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Fertility of Fresh and Frozen Rabbit Semen Inseminated at Different Times Is Indicative of Male Differences in Capacitation Time[1]

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ABSTRACT

Some reports indicate that sperm from different males differ in capacitation time, and other reports suggest that freezing sperm may affect their capacitation time. These two variables were specifically studied in rabbits in a fertility trial with 96 does inseminated with approximately 1.6 million motile fresh or frozen sperm from three different bucks at 15, 10, 5, and 0 h before expected ovulation. Fresh semen averaged 84% live (unstained) sperm and 88% had normal acrosomes; corresponding values for frozen sperm were 44% and 54%. On the basis of does that became pregnant, average litter size with fresh semen was 5.5 and with frozen semen was 4.8 ($p>0.05$), but overall, does bred with frozen semen produced fewer young ($p<0.05$). On the basis of total does and total semen, average litter size from insemination at 15, 10, 5, and 0 h was 2.8, 4.2, 3.8, and 1.7, and average litter size for the three bucks was 4.0, 1.8, and 3.6. There was no interaction of type of semen (fresh or frozen) with the other variables in the model ($p>0.05$). Bucks and time of insemination affected both the proportion of does that were pregnant and litter size ($p<0.01$). A major interaction between buck and time of insemination ($p<0.01$) was due apparently to both differential sperm survival and probable capacitation time among bucks. This major interaction should be considered in designing in vitro and in vivo fertility studies, and for selecting males for use in artificial insemination.

INTRODUCTION

Rabbits are a useful model for studying various aspects of sperm physiology and fertilization because semen can be collected easily (Bredderman et al., 1964), and timing of insemination can be controlled with respect to ovulation by treatment with luteinizing hormone (Kennelly and Foote, 1965) or gonadotropic-releasing hormone. This control of the interval from insemination to ovulation is critical in studies involving capacitation (Austin, 1951; Chang, 1951).

Good fertility results have been reported with frozen rabbit semen (Maurer et al., 1976; Hanada and Nagase, 1980; Arriola, 1982; Weitze, 1977; Weitze et al., 1982; Chen et al., 1989). Parrish and Foote (1986) obtained evidence that suggested that freezing and thawing rabbit spermatozoa might decrease the time required for capacitation of sperm. However, Chen et al. (1989) did not observe any difference in fertility between fresh and frozen semen inseminated at different times. The experiments reported by Chen et al. (1989) involved pooled semen from several males, which could have partially masked any individual buck differences in capacitation time, as reported by Kim et al. (1989) for fresh semen. Furthermore, the times chosen for insemination and the number of sperm used for insemination may not have covered a range that would allow detection of subtle effects.

The objectives of the present experiments were (1) to examine the fertility of fresh and frozen sperm that had resided in the female for longer or shorter times than normal, (2) to compare fertility of fresh and frozen sperm following variable exposure to the female tract before ovulation as an indication of capacitation time, and (3) to assess the male effect on pregnancy rates and litter size resulting from fresh and frozen sperm from different males. Relatively few sperm were inseminated to increase the sensitivity of the test. Also, staining procedures were developed to assess acrosomal changes in rabbit sperm.

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MATERIALS AND METHODS

Experimental Design

A 3 × 2 × 4 factorial arrangement, consisting of semen collected from three males that had been extended and used as fresh versus frozen-thawed semen and inseminated at four different times relative to ovulation, was employed. Each subclass was replicated with four does. Thus, 96 females were used (3 × 2 × 4 × 4).

Animals

All males and females were sexually mature Dutch-belted rabbits raised in our colony. They were housed in animal rooms maintained at about 20°C, with lighting controlled by a time clock at 12 h of artificial light.

Semen Collection and Evaluation

The males chosen as semen donors produced semen with good sperm concentration and motility. They were ejaculated on a regular schedule two to three times per week, with two ejaculates collected on any single day (Bredderman et al., 1964). After semen collection, any gel plug was removed with forceps. The volume of the semen was measured in a calibrated semen collection tube. Sperm concentration was estimated with a hemacytometer. The percentage of motile sperm in fresh semen was estimated subjectively by diluting the semen, placing a drop on a microscope slide at 37°C, and determining the progressively motile sperm in three to five fields with a video camera connected to the microscope and a television monitor.

The proportion of live and dead sperm was estimated by staining them with trypan blue. These two classes of sperm were further divided into those with normal intact or abnormal (loose, deformed, or lost) acrosomes, which could be seen clearly by applying Giemsa stain to the slides first stained with trypan blue. The method used in the present study was a modification of the procedure described by Didion et al. (1989). We found that various combinations of trypan blue in defined medium (Brackett and Oliphant, 1975) and in fertilization medium (Dodds and Seidel, 1984) with bovine serum albumin caused clumping of sperm and damaged the acrosome. Replacement of bovine serum albumin with polyvinyl alcohol or by reducing the centrifugal force used in washing sperm, from 700 × *g* to 500 × *g*, prevented clumping. Also, egg yolk in the egg yolk-acetamide extender used for freezing rabbit sperm interfered with staining, so this component was removed by washing the sperm. The procedure finally adopted is described briefly.

Fresh or frozen-thawed sperm were washed by centrifugation with 1 M acetamide without egg yolk (pH = 7.2) and resuspended. Equal parts of semen and 1% trypan blue were incubated for 10 min at 37°C in a water bath. Sperm were further extended with 1 M acetamide and centrifuged at 500 × *g* for 6 min to remove the background trypan blue. After resuspension in 1 M acetamide, this sperm suspension was smeared on a glass slide, dried, and fixed with 10% formalin for 30 min.

Slides were placed in a 10% solution of Giemsa stain in distilled water and stored overnight. The slides were rinsed with distilled water, dried, and covered with Permount and a coverslip. One hundred sperm from each ejaculate were counted to determine the proportions of stained sperm with normal or abnormal acrosomes versus unstained sperm with normal or abnormal acrosomes (Didion et al., 1989).

Semen Extension, Freezing, and Thawing

Semen was processed as reported by Chen et al. (1989). The extender consisted of 20% egg yolk (vol/vol) and 1 M acetamide (Hanada and Nagase, 1980: Arriola, 1982; Chen et al., 1989). The pH was adjusted to 7.2 by adding drops of 1 N NaOH. One part of semen was mixed with six parts of extender initially and placed in small conical tubes surrounded by 1-liter water jackets at 37°C and set in a cold room at 4 – 5°C. Cooling the extended semen required 4 h; the extended semen then was packaged in plastic straws containing 0.5 ml. Straws were sealed with polyvinyl powder and held in a 5°C water bath for 30 min to assure complete sealing.

The straws were placed horizontally in a rack approximately 4 cm above a reservoir of liquid nitrogen. After 15 min in this position, straw temperature was about −110°C. Straws then were plunged into liquid nitrogen and later transferred to liquid nitrogen storage units.

Just prior to insemination, the semen was thawed by direct transfer of straws from liquid nitrogen to a water bath at 45°C. After 20 s, the straws were removed from the bath and dried; the contents were emptied into small test tubes prewarmed in the water bath at 37°C. Then, the percentage of motile sperm was estimated as described for fresh semen. Also, aliquots of frozen semen

TABLE 1. Characteristics of fresh and frozen rabbit sperm.

Group	Buck no.	Motile sperm (%)	Live-dead and acrosomal status (%)			
			Live sperm		Dead sperm	
			Normal	Abnormal	Normal	Abnormal
Fresh semen	1	85	83	0	7	10
	2	90	87	2	0	11
	3	85	81	0	7	12
Means		87	83[a]	1[a]	5[a]	11[a]
Frozen semen	1	40	36	16	10	38
	2	40	30	6	23	41
	3	40	36	7	27	30
Means		40	34[b]	10[b]	20[b]	36[b]

[a,b]Means within columns comparing fresh with frozen semen with different superscripts differ, $p<0.05$.

were processed and stained with trypan blue and Giemsa stain, as described previously, to estimate the proportion of live sperm and to evaluate sperm acrosomes.

Insemination and Induction of Ovulation

The fresh semen was further extended so that each milliliter contained 5×10^6 total sperm. By inseminating 0.4 ml of extended semen per doe, approximately 2×10^6 total sperm were inseminated. The frozen-thawed sperm were extended to 10×10^6 total sperm/ml, thus providing 4 million total sperm per insemination. In preliminary studies, freezing appeared to reduce the proportion of motile sperm to about half of the original number, consistent with an earlier report (Chen et al., 1989). Thus, it was expected that nearly equal numbers of fresh and frozen-thawed motile sperm would be inseminated.

To induce ovulation, does were injected i.m. with 0.3 μg of gonadotropin-releasing hormone (Buserelin, Hoechst-Roussel Agri-Vet Co., Somerville, NJ) at four different times, 10 or 5 h before insemination, at the time of insemination, or 5 h after insemination. Insemination was performed at one time. The resulting intervals from insemination to ovulation were expected to be 0, 5, 10, and 15 h, assuming 10 h would elapse from Buserelin injection to ovulation. This series of time intervals was selected to determine both the relative maintenance of fertilizing potential of fresh and frozen sperm from different males and the ability of fresh and frozen sperm from different males to capacitate quickly and fertilize eggs while they were still viable. After insemination, the females were allowed to complete a pregnancy. Nestboxes were provided, and the number and condition of each offspring at birth was recorded.

Statistical Analysis

The frozen versus fresh semen and four times of insemination were considered "fixed" and animals were considered "random" in a mixed model (SAS, 1985) package used for analysis of variance. Females within the smallest subclasses formed the residual error term. Differences among individual treatment means for litter size were tested for statistical significance by Duncan's multiple range test. Differences in the proportion of does pregnant were tested by chi square analysis. Probabilities of $p \leq 0.05$ were considered to be statistically significant; when the p values from the analysis of variance were >0.05, the actual p values are given.

RESULTS

Semen collected from the three males ranged in sperm concentration from 220×10^6 to 630×10^6 sperm/ml. Consequently, the final extension rates to yield 5×10^6 and 10×10^6 sperm per ml (2×10^6 vs. 4×10^6 total sperm inseminated in 0.4 ml) were high.

The results of subjective estimation of sperm motility, objective estimates of the proportion of stained sperm, and condition of the acrosomes are shown in Table 1. It is clear from Table 1 that the percentage of motile sperm and unstained sperm were reduced by freezing and thawing to approximately one-half of the original values. Also, there was a significant ($p<0.01$) increase in sperm with abnormal or lost acrosomes after freezing.

Pregnancy results and litter size following the insemination of 96 does, summarized by bucks and times of insemination with fresh or frozen semen, are in Tables 2, 3, and 4. The main sources of variation in the model, males and time of insemination, were significant (p =

TABLE 2. Effect of bucks and time of insemination with fresh and frozen semen on the proportion of does giving birth.

Type of semen	Buck no.	Does giving birth after being inseminated at different times before ovulation (h) 15	10	5	0	Overall
Fresh	1	4/4	4/4	4/4	0/4	12/16
	2	1/4	1/4	4/4	3/4	9/16
	3	3/4	4/4	2/4	2/4	11/16
Overall		8/12	9/12	10/12	5/12	32/48
Frozen	1	2/4	3/4	3/4	0/4	8/16
	2	0/4	1/4	2/4	3/4	6/16
	3	2/4	4/4	3/4	3/4	12/16
Overall		4/12	8/12	8/12	6/12	26/48
Combined	1	6/8	7/8	7/8	0/8	$20/32^{ab}$
	2	1/8	2/8	6/8	6/8	$15/32^{b}$
	3	5/8	8/8	5/8	5/8	$23/32^{a}$
Overall		$12/24^{A}$	$17/24^{B}$	$18/24^{B}$	$11/24^{A}$	58/96

[ab]Buck means with different superscripts are different, $p<0.05$.

[AB]Time of insemination means with different superscripts are different, $p<0.05$.

0.03 to $p<0.001$) on the basis of both pregnancy rate (Table 2) and litter size (Tables 3 and 4). Also, the interaction between males and time of insemination was significant ($p<0.001$). This interaction was due to the fact that sperm from one male were more fertile than other sperm for longer periods before ovulation, and sperm from another male were more fertile when insemination occurred within 5 h of ovulation. From the analysis of variance, probabilities obtained for interactions were as follows: for males × type of semen (fresh vs. frozen), $p = 0.16$; for type of semen and time of insemination, $p = 0.62$; and for the three-way interaction of these variables, $p = 0.67$. Because these interactions were not significant, they were pooled, and main effects were tested for statistical significance, as indicated in the tables. There was no difference in litter size resulting from fresh versus frozen semen when only pregnant does were considered (Table 4), but the difference was significant when all does were included ($p<0.05$).

DISCUSSION

Viability and Acrosomal Morphology of Sperm

Procedures for supravital staining of sperm combined with Giemsa staining of the acrosome developed for other species (Watson, 1975; Didion et al., 1989) were not completely suitable for rabbit sperm. Procedures were developed for both fresh and frozen rabbit sperm that were useful in identifying live and dead cells and the conditions of the acrosome.

Freezing rabbit spermatozoa caused a marked reduction in the proportion of progressively motile sperm and

TABLE 3. The differential effect of time of insemination with different bucks on litter size computed for all does and for pregnant does only.

Buck no.	Means based on	Litter size following different insemination times (h)[a] 15	10	5	0	Overall buck means
1	All does	4.6	6.5	5.0	0.0	4.0^{b}
2		0.5	0.9	3.0	2.7	1.8^{c}
3		3.1	5.4	3.5	2.4	3.6^{b}
Overall		2.8^{AB}	4.2^{A}	3.8^{A}	1.7^{B}	—
1	Pregnant does only	6.2	7.4	5.7	0.0	6.5^{b}
2		4.0	3.5	4.0	3.7	3.8^{c}
3		5.0	5.4	5.6	3.8	5.0_{bc}
Overall		5.5^{AB}	6.0^{A}	5.1^{AB}	3.7^{B}	—

[a]Times are hours from insemination to expected ovulation.

[bc]Buck means with different superscripts within the two groups of does are different ($p<0.01$).

[AB]Means for insemination times with different superscripts are different.

TABLE 4. Litter size with fresh and frozen semen inseminated at different times, based on pregnant does and all does.

Group	Insemination to ovulation (h)	Mean litter size		
		Fresh	Frozen	Overall
All does	15	3.8	1.8	2.8[ab]
	10	4.8	3.8	4.3[a]
	5	4.5	3.2	3.8[a]
	0	1.8	1.7	1.7[b]
Overall		3.7[a]	2.6[b]	—
Pregnant does	15	5.6	5.2	5.5[ab]
	10	6.3	5.6	6.0[a]
	5	5.4	4.7	5.1[a]
	0	4.2	3.3	3.7[b]
Overall		5.5[a]	4.8[a]	—

[ab]Means for fresh and frozen semen within groups differ ($p<0.05$).

in the proportion of unstained sperm ($p<0.01$), in agreement with results reported previously (Chen et al., 1989). Also, the proportion of sperm with abnormal and lost acrosomes after freezing and thawing was significantly increased for all males ($p<0.01$). There was a nonsignificant trend for sperm from different males to be more severely affected by freezing then others (Table 1), but differences were smaller than those obtained from the breeding trial (Tables 2 – 4). It is possible that incubating fresh and frozen sperm and testing the changes in sperm quality during incubation would provide a more sensitive indication of the relative fertility of different males when semen is inseminated at different times. In subsequent studies with these males, sperm from the buck with lower fertility (Buck 2, Table 3) did not survive well when incubated for a few hours.

Pregnancy Rates and Litter Size

The pregnancy rate was different for the three males and also at the different insemination times (Table 2). Furthermore, 26 of 48 does inseminated with frozen semen were pregnant and produced 127 young. In contrast, 32 of 48 does inseminated with fresh semen were pregnant and produced 175 young. The difference in number of young born as a result of inseminating an equal number of does with fresh and frozen semen is primarily due to the pregnancy rate. Although average litter size based on all does was smaller ($p = 0.03$) with frozen semen (Table 4), the average litter size for pregnant does was 5.5 for fresh semen and 4.8 for frozen semen ($p = 0.23$).

Buck 2 was less fertile than the other two males (Table 3), although initial semen quality was similar. The pregnancy rate (Table 2), as well as litter size, was less than was obtained with semen from the other two males. This male did have the highest proportion of stained sperm following freezing. When litter sizes from pregnant does only (Table 3) was summarized separately for frozen versus fresh semen, the litter size associated with Bucks 1, 2, and 3 for fresh semen was 6.7, 4.7, and 5.0, and for frozen semen was 6.1, 2.5, and 5.0. The litter size achieved with frozen semen from Buck 2 was particularly low, but the interaction of bucks × types of semen was not statistically significant ($p = 0.16$). Nevertheless, this result suggests that to obtain best results with frozen semen, it would be helpful to select males pretested to produce high pregnancy rates and normal litter size. Optimal thaw rates also need to be established (Bamba and Cran, 1988). Differences in fertility (Maurer et al., 1976) or lack of differences in fertility between fresh and frozen semen (Arriola, 1982; Weitze et al., 1982; Chen et al., 1989) may have been associated with the bucks used. Chen et al. (1989) used mixed semen from several males. In this situation, with more than enough sperm inseminated, most of the pregnancies could have resulted from the sperm produced by the most fertile buck in the pooled semen.

Parrish and Foote (1986) obtained data indicating that capacitation time in rabbits can be altered by freezing. Chen et al. (1989) found that freezing sperm increased the proportion of acrosome-altered sperm but that any change in capacitation time associated with freezing was not detectable by inseminating does at different time intervals and examining fertility results. The present experiment provided a more sensitive test of this hypothesis. Fewer sperm were used for insemination, more closely spaced intervals from insemination to ovulation were tested, and semen from the three

males was processed and used separately. A very high interaction between bucks and time of insemination resulted (Table 3). Buck 1 produced sperm that fertilized a high proportion of oocytes 15, 10, and 5 h before ovulation, but evidently sperm could not capacitate quickly enough to fertilize oocytes ovulated about the same time as insemination (Table 2). Few does became pregnant after insemination with sperm from Buck 2 that were placed in the female tract 15 and 10 h before ovulation, but pregnancy rates were satisfactory after insemination 5 and 0 h before ovulation. Thus, Buck 2 appeared to produce sperm that could not survive for a long time in the female, but the sperm could capacitate rapidly and fertilize the oocyte while the oocytes remained viable enough to sustain a pregnancy. Finally, Buck 3 appeared to produce sperm capable of retaining fertilizing ability for extended periods (15 h) in the female as well as sperm that capacitated quickly. This result is consistent with previous studies which indicated that sperm from bucks differed in capacitation time (Parrish and Foote, 1986; Kim et al., 1989).

If the semen from the three bucks had been mixed, as was done previously (Chen et al., 1989), fertility results at early and late insemination might not have been greatly different. Sperm from Bucks 1 and 3 could fertilize most of the oocytes 15 h before ovulation and sperm from Bucks 2 and 3 could fertilize many oocytes when insemination occurred near the time of ovulation. This provides the basis for speculating that possible in vitro treatments to delay capacitation and other treatments to partially capacitate sperm could have practical application in livestock breeding by artificial insemination. Providing such a mixture of sperm cells for insemination occurring "early" and "late" relative to ovulation may improve fertility results.

All of the fertility comparisons made here were based upon total number of pregnancies. No attempt was made to measure the proportion of oocytes fertilized that could have died, with the result that some does did not produce litters or produced small litters. Maurer et al. (1969) obtained fertilized eggs from some does inseminated as late as 10 h after expected ovulation. However, when these eggs were placed in culture, none developed to the blastocyst stage. The present results are consistent with the earlier in vitro work.

From these studies, we have concluded that it is important not to pool semen in reproductive studies of this type because important major effects and interactions may be masked. Bucks differed greatly in fertility, and this difference tended to be increased by freezing sperm. Also, bucks interacted in a major way with time of insemination, indicative of differences in sperm capacitation times. The use of low sperm numbers probably increased the sensitivity of the test for treatment effects. Our findings have implications for treating semen and improving fertility of livestock when insemination at precise times relative to ovulation is not possible.

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