Identification of the Capacitating Agent for Bovine Sperm in Egg Yolk-TEST Semen Extender[1]

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ABSTRACT

Bovine sperm can be capacitated in egg yolk-TEST buffer. To determine what constituent of the buffer was responsible, ejaculated semen was diluted 1:10 at 37°C with the following 20% egg yolk (vol/vol)-containing buffers: TES-Tris, TES-tetramethylammonium hydroxide, taurine-Tris, citric acid-Tris, citrate, egg yolk salts, egg yolk proteins Tris, and citrate-taurine. Buffers were pH 7.6 and 321 to 325 mOsmol/kg. Extended semen was cooled slowly to 4°C and stored 8 h. Sperm taken at 0 and 8 h were washed in pH 7.6 bovine serum albumin-saline and assessed for motility and capacitation using zona-free hamster eggs. Sperm motilities at 0 and 8 h were similar (60 to 73%) in all extenders except citric acid-Tris (54%) and egg yolk proteins Tris (15%). Bull sperm, stored 8 h in egg yolk-TEST, became capacitated. Because sperm storage in egg yolk-citrate did not result in penetration, both egg yolk and citrate were ruled out as capacitating agents. Capacitating activity resided in the TES and Tris molecules. The TES molecule contains a Tris component and this capacitated bull sperm. The TES molecule also contains a taurine component. However, taurine was not a capacitating agent for bull sperm. In conclusion, both TES- and Tris-containing buffers, alone or together (TEST), were equally effective in capacitating bull sperm.

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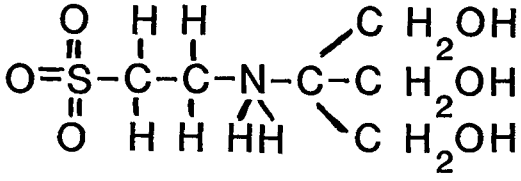
INTRODUCTION

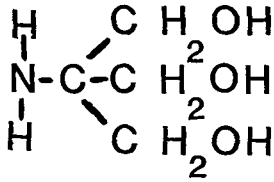
Most recent systems developed to capacitate bull sperm involve preincubation of sperm for a time at 37 or 39°C (13, 20, 24). However, egg yolk-TEST buffer (EY-TEST) has allowed human sperm to be stored at 4°C for up to 48 h without glycerol or antibiotics for human fertility studies (17, 30). During this 4°C storage, the human sperm became capacitated (3, 5, 19). Ijaz and Hunter (16) reported similar findings for cattle.

The buffer EY-TEST is composed of EY and two Good's buffers, namely N-Tris [hydroxymethyl] methyl -2 -aminomethane-sulphonic acid, abbreviated TES, and Tris [hydroxylmethyl]aminomethane, abbreviated Tris. Good et al. (12) developed a series of zwitterion buffers between pK 6 and pK 8. The buffers with the low salt concentrations, TES and Tris, were the buffers of choice for bull semen storage (14). Among these, EY-TEST was more effective in preserving sperm motility at 4°C for 48 h and at −196°C for longer periods. Storage of human spermatozoa at 4°C for at least 18 h in EY-TEST buffer preserved sperm motility (17, 30) and increased sperm penetration into zona-free hamster oocytes (ZFHO) (3, 5, 19). Sperm capacitation and the acrosome reaction are required for the penetration of ZFHO (27, 28). The penetration of ZFHO by human spermatozoa capacitated by EY-TEST buffer has been used in many laboratories to predict male " fertility potentials" and as a research tool for investigating the mechanisms of fertilization and investigating chromosomal anomalies associated with the male gamete (4, 19).

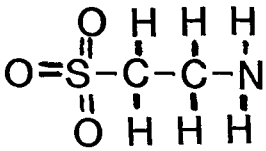
Previous work (16) from this laboratory demonstrated that storage of bovine sperm in EY-TEST buffer at 4°C preserved sperm motility, acrosome integrity, and fertilizing ability for at least 48 h. Moreover, EY-TEST buffer induced capacitation as early as 4 h, and the low percentage of acrosome-reacted sperm in



TES



Tris



Taurine

Figure 1. Structure of the zwitterions TES and Tris and the amino acid taurine used for the egg yolk-TEST buffer.

this population allowed these capacitated cells to retain penetrability for at least 48 h (16).

The EY-TEST buffer consists of 1) chicken egg yolk (EY), 2) TES (a molecule combining Tris and taurine) and 3) Tris. See Figure 1 for structure. The purpose of these investigations was to determine what chemical entity or entities in the EY-TEST buffer possessed the ability to capacitate bovine-ejaculated sperm.

MATERIALS AND METHODS

Preparation of Buffers

Egg Yolk-TES-Tris. For EY-TEST, (N-Tris [hydroxymethyl]methyl-2-aminomethane-sulphonic acid) (Sigma Chemicals, St. Louis, MO) (22.35 g in 300 ml H_2O, 327 mOsmol/kg was titrated against Tris (hydroxymethyl) aminomethane (Tris; 7.8 g in 200 ml H_2O, 325 mOsmol/kg) to pH 7.6. Chicken EY (20% vol/vol) was added, the mixture centrifuged (12,000 × *g*; 10 min), and the supernatant fluid used for semen extension. Final osmotic pressure was 325 mOsmol/kg.

Egg Yolk-TES-Tetramethylammonium Hydroxide. For EY-TES-TETRA, TES (22.35 g in 300 ml H_2O) (Sigma) was titrated against tetramethylammonium hydroxide penthydrate (TETRA; 2.9 g in 100 ml H_2O, 325 mOsmol/kg) to pH 7.6. Egg yolk was added and processed as described. Final osmotic pressure was 322 mOsmol/kg.

Egg Yolk-Taurine-Tris. For EY-TauT, taurine (4.1 g in 100 ml H_2O, 324 mOsmol/kg) (ICN Nutritional Biochemicals, Cleveland, OH) was titrated against Tris (7.8 g in 200 ml) to pH 7.6. Egg yolk was added and processed as described. Final osmotic pressure was 321 mOsmol/kg.

Egg Yolk-Citric Acid-Tris. For EY-CitT, citric acid (5.7 g in 100 ml H_2O, 325 mOsmol/kg, pH 2.2) (Fisher Scientific Co., St. Louis, MO) was titrated against Tris (7.8 g in 100 ml H_2O) to pH 7.6. Egg yolk was added and processed as described. Final osmotic pressure was 323 mOsmol/kg.

Egg Yolk-Citrate. Trisodium citrate dihydrate (3.185 g in 100 ml H_2O, 321 mOsmol/kg) (Mallinckrodt, St. Louis, MO) was adjusted to pH 7.6 with 2 N NaOH. Egg yolk was added and processed as described. Final osmotic pressure was 324 mOsmol/kg.

Egg Yolk-Egg Yolk "Salts". The EY (250 ml) was dialyzed in cellulose dialysis tubing (Spectra/Por No. 132697, Spectrum Medical Industries, Inc., Los Angeles, CA) against 1 L H_2O at 5°C for 24 h to remove molecules less than 12,000 to 14,000 MW. The dialysate (700 ml, 55 mOsmol/kg, pH 8.0) was lyophilized for 24 h to concentrate EY salts (70 ml, 601 mOsmol/kg). The concentrated dialysate was adjusted to 325 mOsmol/kg with distilled H_2O and adjusted to pH 7.6 with 2 N NaOH. To the EY "salts" solution was added EY (20% vol/vol). The pH was 6.8 and was adjusted to pH 7.6 with 2 N NaOH. The suspension was then processed as described.

EY Protein-Tris. The EY retentate (proteins minus "salts") was dialyzed against 1 L of Tris (3.9 g in 100 ml H_2O) for 24 h and lyophilized

TABLE 1. Motility and capacitation status of bovine ejaculated sperm[1] after storage at 4°C in egg yolk-TEST extender.

Time stored at 4°C	% Motility		Penetration rate after storage	
	Before washing	After washing		
(h)			(no.)[2]	(%)
0	70.0^{A}	66.7^{B,a}	1/63	1.6^{a}
2	68.3^{A}	63.3^{B,a}	0/69	0^{a}
4	68.3^{A}	63.3^{B,a}	27/86	31.4^{b}
6	70.0^{A}	63.3^{B,a}	36/118	30.5^{b}
8	64.0^{A}	54.4^{B,b}	20/55	36.4^{b}

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

^{A,B}Denote differences within rows ($P<.05$). [1]Three replicates per treatment.

[2]Number of eggs penetrated/total number of eggs.

for 24 h to concentrate EY "proteins". This protein concentrate (25 ml) was added to 75 ml of Tris (325 mOsmol/kg) to produce the EY protein-Tris extender (pH 7.85, 325 mOsmol/kg). The buffer was centrifuged as described earlier.

Egg Yolk-Citrate-Taurine. For EY-Cit-Tau, trisodium citrate dihydrate (3.185 g in 100 ml H_2O) was mixed (1:1) with taurine (4.1 g in 100 ml H_2O). The pH was adjusted to 7.6 with 2 N NaOH. Egg yolk was added and processed as described. Final osmotic pressure was 321 mOsmol/kg. All buffers were aliquoted in borosilicate disposable tubes and were stored frozen at –20°C until used.

Semen Collection and Sperm Preparation

Ejaculated semen was obtained by artificial vagina from two mature bulls (Holstein; 2 to 3 yr). Ejaculates were maintained at 37°C and returned to the laboratory 5 to 10 min after collection. Motility was scored microscopically on a warm (37°C) slide. Semen was diluted 1:10 in each of these above buffers (37°C), cooled slowly to 4°C (approximately 2.5 h; 0 h sample), and stored at 4°C for up to 8 h unless stated otherwise.

Experimental Design

At the conclusion of storage, an aliquot (1 ml) was taken from each 4°C extended semen sample and washed in 5 ml of 37°C, pH 7.6, .15 *M* NaCl containing .1% bovine serum albumin, Fraction V (BSA-saline). Each aliquot was washed 3 times (1000 × *g*; 5 min). The final sperm pellets were resuspended in pH 7.6 BSA-saline (37°C) to 2×10^6 sperm/ml and analyzed for motility with phase microscopy at 400× and for capacitation (ability to penetrate zona-free hamster oocytes) as described by Ijaz and Hunter (16). Three to six replicates per treatment were run.

Statistical Analysis

Analysis of variance (25) was performed on motility and oocyte penetration data. Means from analyses that were different at $P<.05$ were tested using Duncan's multiple range test (9).

RESULTS AND DISCUSSION

Experiment 1

Storage of Bull Sperm in Egg Yolk-TEST Buffer for Sperm Capacitation. Sperm, stored at 4°C for up to 8 h in EY-TEST buffer, retained excellent motility (Table 1), both after storage and after washing in BSA-saline in preparation for testing of capacitation status. Sperm stored at 4°C for 4 to 8 h in EY-TEST buffer had become capacitated as documented by ability to penetrate ZFHO (Table 1).

Experiment 2

Role of Egg Yolk in Capacitating Bull Sperm. Table 2 presents the sperm motilities after 0 to 48 h storage in EY-TEST, EY-EY salts, EY-citrate, and EY protein-Tris buffers. All buffers except EY protein-Tris maintained

TABLE 2. Effect of various extenders containing individual components of EY-TEST semen extender and time on motility[1] of bovine sperm stored at 4°C.

Time	% Sperm motility in extender			
	EY-TEST	EY-EY Salts	EY-Citrate	EY-Proteins Tris
(h)				
0	$67.5^{B,a}$	$73.8^{AB,a}$	$76.3^{A,a}$	$72.5^{AB,a}$
8	$60.0^{B,ab}$	$68.8^{A,a}$	$72.5^{A,ab}$	$15.0^{C,b}$
24	$52.5^{B,b}$	$53.8^{B,b}$	$66.3^{A,b}$	$0^{C,c}$
48	$41.3^{A,c}$	$23.8^{B,c}$	$45.0^{A,c}$	$0^{C,c}$
$\bar{X}$	55.3^{B}	55.0^{B}	65.0^{A}	21.9^{C}

^{A,B,C}Denote difference within rows ($P<.05$).

^{a,b,c}Denote difference within columns ($P<.05$). [1]Four replicates per treatment per cell.

good to excellent sperm motility for up to 24 h. Sperm, suspended in EY protein-Tris buffer, were mainly immotile by 8 h. Therefore, the EY buffers that maintained sperm motility for 8 h could be tested for capacitating activity without having to worry about poor motility causing reduced fertility.

Table 3 presents the sperm penetration of ZFHO after 8-h storage in the EY-TEST, EY-EY salts, EY-citrate, and EY protein-Tris buffers. The EY-citrate buffer did not capacitate sperm while EY-TEST buffer resulted in sperm capacitation as measured by ability to penetrate ZFHO. Neither storage in the EY-EY salts nor in the EY protein-Tris buffer capacitated sperm. Therefore, sperm-capacitating activity does not reside in the EY component of the buffer. Even though Tris was present in the EY protein buffer, the poor sperm motility in this buffer was probably the cause for penetration failure and not failure to capacitate.

Experiment 3

Component of TEST Responsible for Capacitating Bull Sperm. Having ruled out EY as a capacitating agent (Table 3), the separate constituents of TEST, namely TES and Tris, were evaluated for capacitating activity. To do this, EY-TEST was compared with the following EY buffers: TES-TETRA, taurine-Tris, and citric acid-Tris. Sperm retained good motility for 8 h at 4°C in these buffers (Table 4). However, after washing in BSA-saline in preparation for testing of capacitation status, the sperm that had been stored in EY-citric acid-Tris buffer had poor motility (Table 4). Nevertheless, all four buffers induced sperm capacitation as documented by ability to penetrate ZFHO. Because all four buffers contain TES, or Tris, or TES and Tris, both these chemicals can induce capacitation in bull sperm.

Experiment 4

Role of Taurine, a Component of the TES Molecule, in Capacitating Bull Sperm. Egg yolk-TEST was compared with the EY-citrate-taurine buffer. Sperm motility and the capacitation data are presented in Table 5. Sperm motility was higher ($P<.05$) in the EY-citrate-taurine buffer at all storage times out to 48 h compared with motility of sperm stored in EY-TEST. Both buffers did an excellent job of maintaining sperm motility.

Sperm, stored 8 h in EY-TEST, became

TABLE 3. Effect of various extenders containing the individual components of the egg yolk-(EY)-TEST semen extender on ability of bull sperm stored for 8 h at 4°C to penetrate zona-free hamster oocytes.[1]

Extender	Penetration	
	(no.)[2]	(%)
EY-TEST	20/55	36.4^{a}
EY-EY Salts	8/121	6.6^{b}
EY-Citrate	3/106	2.8^{b}
EY-Proteins Tris	4/103	3.8^{b}

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

[1]Four replicates per treatment.

[2]Number of eggs penetrated/total number of eggs.

TABLE 4. Effect of extenders containing individual buffering components of the egg yolk (EY)-TEST buffer on bull sperm motility and ability to penetrate zona-free hamster oocytes[1] after storage at 4°C.

Extender	% Motility			Penetration rate at 8 h	
	0 h	8 h	After 3× washing at 8 h	(no.)[2]	(%)
EY-TES-Tris	63.3^{A}	65.0^{A,a}	35.0^{B,ab}	50/124	40.3
EY-TES-Tetramethyl ammonium hydroxide	68.3^{A}	72.5^{A,a}	48.3^{B,a}	53/130	40.7
EY-Taurine-Tris	70.8^{A}	69.2^{A,a}	45.8^{B,a}	49/166	29.5
EY-Citric acid-Tris	66.6^{A}	50.0^{B,b}	19.2^{C,b}	58/155	37.4

^{A,B,C}Denote differences within row ($P<.05$).

^{a,b}Denote differences within columns ($P<.05$).

[1]Six replicates per treatment.

[2]Number of eggs penetrated/total number of eggs.

capacitated and penetrated ZFHO while those stored in EY-citrate-taurine did not become capacitated ($P<.001$). The low 5.4% oocyte penetration with sperm stored in EY-citrate-Taurine (Table 5), was equivalent to that reported in Table 3 for the noncapacitating EY-EY salts and EY-citrate buffers. The parthenogenetic control for this system is approximately 2% (16). Therefore, the capacitating activity of EY-TEST buffer does not reside in the taurine moiety of the TES molecule.

DISCUSSION

Storage of bovine sperm in egg yolk-TEST buffer resulted in capacitation of bovine sperm (Table 1). The zwitterions, TES and Tris, were the constituents in the EY-TEST buffer that were the potent stimulators of bovine sperm capacitation. Capacitation begins with the removal of seminal coating proteins, acquired by the sperm cell during epididymal transit and during ejaculation (15, 18, 21, 29). Removal of adsorbed coating proteins from the sperm membrane may lead to altered fluidity of the sperm plasma membrane allowing the sperm to undergo the acrosome reaction and penetrate an oocyte. Simply washing bull sperm in BSA-saline does not capacitate sperm (16). Sperm capacitation requires a species-dependent time interval during which changes occur that promote the sperm's ability to fertilize an oocyte (29). The 8-h capacitation interval was chosen because it yielded maximum penetration of ZFHO by sperm stored in EY-TEST buffer (16). During this 8-h preincubation, the zwitterions TES and Tris somehow altered the sperm's

TABLE 5. Effect of taurine-containing bovine semen extenders on bovine sperm motility and ability to penetrate zona-free hamster oocytes after storage[1] at 4°C.

Extender	% Sperm motility after storage for:					Penetration at 8 h	
	At dilution	0 h	8 h	24 h	48 h	(no.)[2]	(%)
EY-TEST	71.3	70.0	66.3^{a}	63.8^{a}	53.8^{a}	19/56	33.9^{a}
EY-Citrate-Taurine	76.3	76.3	72.5^{b}	67.5^{b}	60.0^{b}	11/184	5.4^{b}

^{a,b}Denote differences within columns ($P<.05$).

[1]Four replicates/treatment/cell.

[2]Number of eggs penetrated/total number of eggs.

plasma membrane such that capacitation occurred as documented by penetration of ZFHO. Alterations in the form of rearrangement of antigenic sites on rabbit sperm membranes have been reported during capacitation (18). O'Rand (22) observed decreased lateral mobility of sperm surface glycoproteins, possibly related to Ca^{++} influx during capacitation and acrosome reaction. O'Rand (22, 23) further investigated the restriction of intrinsic sperm membrane proteins using immunofluorescent assays. A change in antigenic labeling pattern was observed as sperm were capacitated in utero. Loss of some intrinsic proteins was also postulated.

O'Rand (22, 23) suggested the restricted mobility of intrinsic sperm membrane proteins resulted from a segregation of the lipids within the membrane. Friend (11) noted formation of sterol-free areas in the acrosomal region of the plasma membrane. Capacitation-associated decrease in membrane cholesterol, and a decrease in the cholesterol:phospholipid ratio, was observed by Davis (7). Cholesterol is removed from the sperm cell plasma membrane in vivo by action of the uterine fluids (8). Cholesterol removal can be effected in vitro with addition of high density lipoproteins, lysophosphatidylcholine and albumin, to sperm culture medium (21). Langlais and Roberts (21) suggested that loss of cholesterol may result in increased permeability of the sperm membrane to extracellular calcium, which would trigger the acrosome reaction.

Figure 1 reveals that TES and Tris share the same primary aliphatic amine structure. Both were equally effective in capacitating sperm. TES is a zwitterionic amino acid with a N-substituted taurine. Although taurine stimulates sperm motility (Table 5), it did not capacitate bull sperm (Table 5). Fleming and Armstrong (10) reported that polyamines were potent stimulators of guinea pig capacitation.

Figure 1 also reveals that TES and Tris share a terminal glycerol-like structure. Slavik (26) showed that glycerol accelerated the capacitation events in ram sperm as documented by their ability to penetrate ZFHO. However, unlike glycerols effect on ram sperm, the EY-TEST buffer maintained bull sperm's ability to penetrate ZFHO for up to 48 h (16). Although chicken EY had no effect on capacitation, it was required as a cryoprotective agent to prevent cold shocking the sperm during cooling and storage at 4°C.

The TES and Tris do not bind Mg^{++} or Ca^{++}, which are involved in the acrosome reaction and $Na^{+}K^{+}$ ATPase pump (29). Because the zwitterions TES and Tris are very soluble in water but impermeable to biological membranes (12), any effect they have on sperm must begin outside the cell.

During sperm storage at 4°C in EY-Tris-fructose medium, acids are produced that decrease the medium pH about 1 unit within the first 2 h of slow cooling (1). This can directly influence the cytosolic pH of bovine sperm (2) and is associated with a swelling of the sperm's apical ridge, which was considered potentially detrimental to fertility (1). Crabo et al. (6) reported increased osmotic resistance of cattle erythrocytes in hypotonic solutions of TES buffer. They attributed this to an increased mechanical stability of the cell membrane or to a changed cell permeability for water or electrolytes. This might help explain why sperm motility and acrosome integrity were maintained for up to 48 h at 4°C in EY-TEST buffer (16). In addition, the presence of TEST would neutralize the acids generated during storage and thus prevent acrosomal swelling.

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