Liner Collection Cone and pH Effects on Postthaw Motility, Staining, and Acrosomes of Bovine Spermatozoa[1]

J. F. SMITH and C. P. MERILAN
Department of Dairy Science
University of Missouri
Columbia 65211

ABSTRACT

Sixteen ejaculates were collected, four each from four bulls, using artificial vaginas with polyethylene or rubber liner collection cones in a crossover design experiment. The ejaculates were diluted with egg yolk-citrate extender at pH 6.4 or 7.2, cooled, glycerolated, equilibrated, packaged in .5-ml French straws, frozen in nitrogen vapor, and stored in liquid nitrogen. Thirty frozen straws from each ejaculate were thawed rapidly (46.5°C for 12 s), pooled, and then incubated at 46.5°C for periodic evaluation of progressive motility, differential staining, and acrosome morphology under thermal stress conditions. The postthaw motility of spermatozoa and percentage of unstained cells were higher both when collected in polyethylene than in rubber and when extended at pH 7.2 vs. 6.4, but no interaction was found between liner collection cone composition and pH for postthaw motility. Retention of spermatozoan motility during incubation under thermal stress was greater for cells collected in polyethylene, but not different due to pH. Neither pH nor composition of liner collection cone had an effect on postthaw acrosomal scores, but the time required for a 50% increase in severely damaged acrosomes was greater for spermatozoa collected in polyethylene than in rubber liner collection cones.
(Key words: acrosomes, liner collection cones, motility)

INTRODUCTION

A number of studies have examined the effects of polymers on gametes (2, 3, 5, 9, 10, 11, 14). In general, contact with rubber is detrimental to both spermatozoan and embryo survival. Effects of pH on spermatozoa have been of interest for many years. Although the optimal pH range seems to be dependent on the type of buffer and temperature (6, 8, 13), Colborn et al. (4) found that both pH and temperature influenced the toxicity of rubber for equine spermatozoa. Thermal stress, 46.5°C, has been reported (1) to permit more rapid evaluation of semen quality. The objective of this study was to examine the effects of rubber exposure and extender pH on the postthaw motility, percentage of unstained cells, and acrosome morphology of bovine spermatozoa under thermal stress.

MATERIALS AND METHODS

Sixteen ejaculates, four from each of four mature Holstein bulls, were used to determine the effects of liner collection cone composition, pH, and temperature on the postthaw motility, percentage of unstained cells, and acrosome morphology. Ejaculates were collected in a crossover design alternating polyethylene and rubber collection cones on the first and second ejaculates. Bulls were allowed to false mount a teaser cow twice and were collected on the third mount with an artificial vagina using no lubricant.

Polyethylene liner collection cones were fabricated from .1 mm polyethylene to the same specifications as the rubber liner collection cones (63.5 cm in length). Artificial vaginas were assembled in double liner configuration. A 40-cm shell was used, and collection cones were allowed to extend 10 cm beyond the shell. A 15-ml polystyrene centrifuge tube was attached to the end of the collection cone.

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Undiluted semen was diluted (1:10) with 20% egg yolk-citrate at a pH of 6.4 or 7.2. Diluted samples were cooled to 5°C in 3 h and glycerolated in four steps to yield an 8% glycerol concentration. Glycerolated samples were equilibrated for 2 h, packaged in .5-ml French straws, and sealed with polyalcohol. Thirty straws from each sample were frozen 5 cm above a static liquid nitrogen bath for 9 min and stored at −196°C for 24 to 72 h.

Frozen straws from each sample were thawed in a water bath (46.5°C for 12 s), pooled (30 straws), and incubated at 46.5°C. All samples were evaluated every 15 min for motility and percentage of unstained cells for 1 h. Acrosome morphology was evaluated every 60 min for 4 h.

The percentage of motile spermatozoa was determined at 37°C using a heated stage, phase contrast microscope (100×). The percentage of unstained cells was determined by differential staining (2.0 g of fast green FCF and .8 g of eosin B in 100 ml of M/8 phosphate buffer) (7). Acrosomal staining was performed with Giemsa stain, and both percentage of normal acrosomes and acrosomal scores (0 = undamaged; 1 = slight damage; 2 = severe damage; and 3 = acrosome missing) were determined (15). The time required for a 50% change, increase or decrease, in an attribute was calculated by fitting polynomials (first, second, and third degree) to the mean data string for a data set. Choice of the appropriate polynomial was based on length of the data string and reduction in variance between fitted and actual data points. Subsequently, each individual data string in the set was fitted with the chosen polynomial, and the roots of the polynomial were then used to calculate the $T_{.50}$ increase or decrease. The mean of these individual $T_{.50}$ values was reported as the measure for that data set.

Data were analyzed as a split-plot design utilizing two-way ANOVA (12). The main plot was a crossover design used to examine the effect of collection cone composition. The subplot was a factorial arrangement used to determine the effects of temperature and pH. Means were evaluated using Fisher's least significant difference at 5%.

RESULTS AND DISCUSSIONS

A preliminary trial involving four ejaculates, one from each of the four bulls using rubber liner collection cones, was conducted to verify the time:temperature relationships reported by Beck and Salisbury (1), specifically for 39 vs. 46.5°C incubation temperatures and with rate of change in spermatozoan progressive motility denoted by the time required for a 50% change ($T_{.50}$) in the rating. The results for spermatozoa in egg yolk-citrate extender were $T_{.50}$ decreases of 470.4 and 37.5 min for aliquots incubated at 39 and 46.5°C, respectively. The roots of second degree polynomials fitted to the data were used for $T_{.50}$ calculations. The results supported the principles advanced by Beck and Salisbury (1); thus, the 46.5°C incubation and the $T_{.50}$ concept were utilized for all subsequent phases of the study.

The ANOVA showed differences in postthaw motility due to composition of liner collection cone ($P < .04$) and pH ($P < .003$) of the extender, but no interaction ($P > .10$) between liner type and pH was detected (Table 1). Survival during incubation at 46.5°C, as measured by time required for a 50% decline ($T_{.50}$) in progressive motility, differed with liner collection cone type ($P < .02$) but not with pH ($P > .10$). Means for progressive motilities and survival half-lives are presented in Table 1. The decrease in postthaw motility due to rubber exposure agrees with results previously reported (5, 11, 13); however, the differences in survival half-life indicate that survival under accelerated aging conditions, 46.5°C, could be a better estimator of rubber toxicity than initial postthaw progressive motility, because effects of rubber toxicity on spermatozoa are accentuated by aging.

An interaction ($P < .0008$) was found between liner collection cone composition and pH for percentage of unstained cells. There was no difference ($P > .05$) in the postthaw percentage of unstained cells when semen was collected in rubber and diluted at a pH of 6.4 vs. 7.2 (35.6 vs. 34.6%). However, semen collected in polyethylene and diluted at a pH of 7.2 vs. 6.4 had a higher ($P < .05$) percentage of unstained cells postthaw (47.2 vs. 43.4%). There was no difference $P > .10$) in $T_{.50}$ decrease of percentage of unstained cells with either liner type or pH.

Given the involvement of membranes in the live-dead differential staining process and the interaction between type of liner collection cone and pH for the percentage of unstained cells, it appears that membranes of bovine sper-

TABLE 1. The effect of liner collection cone type and extender pH on least squares means of postthaw motility and percentage of unstained bovine spermatozoa incubated at 46.5°C.

	Liner type				Significance		
	Rubber		Polyethylene				
	pH 6.4	pH 7.2	pH 6.4	pH 7.2	Liner	Interaction	pH
Progressive motility, %	32.9	37.4	37.4	42.6	.04	NS	.003
$T_{.50}$ Decrease[1]	32.3	32.3	38.5	39.4	.02	NS	NS
Unstained cells, %	35.6	34.6	43.4	47.2	.0003	.0008	.05
$T_{.50}$ Decrease[1]	70.8	75.9	86.6	84.2	NS	NS	NS

[1]$T_{.50}$ = Minutes required for 50% change.

matozoa collected in polyethylene retained their semipermeability characteristics during freezing and thawing much better at a higher pH than did those cells collected in rubber. However, subsequent rate of loss of semipermeability was not affected by either composition of liner collection cone or pH.

No differences ($P > .10$) were found by ANOVA to be associated with the effects of pH (6.4 vs. 7.2) or liner type (polyethylene versus rubber) on acrosomal score, percentage of normal acrosomes, or $T_{.50}$ decrease in percentage of normal acrosomes (Table 2). Composition of liner collection cone was different $P < .10$), but pH was not different ($P > .10$), in effect on $T_{.50}$ increase in acrosomal score during incubation at 46.5°C. Means of these data are presented in Table 2.

Watson's normal acrosome category (15) pooled classes 0 and 1, thus placing cells with detectable acrosome damage in the normal group. Therefore, we made additional evaluations of the dynamics of relative changes in acrosome morphology classes during incubation at 46.5°C. As with the motility and unstained cell data, second degree polynomials were fitted to the individual data obtained for classes 0, 1, and 2 during the incubation periods, and $T_{.50}$ changes were calculated. These data are given in Table 3. The ANOVA revealed no differences ($P > .10$) associated with pH or composition of liner collection cone for either the Y-intercept or slope coefficients of the polynomials or for the $T_{.50}$ decreases in undamaged or slightly damaged acrosomes, classes 0 and 1, respectively. For class 2 acrosomes, those with substantial damage, the $T_{.50}$ increase was different ($P < .03$), but this difference was only associated with liner collection cone type effects.

The different effects and interactions of composition of liner collection cone and pH upon progressive motility, survival half-life, differential live-dead staining, and acrosomal morphology illustrate the point that exposure to rubber causes several physiological changes in bovine spermatozoa. Further, the use of accelerated aging and thermal stress testing provides a

TABLE 2. The effect of liner collection cone type and extender pH on least squares means of postthaw percentage of normal acrosomes and acrosome scores of bovine spermatozoa incubated at 46.5°C.

	Liner type			
	Rubber		Polyethylene	
	pH 6.4	pH 7.2	pH 6.4	pH 7.2
Normal acrosomes, %	89.5	91.5	90.1	89.3
$T_{.50}$ Decrease[1]	303.0	308.4	267.3	374.7
Acrosome score	.90	.87	.87	.87
$T_{.50}$ Increase[1,2]	146.0	141.3	202.2	181.2

[1]$T_{.50}$ = Minutes required for 50% change.

[2]Difference ($P < .10$) for liner collection cone effects.

TABLE 3. The effect of liner collection cone type and extender pH on least squares means of postthaw acrosomal classes for bovine spermatozoa incubated at 46.5°C.

	Liner type			
	Rubber		Polyethylene	
	pH 6.4	pH 7.2	pH 6.4	pH 7.2
Acrosome class 0				
Percentage	21.0	22.0	22.7	21.8
$T_{.50}$ Decrease[1]	79.5	95.6	91.6	108.2
Acrosome class 1				
Percentage	68.5	69.5	67.4	67.5
$T_{.50}$ Decrease[1]	452.2	254.8	339.3	350.0
Acrosome class 2				
Percentage	10.5	8.5	9.9	10.7
$T_{.50}$ Increase[1,2]	22.6	17.9	32.3	28.5
Acrosome class 3				
Percentage	<.1	<.1	<.1	<.1

[1]$T_{.50}$ = Minutes required for 50% change.

[2]Difference ($P < .03$) for liner collection cone effects.

basis for evaluating the dynamics of these and other time-related changes in spermatozoa.

CONCLUSIONS

Collection of spermatozoa in polyethylene liner collection cones and dilution with an extender at pH 7.2 resulted in increased percentages of progressive motility, unstained cells, and longer retention of motility under thermal stress compared with cells collected in rubber or extended at a pH of 6.4. An interaction between composition of liner collection cone and pH was found only for percentage of unstained cells. No effects of liner collection cone type or pH were identified for postthaw percentage of normal acrosomes, acrosome score, or acrosome class. However, during exposure to thermal stress, the rate of change in both acrosome score and class 2 acrosomes was slower for sperm cells collected in polyethylene than in rubber, thus indicating an increased resistance to thermal stress. Incubation at 46.5°C and use of $T_{.50}$ calculations facilitate the conduct of stress tests on spermatozoa.

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