull				
#36	39.3	17.92		
#1073	36.0	17.47		
Wash				
1	53.6[a]	8.03		
3	21.7[b]	6.71		
Time, h				
0	41.6[b]	20.22		
4	45.9[a]	16.55		
8	38.4[b]	18.86		
16	34.7[c]	18.30		
24	34.1[c]	15.51		
48	31.3[c]	14.43		

[a,b,c]Superscripts denote differences within column ($P<.05$).

*Significant ($P<.05$).

**Highly significant ($P<.01$).

TABLE 3. Effect of sperm storage length in TEST-yolk at 4°C and one or three bovine serum albumin-saline washes on bull sperm acrosomal integrity.

	% Acrosome-reacted	
Storage	One wash	Three washes
(h)		
0	8.8[a]	9.8[a]
4	17.1[b]	17.0[b]
8	15.4[b]	18.0[b]
16	16.3[b]	20.1[b]
24	16.0[b]	19.3[b]
48	25.6[c]	29.0[c]
Average	16.5[A]	18.9[B]

[a,b,c]Superscripts denote differences within column ($P<.05$).

[A,B]Superscripts denote differences between columns($P<.05$).

RESULTS

Motility

Effects of length of incubation time in TEST-yolk buffer and number of washes on bovine sperm motility are presented in Table 1 and the results of the ANOVA in Table 2. No difference ($P>.05$) in sperm motility was observed between the bulls (39.3 vs. 36.0%). Motility was higher ($P<.01$) before washing (64.9%; Table 1) in BSA-saline. However, difference ($P<.01$) in motility was great between one wash and three washes (53.7 vs. 21.7%) throughout the incubation (Table 1). All the incubations showed some head-to-head agglutination following three washings in BSA-saline, usually in the form of two or three spermatozoa adhering together. After three washes in BSA-saline, sperm motility was highest ($P<.05$) at 4 h and then it started dropping. However, there was no difference ($P>.05$) in motility among 16, 24, or 48 h of incubation.

Acrosome Status

Effects of bull, length of incubation in TEST-yolk buffer, and number of washes on bovine sperm integrity are presented in Tables 3 and 4. There was no difference ($P>.05$) in the number of acrosome-reacted sperm between the bulls (17.0 vs. 18.4%). However, a significant difference ($P<.05$) was observed in the number of acrosome-reacted sperm between one wash and three washes (Table 3). The latter produced higher numbers of acrosome-reacted sperm over time. There was an increase ($P<.05$) in the number of acrosome-reacted sperm by 4 h of incubation (Table 3). No difference ($P>.05$) was observed among 4, 8, 16 and 24 h of incubation, but a significant ($P<.05$) increase in the number of acrosome-reacted sperm was noted at 48 h of incubation (Table 3). However, even at 48 h, only 25 to 29% of the sperm population had undergone an acrosome reaction.

Penetration of Zona-Free Hamster Oocytes

Bull, length of incubation in TEST-yolk buffer, and number of washes effects on bovine sperm fertilizability are presented in Tables 5 and 6. After 4 h of storage in TEST-yolk at 4°C, bovine sperm became capacitated and acquired the ability to fertilize ZFHO (Table 5).

TABLE 4. Analyses of variance for the effect of bull, wash procedure, and length of incubation time on sperm acrosome reaction in TEST-yolk buffer at 4°C.

Factor	df	SS	MS	F Value
Replicate	3	249.4	83.1	1.44
Bull	1	48.2	48.2	.84
Error 1	3	172.8	57.6	
Time	5	2647.6	529.5	16.67**
Bull × time	5	271.8	54.4	1.71
Error 2	30	951.9	31.7	
Wash	1	130.7	130.7	7.31*
Bull × wash	1	0	0	.00
Bull × time × wash	10	86.8	8.7	.49*
Error 3	36	843.5	17.9	
Total	95			
	Avg.	Within cell SD		
Bull				
#36	17.0	7.65		
#1073	18.4	7.15		
Wash				
1	16.5[a]	7.11		
3	18.9[b]	7.56		
Time, h				
0	9.3[b]	3.75		
4	17.1[b]	2.93		
8	16.7[b]	4.77		
16	18.2[b]	6.16		
24	17.7[b]	5.07		
48	27.3[c]	7.82		

[a,b,c]Superscripts denote differences within column ($P<.05$).

*Significant ($P<.05$).

**Highly significant ($P<.01$).

TABLE 5. Effect of sperm storage length in TEST-yolk at 4°C and one or three bovine serum albumin-saline washes on ability to penetrate zona-free hamster oocytes.[1]

Storage	Penetration			
	One wash		Three washes	
(h)	(Penetrated/ total)	(%)	(Penetrated/ total)	(%)[2]
0	0/121	0[a]	0/124	0[a]
4	32/99	32.6[b]	32/105	31.6[b]
8	64/168	37.5[b]	46/144	29.2[b]
16	27/108	26.4[c]	44/184	24.3[c]
24	28/122	23.7[c]	48/194	26.6[c]
48	28/128	22.5[c]	30/135	22.6[c]
Average		28.5		26.9

[a,b,c]Superscripts denote differences within column at ($P<.05$).

[1]Eight replicates for each time except 0 h had 6 replicates.

[2]Values differ slightly from arithmetic mean as average of replicates from ANOVA.

No difference ($P>.05$) in penetration rates was observed between the bulls (29.1 vs. 25.2%; Table 6) or between one wash and three washes (28.5 vs. 26.9%; Table 5). Highest ($P<.05$) penetration rates were observed at 4 and 8 h, 32.1 and 33.4%, respectively (Table 6). No difference in penetration rate was observed with sperm incubated 16, 24, or 48 h at 4°C in TEST-yolk. Data eliminating parthenogenetic activation of eggs and the BSA-saline washes as causing pronuclei formation are presented in Table 7.

DISCUSSION

Motility

Bovine sperm, stored in TEST-yolk buffer at 4°C possessed motility, acrosomal integrity, and fertilizing ability for at least 48 h. The results of this experiment also demonstrate that centrifugation and washing 1× or 3× to remove extender depressed sperm motility. Similar results have been obtained in the bovine (4, 33), human (31, 39) and ovine (14, 40). The drastic decrease in the motility of sperm that had been washed 3× may have been due to intracellular damage or leakage of vital components necessary for motility as the result of centrifugation (27, 31, 36). However, even though the 3× washed sperm motility at 4 h was half that of the 1× sperm (31 vs. 61%), their acrosomal integrity and ability to penetrate ZFHO were identical. This suggests that most of the immotile sperm were not dead.

Acrosome Reaction

The number of sperm within any given sperm population that undergo a "true acrosome reaction" is asynchronous over time (2). Sperm have a limited life span following the "true acrosome reaction", and the time of ovulation in respect to sperm deposition is variable in all species (2). Under the TEST-yolk storage conditions, however, the acrosomal integrity was conserved over time as only 15 to 20% of the population were acrosome reacted (either a true or false reaction) after 24 h of storage (Table 3). Johnson et al. (26) hypothesized that incubation of human sperm in TEST-yolk buffer at 4°C would permit capacitation but

TABLE 6. Analyses of variance table for the effect of bull, wash procedure, and length of incubation time on sperm penetrability in TEST-yolk buffer at 4°C.

Factor	df	SS	MS	F-Value
Replicate	3	1465.9	488.6	7.91
Bull	1	169.4	109.4	2.74
Error 1	3	185.4	61.8	
Time	5	1446.8	361.7	4.37**
Bull × time	5	409.3	102.3	1.24
Error 2	30	1985.9	82.7	
Wash	1	55.3	55.3	1.72
Bull × wash	1	272.0	68.0	2.12
Bull × time × wash	10	197.3	39.5	1.23*
Error 3	36	964.5	32.2	
Total	95			
	Avg.	Within-cell SD		
Bull				
#36	29.1	8.81		
#1073	25.2	10.06		
Wash				
1	28.5	10.23		
3	26.9	8.78		
Time				
0	0[a]	0		
4	32.1[b]	8.62		
8	33.4[b]	12.47		
16	25.3[c]	7.49		
24	25.3[c]	8.08		
48	22.5[c]	5.35		

[a,b,c]Superscripts denote differences within column ($P<.05$).

*Significant ($P<.05$).

**Highly significant ($P<.01$).

would not allow sperm to undergo the acrosome reaction. As shown in Table 3, the low number of sperm that had undergone an acrosome reaction even after 48 h of storage, support that hypothesis.

Penetration of Zona-Free Hamster Oocytes

The penetration of ZFHO by sperm has been used routinely to predict male "fertility potential" and as a research tool for studying fertilization (6, 18, 38, 42, 43, 44). Sperm capacitation and the acrosome reaction are required for the penetration of ZFHO (38, 43).

In human fertility clinics, TEST-yolk buffer without glycerol and antibiotics was first used for short-term storage of human sperm at 2 to 5°C (24). Motility of human sperm was preserved for up to 72 h (24, 46) similar to what occurs with bull sperm (17). Bolanos et al. (5) found that human sperm, stored in TEST-yolk buffer at 4°C for 48 h, resulted in more ($P<.05$) ZFHO being penetrated than paired samples stored in Biggers, Whitten, and Whittingham medium. Johnson et al. (26) reported that human sperm, stored in TEST-yolk buffer at 4°C for 42 h, subsequently penetrated more ZFHO and produced more penetrations per ova than sperm stored for 18 h. The TEST-yolk buffer appeared to preserve sperm motility over prolonged periods during which increased capacitation of the total sperm population was achieved (26). Chan et al. (11) evaluated the effect of 24-h storage of sperm at 4°C in TEST-yolk buffer on penetration of ZFHO using semen samples from patients attending an infertility clinic. Penetration rates of samples improved significantly after storage at 4°C for 24 h in TEST-yolk buffer.

Hensleigh and Hunter (20) achieved in vitro fertilization of bovine cumulus-enclosed oocytes with bull sperm that had been stored 24 to 48 h at 4°C in TEST-yolk buffer. All of these studies required that capacitation had occurred for penetration of oocytes.

The present study confirms that storage of bull sperm at 4°C in TEST-yolk buffer results in capacitation. The data extend penetration findings with human sperm to the bovine. The data show that bovine sperm behave differently than human sperm in TEST-yolk buffer. Whereas human sperm penetrability of ZFHO increased significantly with storage (26), bovine sperm penetrability peaked at 8 h and decreased slightly out to 48 h (Table 5).

Under in vitro conditions, bovine sperm require 3 to 4 h to capacitate (23) and human sperm also require 3 to 5 h (3). Preincubation of bovine sperm in TEST-yolk buffer at 4°C for 4 h resulted in fertilization (Table 5), thus confirming the short time needed for in vitro capacitation of bull sperm

Capacitation involves sperm plasma membrane alterations in preparation for the acrosome reaction (28). Capacitation begins with the removal of seminal coating proteins, acquired by the sperm cell during epididymal transit or absorbed from seminal plasma at the time of ejaculation (21, 25, 45). These seminal plasma proteins (decapacitation factors) prevent capacitation from being initiated (13, 15, 21,

TABLE 7. Effect of sperm storage time in bovine serum albumin (BSA)-saline on sperm motility and ability to penetrate zona-free hamster oocytes.

Treatment	% Motility		Penetration[1]	
	0 h	4 h	0 h	4 h
Undiluted semen 37°C, 4 h, then washed 3× in BSA-saline	82.5	7.5	1/20	0/38
Semen diluted 1:3 with BSA-saline 37°C, 4 h, then washed 3× in BSA-saline	82.5	57.5	0/20	0/39
Semen immediately washed 3× in BSA-saline 37°C, 4 h	52.5	10	2/20	0/38
Semen diluted 1:10 with BSA-saline 4°C, 4 h, then washed 1× in BSA-saline	30	0	0/7	0/37

[1]Number penetrated/total number.

34). The molecular changes associated with capacitation are not well-defined (28). The active component or factor in the TEST-yolk buffer capacitating system could be Tes, Tris, egg yolk, pH, or temperature. The data in Table 7 eliminate BSA-saline wash as causing capacitation. Preliminary findings suggest that the capacitation activity resides in the Good's buffers Tris and Tes (22).

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