Effect of Washing and Capacitating Media pH on Bull Sperm Motility, Acrosome Integrity, and Ability to Penetrate Zona-Free Hamster Oocytes

A. IJAZ and A. G. HUNTER
Department of Animal Science and Large Animal Clinical Sciences
University of Minnesota
St. Paul 55108

ABSTRACT

Bovine-ejaculated sperm were washed thrice in bovine serum albumin-saline media, pH 7.2 to 8.4, and incubated at 37°C in Ca^{++}-free Tyrode's media, pH 7.2 to 8.4, for 0, 2, 4, 6, and 8 h. Motility was highest when sperm were washed in pH 7.2 medium and incubated in pH 8.0 or 8.4 media. Motility remained above 50% until 8 h. Washing in pH 7.6, 8.0, or 8.4 media induced more acrosome reactions after incubation than washing at pH 7.2. Percentage of acrosome-reacted sperm increased at each successive time period. Sperm penetrated more oocytes at 4, 6, and 8 h when wash medium pH was fixed at 7.2 and capacitating media pH was raised at .4 unit increments from 7.2 to 8.4. When sperm were washed in pH 7.2 medium, the postincubation penetration rates peaked at 8 h. With wash media of pH 7.6, 8.0, or 8.4, the postincubation penetration rates peaked at 4 h and then gradually declined. In conclusion, the most effective system for capacitating bull sperm was a pH 7.6 wash followed by capacitation in pH 7.6 medium for 4 to 8 h and this system resulted in the highest penetration rates. Wash media pH hastened capacitation but was not a capacitating agent.

INTRODUCTION

To fertilize an egg, the sperm must be highly motile, capable of undergoing the acrosome reaction (AR), and capable of penetrating the egg investments and fusing with the egg proper (29). Successful fertilization in vitro implies the sperm underwent capacitation successfully (29). Capacitation is characterized by the removal of coating materials from the sperm surface (11, 12, 29), resulting in an increased permeability of the plasma membrane to Ca^{++} ions, which allows the sperm to undergo the AR (24, 29, 30). Usually hyperactivated motility is associated with capacitation (1, 29).

The ionic components of the culture medium influence mammalian sperm motility (17, 30), capacitation (29, 30), acrosomal integrity (29), and a sperm's ability to penetrate an oocyte (20, 29). The pH of the medium effects the ionization of substances within it (13), including proteins intrinsic to the sperm membrane and extrinsic, adsorbed seminal plasma proteins (12, 13). The pH of the medium determines many important aspects of the structure and function of biological macromolecules, including enzyme activity, and thus determines the behavior of cells (13). The pH of bovine estrual uterine fluid is 7.91 (7), rabbit oviductal fluid 7.8 (8), and sow oviductal fluid 8.4 (6). However, most in vitro fertilization procedures are performed between pH 7.2 and 7.8 (20).

Calcium^{++}-free Tyrode's solution (CFT) is a chemically defined medium mimicking the bovine sperm capacitating action of the female tract (2, 6). Although it is usually used at pH 7.6, Cheng (6) raised its pH to 8.5 to capacitate ejaculated bull sperm and obtained a higher fertilization rate of bovine oocytes than that achieved using other systems (3, 10). In addition, Behnke (2) found that adjusting the pH of the bovine serum albumin-saline (BSA-saline) sperm washing medium to pH 8.5 hastened the bull sperm capacitating activity of the pH 8.5 CFT.

The objective of this study was to determine the effect of varying the pH of BSA-saline

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sperm washing media and sperm-capacitating CFT media on bovine sperm motility, acrosomal integrity, and capacitation as measured by ability to penetrate zona-free hamster oocytes (ZFHO).

MATERIALS AND METHODS

Preparation of Media

The CFT was composed of 105.05 m*M* NaCl, 2.3 m*M* KCl, .49 m*M* $MgCl_2 \cdot 6H_2O$, 25.0 m*M* $NaHCO_3$, 1 m*M* sodium pyruvate, 13.87 m*M* glucose, 5.04 m*M* Hepes, 112 μl sodium lactate (60% syrup) per 100 ml, and .6% BSA (Fraction V, Sigma). The osmolality was 308 mOsmol/kg.

The BSA-saline wash solution was composed of 150 m*M* NaCl and .1% BSA (Fraction V, Sigma). Its osmolality was 308 mOsmol/kg. Media were prepared immediately before use and were filtered through .2-μ filters. Phenol red (.2%, 100 μl/100 ml) was added as indicator of pH. Wash and incubation media, respectively, were adjusted to the following pH combinations with 2.0 N NaOH: 7.2, 7.2; 7.2, 7.6; 7.2, 8.0; 7.2, 8.4; 7.6, 7.6; 8.0, 8.0; and 8.4, 8.4.

Semen Collection and Preparation of Sperm

Bovine-ejaculated semen was collected by artificial vagina from three dairy bulls and transported to the laboratory in a 37°C insulated container within 10 min of collection. A pH combination for wash and incubation media was selected. Semen was diluted 1:10 in 37°C BSA-saline of a specific pH, centrifuged for 5 min at 1000 × *g*, and supernatant discarded. Washing was repeated twice in 5 ml BSA-saline and final resuspension done in 37°C CFT of a specific pH to obtain 2×10^8 sperm/ml. Sperm were incubated in a 5% CO_2, 37°C humid environment in 35 × 10 mm polystyrene culture dishes for 0, 2, 4, 6, and 8 h.

Collection of Zona-Free Hamster Ova

The ZFHO were obtained by the procedure described by Byers et al. (4). Briefly, immature female golden Syrian hamsters were superovulated with 40 IU pregnant mare serum gonadotropin (PMSG, Sigma Chemical, St. Louis, MO) followed 55 h later by 40 IU human chorionic gonadotropin (hCG, Sigma). Hamsters were killed 18 to 20 h post-hCG injection and the ovaries and oviducts transferred to a 60- × 15-mm culture dish containing 5 ml, pH 8.5, CFT. The oviducts were opened and the cumulus masses were removed. The cumulus cells were removed after 5 to 10 min incubation in .1% hyaluronidase (Sigma) in CFT. Oocytes were washed twice in CFT without hyaluronidase. Oocytes were then transferred to .03% trypsin (Sigma) in pH 8.5 CFT to remove the zona. This was done while observing through a stereoscope. Oocytes were transferred to trypsin-free CFT as soon as the zonae became swollen in appearance, approximately 5 to 7 min after trypsin exposure. The zona-free oocytes were washed 3× in CFT at 37°C and used for the sperm penetration assay.

Coincubation of Sperm and Oocytes

At 0, 2, 4, 6, and 8 h, sperm previously incubated at a concentration of 2×10^8 sperm/ml were further diluted to 2×10^6 sperm/ml in CFT, and 50-μl droplets were placed in a 60- × 15-mm culture dish beneath a layer of paraffin oil. The ZFHO were added to the droplets (10 oocytes/drop) and incubated for 3 h in a 37°C, 5% CO_2 humid environment.

Measurements of Motility, Acrosome Reaction, and Penetration

Aliquots (10 μl) were taken at 0, 2, 4, 6, and 8 h of sperm incubation for estimation of sperm motility and AR. Motility was subjectively scored microscopically on a warm (37°C) slide using phase optics at 400×. Slides for AR estimates were prepared by smearing 10 μl of sperm on a glass slide with a Pasteur pipette and drying them for 48 h. The AR status was determined by the procedure of Lenz et al. (14). Sperm so prepared were examined by using phase microscopy and 200 sperm/slide were classified for AR. An intact acrosome was bright cherry red with a densely stained apical ridge and a reacted acrosome was uncolored or dull gray.

Evaluation of the oocytes for sperm penetration was done at completion of the 3-h coincu-

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

Statistical Analysis

Data on percentage of acrosome-reacted sperm and percentage of penetrated eggs were transformed to arc sins and ANOVA was carried out employing a split-plot design (21). The ANOVA was based on the model:

$$Y_{ijk} = \mu + R_k + P_i + (R \times P)_{ki} + T_j + (P \times T)_{ij} + E_{ijk}$$

where:

μ = overall mean,
R_k = replicate effect,
P_i = pH effect,
$(R \times P)_{ki}$ = interaction of replicate and pH (Error 1),
T_j = time effect,
$(P \times T)_{ij}$ = interaction of pH and time (Error 2), and
E_{ijk} = residuals.

Means that were different at $P<.05$ were tested using Duncan's multiple range test (21). Ejaculates (and days) were considered as replicates and were treated as random variables; treatments were considered fixed. Linear regression was used, and penetration rate was regressed on motility and AR. Correlations were used to determine the closeness of linear relationships between motility and AR, motility and penetration, and AR and penetration.

RESULTS

Motility

Effects of various pH wash media and capacitation media on sperm motility are presented in Table 1 and the ANOVA in Table 2. Motility was highest ($P<.05$) when sperm were washed in pH 7.2 BSA-saline and incubated in pH 7.2, 8.0, or 8.4 CFT. Sperm motility after washing and incubating in media of the same pH was not different between pH 7.6 and pH 8.4.

Sperm motility was not different ($P>.05$) between the 0 h and the 2 h or 4 h of incubation (Table 1 and 2). However, mean motility decreased ($P<.05$) after 4 h of incubation but remained above 50% until 8 h (Table 2). No interaction was observed ($P>.05$) between media pH and incubation time in terms of an increase or decrease in sperm motility (Table 2).

Acrosome Reaction

Effects of various pH wash media and capacitation media on sperm acrosomal integrity are presented in Table 3 and the ANOVA in Table 4. No difference ($P>.05$) was observed in percentage of acrosome-reacted sperm when sperm were washed in pH 7.2 BSA-saline and incubated in pH 7.2, 7.6, 8.0, or 8.4 CFT (Table 4). However, washing in pH 7.6, 8.0, or 8.4 BSA-saline induced more ($P<.05$) AR after incubation than at pH 7.2 (Table 3). In addition, from the ANOVA data, increasing the pH of washing and capacitating media from 7.2 and also the incubation time increased ($P<.01$) number of acrosome-reacted sperm (Table 4).

The percentage of acrosome-reacted sperm increased ($P<.05$) at each successive time (Table 3). However, the main population was still unreacted (Table 4) even after 8 h (27.9% reacted vs.72.1% unreacted).

Penetration

Effects of various pH wash media and capacitation media on bull sperm penetrability of ZFHO are presented in Table 5 and the ANOVA in Table 6. A numerical trend of increased penetration rate at 4, 6, and 8 h was seen when the wash medium pH was fixed at 7.2 and the capacitating media pH were raised at .4 unit increments from 7.2 to 8.4 (Table 5). However, the greatest increase ($P<.05$) in penetration rate occurred when wash medium pH was increased from 7.2 to 7.6 with the capacitating medium pH fixed at 7.6 (Table 5). From the ANOVA data, length of incubation also effected ($P<.01$) the ability of sperm to penetrate ZFHO (Table 6).

TABLE 1. Effect of pH of wash media, capacitating media, and time on bovine sperm motility.

pH BSA-saline/ CFT[1]	% Sperm motility at 0 h	2 h	4 h	6 h	8 h
7.2/7.2	60.6	61.3^{AB}	63.8^{A}	56.9^{AB}	56.3^{ABC}
7.2/7.6	63.1	61.3^{AB}	58.8^{AB}	55.0^{ABC}	51.9^{ABC}
7.2/8.0	61.3	65.6^{A}	61.3^{A}	62.5^{A}	59.4^{A}
7.2/8.4	64.4	68.1^{A}	63.8^{A}	59.4^{AB}	56.9^{AB}
7.6/7.6	51.9	51.3^{B}	47.5^{B}	48.1^{ABC}	43.8^{ABC}
8.0/8.0	53.1	51.3^{B}	46.9^{B}	45.6^{BC}	41.9^{BC}
8.4/8.4	51.9^{ab}	$53.8^{a,B}$	$51.3^{ab,AB}$	$43.8^{ab,C}$	$40.6^{b,C}$

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

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

[1]CFT = Ca^{++}-free Tyrode's solution, eight replicates/cell; BSA-saline = bovine serum albumin-saline.

In pH 7.2 wash medium, penetration of ZFHO increased throughout incubation and peaked at 8 h (Table 5). However, with wash medium of pH 7.6, 8.0, or 8.4, the penetration rate peaked at 4 h and then tended to decrease (Table 5). Sperm, washed in pH 7.6 BSA-saline and incubated in pH 7.6 CFT, penetrated more ($P<.05$) ZFHO than in other treatments at 4, 6, or 8 h (Table 5). The correlation between AR and motility was high (r = −.88). However, penetration of ZFHO was poorly correlated with motility (r = −.46) and AR (r = .54).

DISCUSSION

Effect of Media pH on Sperm Motility

Sperm have to be capacitated and motile to penetrate ZFHO (20). Motilities were not dras-

TABLE 2. Analysis of variance for the effect of media pH and length of incubation time on bovine sperm motility.

Factor	df	SS[1]	MS	F	P
Replicate	7	4632.5	661.8		
pH	6	11,070.0	1845.0	9.94	<.01
Error 1	42	7790.0	185.5		
Time	4	3004.8	751.12	16.69	<.01
Interaction pH/time	24	813.9	33.9	.75	>.05
Error 2	196	8821.2	45.0		
Total	279	36,132.4			
	Value	Average	Within-cell SD		
pH	7.2/7.2	59.75^{ab}	9.26		
	7.2/7.6	58.00^{b}	9.11		
	7.2/8.0	62.00^{a}	9.11		
	7.2/8.4	62.50^{a}	11.49		
	7.6/7.6	48.50^{c}	7.69		
	8.0/8.0	47.75^{c}	9.86		
	8.4/8.4	48.25^{c}	10.09		
Time, h	0	58.03^{ab}	11.14		
	2	58.92^{a}	9.47		
	4	56.16^{b}	10.53		
	6	53.03^{c}	11.42		
	8	50.08^{d}	12.11		

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

[1]Sums of squares.

TABLE 3. Effect of pH of wash media, capacitating media, and time on bovine sperm acrosomal integrity.

pH BSA-saline/ CFT[1]	% Acrosome-reacted sperm at				
	0 h	2 h	4 h	6 h	8 h
7.2/7.2	$11.6^{a,A}$	$14.9^{ab,A}$	$17.4^{ab,A}$	$16.8^{ab,A}$	$18.9^{c,A}$
7.2/7.6	14.0^{AB}	14.3^{A}	15.1^{A}	16.3^{A}	17.5^{A}
7.2/8.0	13.8^{AB}	16.6^{AB}	15.4^{A}	17.0^{A}	19.6^{A}
7.2/8.4	14.5^{AB}	15.3^{A}	16.0^{A}	19.1^{A}	18.9^{A}
7.6/7.6	$17.8^{a,ABC}$	$22.5^{ab,BC}$	$28.1^{b,B}$	$30.3^{bc,B}$	$39.9^{c,B}$
8.0/8.0	$18.6^{a,BC}$	$26.9^{b,C}$	$27.0^{b,B}$	$37.8^{c,BC}$	$41,8^{c,B}$
8.4/8.4	$16.3^{a,C}$	$27.5^{a,C}$	$38.3^{b,C}$	$38.6^{b,C}$	$39.0^{b,B}$

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

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

[1]CFT = Ca^{++}-free Tyrode's solution, eight replicates/cell; BSA-saline = bovine serum albumin-saline.

tically altered by changing the pH of media from 7.2 to 8.4 and ranged from 48 to 63%, although this difference was statistically significant. However, the correlation between motility and penetration of ZFHO was negative and low ($r = -.46$).

The pH in the epididymis, where mammalian spermatozoa are stored before ejaculation, is generally 6.0 or lower in the bull (22). Epididymal spermatozoa are generally motionless or only weakly motile. Upon exposure to seminal plasma or physiological salt solutions with higher pH values, the spermatozoa become highly motile. This initiation of motility is associated with an efflux of intracellular H^+, causing an increase in intracellular pH (26).

TABLE 4. Analysis of variance for the effect of media pH and length of incubation time on bovine sperm acrosomal integrity.

Factor	df	SS[1]	MS	F	P
Replicate	7	.40	.06		
pH	6	2.05	.34	60.35	<.01
Error 1	42	.24	.01		
Time	4	.64	.16	59.26	<.01
Interaction pH/time	24	.30	.01	4.58	<.01
Error 2	196	.53	.00		
Total	279				
	Value	Average	Within-cell SD		
pH	7.2/7.2	15.90^{a}	4.87		
	7.2/7.6	15.42^{a}	4.59		
	7.2/8.0	16.50^{a}	4.04		
	7.2/8.4	16.75^{a}	5.12		
	7.6/7.6	27.50^{b}	10.16		
	8.0/8.0	30.40^{c}	9.75		
	8.4/8.4	33.37^{d}	10.07		
Time, h	0	16.26^{a}	5.38		
	2	19.69^{b}	9.12		
	4	22.46^{c}	9.94		
	6	25.10^{d}	10.66		
	8	27.78^{e}	11..60		

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

[1]Sums of squares.

TABLE 5. Effect of pH of wash media, capacitating media, and time on penetration on zona-free hamster oocytes by bovine sperm.[1]

pH BSA-saline/ CFT[2]	% Zona-free hamster oocytes penetrated at				
	0 h	2 h	4 h	6 h	8 h
7.2/7.2	2.2	6.9	6.7^{A}	9.5^{A}	13.2^{A}
7.2/7.6	0	5.4	9.2^{A}	10.1^{A}	12.7^{A}
7.2/8.0	0^{a}	6.5^{ab}	$16.7^{b,AB}$	$11.4^{ab,A}$	$19.2^{b,B}$
7.2/8.4	0^{a}	6.0^{ab}	$21.6^{c,AB}$	$16.4^{bc,AB}$	$21.7^{c,B}$
7.6/7.6	2.8^{a}	14.7^{ab}	$28.9^{b,B}$	$25.6^{b,B}$	$24.2^{b,B}$
8.0/8.0	0^{a}	13.5^{b}	$18.3^{b,AB}$	$13.3^{b,A}$	$8.6^{ab,A}$
8.4/8.4	0^{a}	15.0^{b}	$27.9^{c,B}$	$18.3^{bc,AB}$	$9.2^{ab,A}$

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

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

[1]Four replicates, approximately 15 to 20 eggs per replicate per treatment.

[2]CFT = Ca^{++}-free Tyrode's solution; BSA-saline = bovine serum albumin-saline.

Washing bull spermatozoa at a pH greater than 7.2 resulted in lower motilities at 4, 6, and 8 h of capacitation (Table 1), perhaps due to the lack of Ca^{++} in the media. Hyperactivated motility is usually associated with capacitation and may provide the sperm with strong thrusting power needed to penetrate the zona pellucida (29). Calcium^{++} is essential for initiation of hyperactivation of bull (24, 25), guinea pig, hamster, and mouse sperm (29). However, hyperactive sperm motility was not achieved when epididymal hamster spermatozoa were expressed directly into Tyrode's solution ranging in pH from 7.2 to 8.4 and incubated from 5 min to 5 h (28).

The CFT media was used in our study to delay or prevent the AR from occurring after capacitation. In this way we were able to separate pH effects on capacitation from those on the AR. Table 3 reveals that at 8 h of sperm incubation, few sperm (18 to 42% of the population, depending on treatment) were acrosome reacted. Singh et al. (24) reported the Ca^{++} influx into bull sperm served to link alterations of the sperm membrane to subsequent activation of sperm motility at capacitation. Therefore, the CFT used in our study may have prevented the hyperactive motility associated with capacitation.

Effect of Media pH on Acrosome Reaction

When sperm were washed in pH 7.2 BSA-saline and incubated in pH 7.2, 7.6, 8.0, or 8.4 CFT, the percentage of acrosome-reacted sperm was almost the same in all treatments (i.e., 15.9, 15.4, 16.5, and 16.8, respectively; Table 4). However, when wash medium pH was more alkaline, i.e., 7.6, 8.0, or 8.4, percentage of AR increased with each increase in pH, i.e., 27.5, 30.4, and 33.4, respectively (Table 4). Two factors may account for these findings. First, Singh et al. (24) found under in vitro capacitating conditions, when lower external Ca^{++} and higher sperm concentrations were used, that the AR was substantially delayed. Our experiment was carried out under similar conditions, and thus, this would explain the low percentage of acrosome-reacted sperm found after the pH 7.2 sperm wash. Second, both Ca^{++}-containing and CFT media are more effective in inducing AR in guinea pig spermatozoa incubated at alkaline pH between 8.2 to 8.4 (17) with the optimum reaction occurring at pH 8.3 (18). In addition, peak acrosome reactivity occurred at pH 8.0 in the rabbit (19) and was lost irreversibly at pH 5. Hamster sperm, incubated in media below pH 6.7, were unable to acrosome react (15). Our data (Table 4) support the idea that more bull sperm AR occur as the pH of the washing and capacitating media was raised from pH 7.2 through pH 8.4. Our results from washing bull sperm with media of pH 7.6, 8.0, or 8.4 might be due to more efficient removal of seminal plasma from the sperm prior to exposure to the capacitating media. The data in Table 3 support that hypothesis since more sperm were acro-

TABLE 6. Analysis of variance for the effect of media pH and length of incubation time on the ability of bovine sperm to penetrate zona-free hamster oocytes.

Factor	df	SS[1]	MS	F	P
Replicate	3	.03	.01		
pH	6	.53	.09	10.80	<.01
Error 1	18	.15	.01		
Time	4	2.89	.72	60.53	<.01
Interaction pH/time	24	.52	.02	1.81	<.01
Error 2	84	1.00	.01		
Total	139				

	Value	Zona-free oocytes	Average	Within-cell SD
pH	7.2/7.2	349	7.71[a]	5.74
	7.2/7.6	348	7.47[a]	6.98
	7.2/8.0	336	10.70[abc]	9.69
	7.2/8.4	318	13.16[abc]	10.66
	7.6/7.6	377	19.24[d]	11.65
	8.0/8.0	367	10.76[abc]	7.67
	8.4/8.4	386	14.11[c]	10.47
Time, h	0	403	71[a]	1.81
	2	524	9.72[b]	6.44
	4	467	18.50[c]	10.43
	6	552	14.92[c]	6.84
	8	535	15.54[c]	9.40

[a,b,c]Denote differences within columns ($P<.05$).

[1]Sums of squares.

some reacted at 0 h following washing at pH 7.6, 8.0, and 8.4 than at pH 7.2.

Washing may increase the permeability of the sperm membranes to Ca^{++}, a crucial component of capacitation, that allows the subsequent occurrence of the AR and motility activation. Because Ca^{++}-free media was used, a low percentage of the sperm population would be expected to have acrosome reacted under the capacitating conditions. Under similar conditions, Babcock et al. (1) found that addition of Ca^{++} rapidly resulted in motility activation of bovine sperm.

Effect of Media pH on Sperm Penetrability

Media pH and time within a specific medium affected the ability of bovine sperm to penetrate ZFHO (Tables 5 and 6). To penetrate ZFHO, sperm must be both motile and capacitated (20). Therefore, both these factors and their interaction were involved in capacitating bovine ejaculated sperm.

The pH of the suspending media affects the charge on proteins (13). Proteins function as dipolar ions mainly due to the ionization of the various R groups of the amino acids, which make up their primary structure (13). Thus, media pH will affect their protein-protein interactions (13). Because capacitation involves the removal of seminal coating proteins adsorbed on the sperm's surface membrane (11, 12, 29), changing the pH of the capacitating media from pH 7.2 through pH 8.4 would be expected to alter the binding of these proteins to the sperm's surface. Indeed, maintaining the wash medium's pH at 7.2 while increasing the pH of the capacitating media to pH 8.4 resulted in more sperm becoming capacitated (Table 5) at 4 h. This agreed with Cheng's finding (6) that more bull sperm penetrated bovine zona-intact oocytes after being capacitated in pH 8.4 CFT medium than in CFT medium of a lower pH.

In the in vivo condition, the pH of the cow vagina is in the range of pH 6 to 7 (23). Sperm do not become capacitated in the vagina (29) but rather in the uterus and oviducts (29). The pH 7.2 wash medium (BSA-saline) will not capacitate sperm under the conditions of our assay (9). Therefore, the pH 7.2 wash medium mimics vaginal pH conditions while the higher

capacitating media pH mimic uterine and tubal pH conditions. However, washing the sperm at pH 7.6, 8.0, or 8.4 before incubating in the identical pH-capacitating media resulted in more sperm becoming capacitated earlier, especially after 2 or 4 h, than did washing at pH 7.2 (Table 5). Behnke (2) reported more bovine sperm were capacitated at 4 h if they had been washed first in pH 8.4 BSA-saline than if they had been washed in pH 7.2 BSA-saline. Again, this pH wash effect might be due to more efficient removal of seminal coating proteins by the higher pH by decreasing the protein-protein interactions between sperm and seminal plasma. During capacitation, a decrease occurs in the net negative charge on the sperm's membrane, possibly due to removal of sialic acid residues (29).

The most effective pH treatment for capacitating high numbers of sperm by 4 h of incubation and for retaining their ability to penetrate for at least up to 8 h of incubation was the pH 7.6 BSA-saline wash followed by pH 7.6 CFT capacitating medium (Table 5). The lower penetrabilities at 8 h after washing and capacitating in pH 8.0 or 8.4 media (Table 5) were probably due to the increased incidence ($P<.05$) of acrosome-reacted sperm found at 2, 4, and 6 h (Table 3). Once sperm are capacitated, they must undergo the AR on the egg's surface in order to penetrate (29). The higher pH resulted in more acrosome-reacted sperm (Tables 3 and 4).

Variations in the extracellular pH are assumed to be directly reflected by internal changes in the pH of sperm (23). Increased pH within the sperm is the final step in capacitation (27). At least a 1 pH unit increase after capacitation and just prior to the AR was shown in hamster sperm (27). The pH increase probably resulted from changes in membrane permeability to K^+ and H^+. High K^+ in the incubation medium stimulated the rise in intraacrosomal pH, but Ca^{++} had no effect on this pH (27). However, high pH may stimulate an influx of Ca^{++}, which in turn stimulates hypermotility and the AR. This Ca^{++} effect is regulated by a Ca^{++}-independent ATPase (18). The Ca^{++} ATPase had peak activity at pH 9, and a Mg^{++} ATPase had maximal activity at pH 8 (5). Most enzymes require a particular pH range for optimum response (13). Stimulation of metabolic activity as reflected by increased sperm motility by alkaline media has long been recognized (23).

This paper documents that by metabolic or physical means, alkaline pH stimulates the capacitation of bovine sperm in CFT media. The alkaline pH specifically affected the capacitation of sperm and not the union of sperm and oocyte. Capacitation allows sperm to penetrate ZFHO (20, 29). However, if the pH of the medium in which the capacitated sperm are exposed to the oocytes is wrong, penetration will not occur. Miyamoto et al. (16) reported the in vitro fertilization rate of hamster oocytes was 90 to 100% in Tyrode's of pH between 6.5 and 8.5. Thus, by adding the ZFHO directly to the capacitated sperm droplets, potential pH shocks to the sperm due to the difference in pH of sperm-egg interaction media and the capacitating media were avoided. This allowed us to separate the effect of pH on capacitation from those dealing with gamete interaction. In addition, comparison of penetration rates in like pH media, but with sperm washed in differing pH media (Table 5), revealed that pH affected sperm capacitation rather than the actual sperm-oocyte interaction step.

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