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SPERMATOZOAL VELOCITY AND MOTILITY AND ITS RELATIONSHIP TO FERTILITY IN THE RABBIT INSEMINATED WITH LOW SPERM NUMBERS

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ABSTRACT: The objective of this study was to examine the relationship between velocity and fertility of rabbit sperm, using low sperm numbers per insemination. Semen was collected weekly for 5 weeks from two fertile males. To study the effect of high dilution of sperm in media without macromolecules, each semen sample was split with one portion retained as whole semen, and the remainder was centrifuged and washed with saline. The washed sperm were resuspended with seminal plasma (SP), phosphate-buffered saline (PBS), or PBS containing 1% (wt/vol) of bovine serum albumin (BSA). Each week two non-lactating does per buck were each inseminated at the cervix with 0.5 X 10^6 total sperm, for a total of 80 does inseminated. This was followed immediately with injection of luteinizing hormone. Sperm were video taped at this time. The velocity of 25 sperm from each semen sample was determined by each of two observers (50 sperm total). The two observers also estimated the percentage of motile sperm. Fertilized and unfertilized oocytes (1635 total) were recovered from the oviducts 42 hours after insemination. Both the percentage of motile sperm and their velocity were greatly affected by the washing and diluting fluid used. The ranges for these two variables, respectively, were 1 to 50% and 11 to 100 Fm/second. The percentage of motile sperm and velocity were highly correlated ($P<0.05$) in the different treatments (BSA=0.77; SP=0.59; PBS=0.81; WS=0.86). Fertility ranged from 42 to 85%. Velocity was not more useful than the percentage of motile sperm in predicting fertility. The importance of including macromolecules in the diluting fluids to maintain motility of highly diluted sperm was obvious from the five-fold or greater improvement in motility and velocity of sperm by adding BSA. Good fertility was obtained with low sperm numbers inseminated on the cervix in media containing macromolecules, even following extensive washing and storage.

INTRODUCTION

The commercial success of artificial insemination (AI) of any species depends on the extensive use of genetically superior males to impregnate a large group of females with relatively low doses of sperm per insemination. This requires high quality semen which can be assessed in various ways (FARRELL *et al.*, 1993; CASTELLINI *et al.*, 1996; CASTELLINI *et al.*, 2000; BRUN *et al.*, 2002; DUCCI *et al.*, 2002). The number of possible inseminations per ejaculate of semen depends on the number of viable sperm in the ejaculate and the number required to inseminate each female. The required number per insemination depends on semen handling (diluters and length of preservation), and on the genetic strain and physiologic state of the female (FARRELL *et al.*, 1993, 1996; CASTELLINI and LATTAIOLI, 1999; CASTELLINI *et al.*, 2000; BRUN *et al.*, 2002). Whereas under commercial conditions up to 13.1 x 10^6 sperm per AI may be required (CASTELLINI and LATTAIOLI, 1999), under experimental conditions with non-lactating Dutch females as few as 0.5 x 10^6 sperm results in a high conception rate (FARRELL *et al.*, 1993).

One of the characteristics of bull sperm which is highly correlated with fertility of bulls used in AI is velocity (FARRELL *et al.*, 1998). Although velocity of rabbit sperm has been measured (FARRELL *et al.*, 1993; DUCCI *et al.*, 2002), only one report relating velocity of rabbit sperm to fertility was found (WILLIAMS *et al.*, 1990). The objectives of the present study were to compare the motility and velocity of rabbit sperm with the fertilization of oocytes obtained following mixing the sperm with various diluters, and inseminating females with relatively few sperm.

MATERIAL AND METHODS

Animals

Sexually mature Dutch rabbits were housed in individual cages in a facility maintained at 21°C, with a 12 hours light:12 hours dark cycle. Animals were

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cared for according to standards established by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC).

Semen collection, processing and insemination

Semen was collected weekly for 5 weeks from two males of known fertility. The gel was removed, and the semen was evaluated for quality as described by FARRELL *et al.* (1993). During processing the semen was held at approximately 25 °C. An aliquot of the ejaculate was retained as whole semen (WS). The remainder of the ejaculate was centrifuged twice and washed with saline to separate the sperm from macromolecules in the seminal plasma. The separated sperm were resuspended in either seminal plasma (SP), phosphate-buffered saline (PBS), or PBS containing 1% bovine serum albumin (BSA) at concentration of 5 x 10^6 cells per ml. The whole semen was similarly extended in saline, as all the separatory procedures were performed in saline. This rigid processing of semen, and insemination with low sperm numbers was employed in an attempt to provide a critical test of the velocity, motility and, fertility of sperm. Two non-lactating does per buck-treatment were inseminated each week with 0.1 ml of each sample. Each doe then received an ovulatory dose of LH, and 42 hours after insemination the does were laparotomized to recover the fertilized and unfertilized oocytes (FARRELL *et al.*, 1993).

Measurement of velocity

To measure *in vitro* semen quality at the time of insemination, immediately after insemination an aliquot of each sample was placed on a slide on a stage warmer at 37 °C and recorded on video tape. Sperm that were not obstructed by other sperm were individually tracked. The sperm selected had to travel at least 20 mm collisions with other sperm to be included. Because of the high dilution and low sperm motility this was done manually. The distance travelled per second was recorded. Two observers each recorded the velocities of 25 different randomly selected sperm for each semen sample. The two observers also determined the percentage of motile sperm. Because of the multiple processing of the sperm the elapsed time from semen collection to video recording was 2 to 2.5 hours, a much longer time than is required for routine semen collection, evaluation and insemination.

Statistical analysis

The experimental design consisted of two randomly selected fertile males with four fixed treatments applied to each of five ejaculates nested within males, used to inseminate 80 randomly selected does. Data were analyzed by analysis of variance (STEEL and TORRIE, 1960). Fisher's LSD method (STEEL and TORRIE, 1960) was used to test differences among means. Correlation coefficients were calculated across the 10 samples of semen.

RESULTS

The percentages of motile cells, spermatozoal velocity, and fertility obtained for treated semen from each male are listed in Table 1. The velocity of spermatozoa averaged over both bucks in BSA was 89.8±3.5 μm/s whereas that of the SP treatment was 54.5±4.9 μm/s and that of the whole semen was 47.1±8.5 μm/s ($P<0.05$). Sperm in PBS had a lower velocity, 13.1±4.0 μm/s, ($P<0.01$) than all other treatments. Corresponding values overall for motility were 34.3±3.5, 39.5±4.9, 4.5±4.0 and 13.6±8.5%.

The percentage of motile sperm and velocity were correspondingly low in PBS, but there was considerable divergence in WS. The correlation coefficients between motility and velocity of sperm in BSA, SP, PBS and WS respectively, were 0.77, 0.59, 0.81, and 0.86 ($P<0.05$). Higher fertility was obtained with the two bucks when semen was highly diluted with PBS plus BSA compared with PBS containing no macromolecules ($P<0.05$), although the effect was only substantial for the first male. The only significant

Table 1: Means (±S.E.) of motility and velocity of 10 samples of rabbit sperm treated four ways and fertility of 80 inseminated does (10 does per treatment per male).

Male	Semen treatment	Motile sperm (%)	Velocity (μm/s)	Oocytes-embryos	
				Number collected	Fertile (%)
1	BSA	42.0 ± 5.1[a]	100 ± 10[a]	143	85.3[a]
	SP	49.5 ± 6.0[a]	65 ± 5[b]	194	68.6[b]
	PBS	8.0 ± 8.0[b]	15 ± 15[c]	229	41.9[c]
	WS	3.7 ± 4.3[b]	53 ± 23[b]	232	63.4[b]
2	BSA	26.6 ± 7.1[c]	79 ± 11[b]	176	63.6[b]
	SP	29.5 ± 10.3[c]	44 ± 19[bc]	177	72.3[ab]
	PBS	1.0 ± 1.0[b]	11 ± 11[c]	280	55.0[bc]
	WS	23.5 ± 10.7[c]	41 ± 15[bc]	205	78.5[ab]

BSA: bovine serum albumin, SP: seminal plasma, BS: phosphate-buffered saline, WS: whole semen. For details, see text.
Within columns, numbers with different superscripts differ, $P<0.05$.

positive correlation between the percentage of motile sperm and fertility of individual semen samples was between motility and fertility of sperm in PBS (r=0.68, $P<0.05$). No increase in hyperactivity of sperm characteristic of capacitation was observed.

DISCUSSION

Although velocity of rabbit sperm has been examined (CASTELLINI *et al.*, 2000; DUCCI *et al.*, 2002), only one study was found in which fertility also was measured (WILLIAMS *et al.*, 1990). In studies with bull semen used for commercial AI, velocity of sperm was found to be significantly correlated with fertility. Combining motility and velocity in a multiple regression equation for bull sperm resulted in a correlation of 0.82, whereas with motility alone the correlation was only 0.58 (FARRELL *et al.*, 1998).

Rabbit sperm velocity was not significantly correlated with fertility in the present studies. There are several possibilities why this was so, in addition to the fact that more bucks could increase the sensitivity of the test. One reason could be the extensive semen processing procedure. In order to obtain the sperm free from the seminal macromolecules and treat the initial semen sample equally before splitting it into individual treatments, it was twice centrifuged to remove seminal plasma. This processing, plus high dilution, placed considerable stress on the sperm, as evidenced by the effects on motility of sperm (Table 1) by the time of insemination. The whole semen was extended with saline which was not buffered during the 2.5 hours of storage.

These studies provide support for the importance of macromolecules in sperm preservation, even for short periods, as motility (%) of sperm from both males and velocity of sperm from one male were much lower in PBS than the sperm in PBS supplemented with BSA. Fertility also was generally better maintained when macromolecules were present. The beneficial effects of macromolecules from BSA or SP is consistent with previous reports (FARRELL *et al.*, 1993, 1996; CASTELLINI and LATTAIOLI, 1999). The fact that fertility differences were less pronounced, is likely due to the presence of many nonmotile sperm in vitro that remained viable.

Observations by FOOTE (1998) revealed that much of the loss in sperm motility during a few hours of storage can be restored by adding PBS-glucose-BSA to sperm in PBS. The female reproductive tract likely could produce the same beneficial effect following insemination.

Under these circumstances, with 1636 eggs collected, but only two males used, no significant positive correlations between velocity and fertility were obtained and only sperm motility in SP was significantly correlated with fertility. However, all correlations between sperm motility and velocity in BSA, SP, PBS and WS, ranging from 0.59 to 0.86, were significant. Thus, under these conditions velocity added little to the information already gained from estimating the percentage of motile sperm.

Another aspect of considerable practical significance is the low number of sperm required for insemination to achieve high fertility. Lactation and sexual receptivity may affect the number of sperm required for optimal fertility (CASTELLINI, 1996; CASTELLINI and LATTAIOLI, 1999). CASTELLINI *et al.* (2000) reported that non-lactating receptive does required from 1.6 to 4.7 x 10^6 motile sperm per insemination, but the numbers inseminated usually are much greater (LÓPEZ and ALVARIÑO, 2000; BRUN *et al.*, 2002).

In our studies over many years mostly with non-lactating Dutch rabbits , but also with New Zealand White rabbits (FOOTE *et al.*, 1963), we have found no difference in fertility of nonlactating rabbits whether they are receptive or not, and FSH-primed or not, provided the does are given LH or GnRH at the time of insemination. Under these circumstances we obtained an 88% kindling rate (FOOTE and SIMKIN, 1993) without selection for receptivity, and a 95% fertilization rate with 1 x 10^6 total sperm inseminated as close to the entrance of the cervical canals as possible (FARRELL *et al.*, 1993). In the present study we used only 0.5 x 10^6 total sperm, which had been stressed by extensive processing. Yet in the best diluting fluid used 72 and 85% fertilization was achieved. WILLIAMS *et al.* (1990) reported no difference in fertility when 1 x10^6 or more motile sperm were inseminated.

At least two important factors are associated with our obtaining high fertility with low sperm numbers in non-lactating does. These were the site of semen deposition and the volume of semen inseminated, as is known from many large studies with cattle and sheep (EVANS and MAXWELL, 1987). Fewer sperm are lost from the vagina when small volumes of semen are inseminated. Originally, different fertility results were obtained in our colony by various inseminators when using low sperm numbers. When 10x10^6 sperm in 1 ml were inseminated on the cervices compared with 1x10^6 sperm in 0.1 ml (Unpublished, FOOTE, 1990),there was no difference in fertility (91% pregnant in both groups). Subsequently we trained all inseminators to pass the catheter a considerable distance beyond the pelvic arch until the tip pressed gently against the cervix (FARRELL *et al.*, 1993). Secondly, we inseminated a small volume of semen with more concentrated sperm (usually 0.2 ml), rather than larger volumes of less concentrated sperm. These procedures routinely practiced in our research colony result in 85-90% kindling in unselected nonlactating does (FOOTE and SIMKIN, 1993). Our research colony also is maintained at 21 °C, avoiding stress of rabbits that can affect fertility when the environmental temperature exceeds 28 °C (FOOTE, 2002).

In lactating rabbits time of insemination post-partum is important (CASTELLINI, 1996). PAUFLER *et al.* (1979) inseminated lactating New Zealand rabbits repeatedly at 32-35-day intervals for one year on Days 1, 2, 3 or 4 postpartm, with GnRH primarily given at the time of insemination. When inseminated on Day 2 postpartum, 78% kindled, whereas only 49% kindled when inseminated on Day 4 postpartum. These

lactating rabbits received no PMSG and were not selected for receptivity. Thus, this simple system of Day 2 post-partum isemination may have commercial application. No hormones were used that cause antibody formation.

The current study is consistent with the literature indicating that macromolecules in the diluter and low dilution rates are desirable (CASTELLINI, 1996). This and previous studies also strongly indicate that sperm numbers required for optimal fertility can be reduced if care is taken to use deep vaginal-cervical insemination, an important factor in commercial AI of rabbits. Likewise, the literature indicates that early postpartum insemination using non-antibody forming GnRH to induce ovulation is beneficial. Velocity measurements did not provide more information to judge fertilizing potential of sperm than estimates of the percentage of motile sperm. However, large tests under conditions not available to the authors, such as those reported by CASTELLINI and LATTAIOLI (1999), LÓPEZ and ALVARIÑO (2000), and BRUN *et al.* (2002) are needed to provide further insight on the relationship of rabbit sperm velocity to fertility, using critical numbers of sperm inseminated in small volumes on the cervices using commercial semen extenders.

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