Fertilizing rabbit spermatozoa are not selected as a special population by the female tract

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Summary. The survival of rabbit embryos from eggs fertilized by spermatozoa passing naturally from the vagina to the ampulla of the oviduct was compared with that of eggs fertilized by 'unselected' spermatozoa inseminated directly into the oviduct. After their transfer at the 2-cell stage to recipient does, embryos derived from fertilization with 'unselected' spermatozoa (57%) survived as well as those eggs fertilized by the natural 'selected' ampullary population (62%). Spermatozoa taken from the uterus 4 h *post coitum* ('selected' by virtue of having passed the cervix) were not competitively superior to an equal number of spermatozoa from the ejaculate of the same buck. The results do not support the suggestion that the reproductive tract of the female rabbit selects a genetically elite subpopulation of spermatozoa from the ejaculate.

Introduction

In most mammals, many millions of spermatozoa are inseminated to ensure the fertilization of a very few ova. Moreover, a variety of studies show that only about 0.001%, or even less, of those spermatozoa reach the fertilization site (Austin, 1948; Braden & Austin, 1954; Mattner, 1963; Overstreet, Cooper & Katz, 1978). The large discrepancy between the number of spermatozoa inseminated and that reaching the ampulla of the oviduct in the rat led Blandau & Odor (1949) to suggest that perhaps the few reaching the site of fertilization may possess special qualities. Cohen (1967, 1973) has postulated that the majority of spermatozoa experience chiasmatic errors during meiosis and are, therefore, genetically unfit. Cohen & McNaughton (1974) concluded from experimental studies that the rabbit female reproductive tract restricts the passage of genetically unfit spermatozoa. Overstreet & Katz (1977) also suggested that an elite sperm population attains the oviduct. There is little question that morphologically abnormal spermatozoa tend to be restricted at the cervix or utero-tubal junction or both (Krzanowska, 1974; de Boer, van der Hoeven & Chardon, 1976; Mortimer, 1977). However, the fitness of all or some of the spermatozoa that reach the ampulla and fertilize is still questionable. The present studies were conducted to determine whether oviducal spermatozoa have a relatively superior ability to fertilize successfully. The ability of spermatozoa recovered from the uterus to compete with comparable numbers of ejaculated spermatozoa from the same male has also been examined.

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Materials and Methods

Animals

Sexually mature, virgin New Zealand White rabbits, at least 7 months old and weighing 3.0–4.5 kg, were purchased from commercial breeders. Animals were housed under standard laboratory conditions. Females were caged individually for at least 18 days before use.

Reagents

Dulbecco's phosphate-buffered saline (PBS) and Ham's F-10 nutrient mixture with L-glutamine were purchased from Gibco, Grand Island, New York. Immediately before use, Ham's medium was equilibrated with a gas mixture consisting of 5% CO₂ in air. Ficoll (Type 400, DL), fluorescein isothiocyanate (FITC), human chorionic gonadotrophin (hCG), K-penicillin G and streptomycin sulphate were purchased from Sigma Chemical Company, St Louis, Missouri.

Genetic fitness of oviducal spermatozoa

To test the hypothesis that spermatozoa reaching the site of fertilization are genetically superior to the majority in the ejaculate, semen was collected with an artificial vagina from mature bucks of proven fertility. To remove the seminal plasma, the ejaculate was layered onto 10 volumes of sterile 10% Ficoll in PBS, centrifuged at ~700 g for 10 min, decanted, resuspended in 1 ml sterile PBS containing 0.1% glucose, and placed in an incubator at 37°C until used. Samples displaying <50% progressive motility, or containing <100 × 10⁶/ml spermatozoa, were discarded.

For each experiment, a rabbit was anaesthetized with pentobarbitone sodium (50 mg/kg, i.v.), one oviduct was exposed through a flank incision under aseptic conditions, and a silk ligature was placed immediately above the uterotubal junction. Sperm numbers ranging from 0.2 to 0.8 × 10⁶ were instilled in approximately 20 µl via the tubal ostium into the ampulla. The remainder of the washed ejaculate was inseminated vaginally as far as the cervix, the time elapsing from collection being 45–60 min. The ligature prevented the vaginally inseminated spermatozoa from migrating into the ipsilateral ampulla, ensuring that ova on that side would be fertilized only by unselected spermatozoa instilled directly. Ova on the contralateral side would be fertilized by a population transported naturally and thereby subjected to possible selection. For convenience, this sperm population is referred to hereafter as 'selected'. After a sham operation was performed on the contralateral side, 75 i.u. hCG were administered to induce ovulation. A second (recipient) doe was given hCG 2–5 h later.

The inseminated doe was killed 26–30 h after surgery. The entire length of the oviducts was flushed with warm (37°C) Ham's F-10 medium containing 100 i.u. K-penicillin G/ml and 50 µg streptomycin sulphate/ml and the ova were transferred to fresh Ham's medium under mineral oil at 37°C. The embryos were only used when at least one 2-cell embryo was obtained from each side and when there was a total of at least 4 from both sides. The recipient doe was anaesthetized and the 2-cell embryos were transferred via flank incisions with a fine pipette into contralateral tubal ampullae. No more than 5 embryos were transferred into one oviduct.

A laparotomy was performed 10–12 days later and the implantation sites in each uterine horn were noted. Finally, the recipients were autopsied at 28–30 days of gestation and the number of live, dead and resorbed fetuses was recorded. The reproductive tract of each fetus was excised and examined under a dissecting microscope to confirm the sex of the fetus.

Competition between selected and unselected spermatozoa

The relative ability of spermatozoa to pass through the reproductive tract and penetrate ova after re-insemination in a second female was compared with that of ejaculated spermatozoa. For

convenience, this comparison is termed 'competition'. Spermatozoa were flushed from the uterus of a donor doe 3.5–4 h after mating or vaginal insemination. This timing ensured the recovery of the vanguard spermatozoa, since very few attain the oviducts before 4 h after insemination (Overstreet *et al.*, 1978). These uterine spermatozoa were then inseminated into the vagina of a recipient doe together with a comparable number taken directly from a fresh ejaculate (Group I, Text-fig. 1).

The donor doe was killed with pentobarbitone sodium given i.v. 3.5–4 h after mating or artificial insemination and haemostats were quickly placed on the utero-tubal junctions. Sterile Dulbecco's PBS with 0.1% glucose and 5% heat-treated rabbit serum (2 ml at 37°C) was injected carefully through the cervix with a blunt needle into the uterine horn and then haemostats were placed at least 2 cm above the cervix. The distended uteri were then excised, thoroughly washed and blotted. This procedure minimized blood contamination, prevented contamination of the uterine populations by cervical spermatozoa, and restricted recovery to the upper two-thirds of the uterus, thus maximizing the recovery of vanguard spermatozoa only. After collecting the fluid contents in a silicone-coated tube, each horn was rinsed with an additional 1 ml PBS. The washings were pooled and centrifuged at 500 *g* for 10 min; the solution was decanted, the pellet resuspended gently in 0.2 ml PBS and placed in an incubator at 37°C until needed. The motile spermatozoa in an aliquant were counted on a haemocytometer slide and samples containing $<1 \times 10^5$ motile spermatozoa were discarded.

Competition between the two samples of spermatozoa was assessed always by using the same buck for both, thereby avoiding the possibility of selective fertilization between males (Overstreet & Adams, 1971). To distinguish the populations, ejaculated spermatozoa were labelled with FITC by the method described by Overstreet & Adams (1971). After first counting the cells in an aliquant of each population with a haemocytometer, ejaculated spermatozoa were mixed with the 'selected' population in a 5:1 ratio. The mixture was then inseminated within 1 h of sperm collection through a small midline abdominal incision directly into the anterior vagina of the anaesthetized recipient doe. Immediately thereafter, 75 i.u. hCG were administered intravenously to induce ovulation.

Text-fig. 1. Experimental design used for preparing the different populations of spermatozoa. In each case, one population was distinguished from the other in mixed inseminations by labelling it with FITC.

When using small numbers of spermatozoa, surgical insemination achieved higher, more consistent fertilization rates than did conventional artificial inseminations. After insemination, 200 spermatozoa were counted in a small aliquant of the mixture to confirm the exact ratio used. The inseminated doe was killed 18–20 h later. The numbers of FITC-labelled and unlabelled spermatozoa recovered from the vagina, uterus and oviducts, and present on the zonae pellucidae and in the peri-vitelline spaces, were compared. All the spermatozoa recovered, or 200 spermatozoa, whichever was the lesser, were counted for each segment of the tract. The FITC label was visualized in a Zeiss phase-contrast microscope equipped with a reflected light fluorescence attachment and filters for FITC.

To ensure that exposure to the uterine or tubal environment itself, as opposed to selection, had no influence on competitive ability, two other experiments (Groups II and III) were included. In Group II, 10×10^6 FITC-labelled spermatozoa were injected into the uterine horns of a mated or artificially inseminated doe approximately 1.5 h before autopsy (Text-fig. 1). Initial attempts to incubate the spermatozoa for longer periods yielded insufficient motile spermatozoa due mainly to extensive formation of sperm-leucocyte clumps after centrifugation. Approximately 4 h after mating, the doe was killed, the uterus flushed and the concentrated sperm preparation, containing about 1×10^6 motile spermatozoa (with approximately a 5:1 ratio of 'selected' to 'unselected' spermatozoa), was inseminated vaginally into a second doe. This doe was killed 18 h later and treated as in Group I. In Group III, a procedure similar to that in Group II was used except that the 'unselected' population consisted of spermatozoa recovered 4 h after being placed into the oviduct of the donor doe near the time of vaginal insemination (Text-fig. 1). This and the 'select' population recovered from the uterus were inseminated vaginally together into a second doe which was killed later.

In at least two trials in all groups, a semen sample was labelled before insemination into the donor doe. This allowed recovery of FITC-labelled, 'selected' spermatozoa, thus controlling for possible effects of the labelling procedure. To establish the equality of spermatozoa in successive ejaculates from the same male and so further validate the experimental design, an additional group (Group IV) was tested in which 2 semen samples were collected consecutively from the same buck. One sample was labelled with FITC and mixed with comparable numbers from the other sample; the mixture was inseminated vaginally into an oestrous doe which was killed 18 h later (Text-fig. 1).

Statistical analysis

The genetic fitness of 'selected' and 'unselected' spermatozoa was compared in 10 trials which were analysed by the Mann-Whitney test. Competition between cervix 'selected' and 'unselected' populations of spermatozoa was evaluated in 6 trials for each group. These data were analysed by the Kruskal-Wallis test.

Results

Genetic fitness of 'selected' and 'unselected' spermatozoa

Ova were equally likely to develop into normal full-term fetuses whether fertilized by selected or unselected spermatozoa (Table 1). There was no significant difference in the ratio of male to female fetuses or in the fertilization rate ('selected' population, 86%; 'unselected', 94%). The percentage of ova recovered, however, was lower on the side receiving tubal inseminations ('selected' population, 88%; 'unselected', 56%; $P < 0.01$). Previous studies suggest that this could be due to the abnormally large numbers of spermatozoa placed in the oviduct and not to the utero-tubal ligation (Adams, 1973). Unilateral tubal insemination also may occasionally result in a fertilized ovum in the contralateral oviduct (Adams, 1973). To test for this possibility, 4 females received unilateral oviducal inseminations and were autopsied 24 h later. All of the 12 ova recovered from the inseminated sides were fertilized, while none of the 13 was fertilized on the sham-operated side.

Table 1. The survival of embryos after fertilization of eggs in the oviduct by 'selected' (vaginally inseminated) or 'unselected' (tubally inseminated) spermatozoa

Sperm population	Total no. of embryos transferred*	Mean % of transferred embryos surviving on:	
		Day 10–12†	Day 30†
Unselected	21	72	57
Selected	32	76	62

* Two-cell embryos were flushed from the oviducts and transferred to synchronized recipients 24 h after insemination. The data represent the results of 10 trials.

† No statistical difference between the two sperm populations ($P > 0.2$, Mann–Whitney test).

Competitive ability of 'selected' and 'unselected' spermatozoa

Comparison of absolute numbers was precluded by considerable variation in the numbers of spermatozoa recovered from the different does inseminated with mixed populations. Therefore, the data were analysed with regard to the ratio of 'unselected' to 'selected' spermatozoa recovered from each region of the female tract. To facilitate this comparison, the ratios in each compartment were normalized to those in the vagina, i.e. the initial ratio of 'unselected' to 'selected' spermatozoa found in the vagina was always expressed as 1.0. A competitive advantage of one population over the other would be reflected in a significant change in the normalized ratio of the two populations recovered from the uterus and oviducts or associated with ova.

The results shown in Table 2 suggest that spermatozoa reaching the oviduct from the vagina possess no competitive advantage over ejaculated spermatozoa. In fact, those that had passed the cervix of one doe appeared less able to reach the ova in a second doe than freshly ejaculated

Table 2. The relative distribution in different regions of the female tract and on tubal ova of 'selected' and 'unselected' spermatozoa after mixed vaginal insemination

Group	Sperm populations compared†	Normalized ratio of 'unselected' to 'selected' spermatozoa‡			
		Vagina	Uterus	Oviducts	Ova-associated
I	Ejaculated : 'selected'	1.0	2.1 ± 0.3	4.6 ± 0.8	9.4* ± 3.2
II	Uterine inseminated : 'selected'	1.0	1.1 ± 0.2	1.6 ± 0.4	3.4 ± 1.6
III	Oviducal inseminated : 'selected'	1.0	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.1
IV	Ejaculated : ejaculated (control)	1.0	0.9 ± 0.1	1.1 ± 0.2	1.2 ± 0.2

* Increase in ratio from vagina to ova is statistically significant ($P < 0.001$ Kruskal–Wallis test). [Changes in other groups are not significant; Group II, $P > 0.1$; III, $P > 0.5$, IV, $P > 0.25$.]

† 'Selected' spermatozoa are those recovered from the uterus 3.5–4 h after natural mating. 'Unselected' spermatozoa are those taken in aliquants from the ejaculate of the same male (Group I), surgically inseminated into the uterus of a donor for 1.5 h before use (Group II), or into the oviducts for 4 h before use (Group III).

‡ Results are expressed as mean ± s.e.m. of 6 trials for each group. Actual ratios varied between 2:1 and 12:1.

spermatozoa since the mean ratio of 'unselected' (freshly ejaculated) to 'selected' spermatozoa increased 9-fold from the vagina to the ova (Table 2). However, if ejaculated spermatozoa had first been exposed to the uterus (Group II) or oviduct (Group III) this effect did not occur. There was virtually no difference in the ratio in any segment of the recipient's reproductive tract (Table 2).

Alteration of the FITC label between sperm populations in different trials did not influence the results (data not shown). Furthermore, two ejaculates collected successively from the same buck competed equally in reaching and penetrating ova (Table 2, Group IV). This confirmed the validity of FITC as a suitable marker, and the qualitative similarity of the sperm population in successive ejaculates.

Discussion

Ova fertilized by spermatozoa placed directly into the oviduct appeared as likely to undergo normal development thereafter as did those fertilized by spermatozoa transported naturally there from the vagina. This speaks against the notion (Cohen, 1967, 1975) that only a very small proportion of the ejaculate are 'perfect' gametes more likely to be selected by the female tract during sperm transport to the tubal ampulla. Nor does it support the possibility that the relatively poor fetal survival observed after fertilization *in vitro* and subsequent embryo transfer in animals is due to fertilization by 'unselected', and so putatively incompetent, spermatozoa (see Overstreet & Katz, 1977). The progressive reduction in numbers during the ascension of spermatozoa through the female reproductive tract does not, therefore, appear to be an important factor for embryonic survival, other than its role, perhaps, in reducing the incidence of polyspermy. Thus, the term 'selected' used here for semantic convenience, is, in fact, inappropriate or irrelevant from a functional standpoint.

Two *in-vitro* studies further contradict the idea that sperm selection is necessary for normal fertilization and embryonic survival. Bavister (1979) has shown that only a few hamster spermatozoa per egg are necessary to effect fertilization *in vitro* and Thadani (1982) has demonstrated that mouse ova fertilized *in vitro* when exposed to a very small number of randomly selected spermatozoa can develop into normal offspring. The random manner in which spermatozoa were chosen in those and the present experiments is the functional equivalent of exposing ova to 'unselected' spermatozoa. These findings all imply strongly that, cell for cell, unselected spermatozoa are able to initiate and support embryonic development as well as the subpopulation transported to the ampulla after natural mating.

The design of the present experiment does not preclude the possible existence of a super-competitive subpopulation of spermatozoa some of which could have been present in the tubally inseminated aliquant. However, assuming that the entire subpopulation is no larger than that normally reaching the ampulla, the number represented in that aliquant would be less than 10. This small number, compared to the total inseminated tubally, minimizes that possibility and the present demonstration that unselected spermatozoa compete equally for ova provides further evidence that such an occurrence would be unlikely.

In addition, the present competition experiments suggest that uterine spermatozoa 'selected' by the cervix, have no greater individual ability to reach the ampulla and penetrate ova than do those alive in the remainder of the ejaculate. These findings again are inconsistent with the conclusion (Cohen & McNaughton, 1974; Cohen & Tyler, 1980) that spermatozoa transported naturally represent an elite population. In both these studies, the competence of naturally transported spermatozoa was compared to that of washed ejaculated spermatozoa following their mixed insemination directly into the uterine horns of a second doe. Other investigators have observed a significant entry of leucocytes within a few hours of uterine insemination in the rabbit (Bedford & Chang, 1962; Howe, 1967; Soupart, 1970) and, although fertilization usually occurs, the percentage of eggs penetrated tends to be lower than after vaginal insemination (Overstreet & Bedford, 1976).

The report on sperm selection by Fischer & Adams (1981) supports the possibility that the results of Cohen & McNaughton (1974) and Cohen & Tyler (1980) reflect an adverse effect of direct uterine insemination on freshly ejaculated spermatozoa. Fischer & Adams (1981) recovered spermatozoa from the uterus of a mated doe and from a doe inseminated *in utero*; neither sperm population displayed a competitive advantage when mixed and re-inseminated together into a recipient a few hours before ovulation.

The results of the present competition experiments also contradict the conclusions of Overstreet & Katz (1977). They inferred a competitive advantage of selected rabbit spermatozoa because spermatozoa recovered from the oviduct after vaginal insemination fertilized a greater proportion of eggs than expected on the basis of the relative number re-inseminated tubally with ejaculated spermatozoa. Those results, however, may reflect the difference in the lower efficiency of capacitation achieved by the oviduct alone compared to that achieved by the uterus and oviduct together. Overstreet & Katz (1977) recovered spermatozoa from the oviducts 16 h after inseminating them into the vagina or oviducts. Since the latter population remained in the oviduct approximately 5 h longer than the minimal capacitation time there (Adams & Chang, 1962), it was assumed that both populations were equally capacitated. The study by Adams & Chang (1962), however, does not necessarily support that assumption. Although they showed that an 11-h incubation capacitates enough spermatozoa to achieve normal fertilization rates, there is no evidence to suggest that any more than a small proportion of the population may be capacitated at the time some members of it first become competent to fertilize (Bedford, 1983). Furthermore, the oviduct alone is less efficient in that respect than the uterus and oviduct together (Bedford, 1969). Therefore, a much smaller proportion of a sperm population inseminated tubally may be capacitated in a given time as compared to that in the population transported there naturally. This likelihood that, quantitatively, both populations were not capacitated to an equivalent degree, could explain the differential performance observed by Overstreet & Katz (1977).

In the present study, the spermatozoa were subjected to various manipulations which could have affected their performance. Since freshly ejaculated spermatozoa in Group I were more competitive than selected spermatozoa, residence in the female reproductive tract or the procedures for recovery, or the combination, may create adverse conditions that put spermatozoa at some disadvantage. The extensive leucocytic infiltration which occurs in the uterus after direct insemination appears to be particularly damaging. The results for Group III, however, in which spermatozoa were surgically deposited into the oviducts, provide additional support for the present conclusions. Although the variables introduced by tubal insemination and their effects upon spermatozoa are not known, their impact upon sperm integrity would not appear very different from that imposed by normal cervical migration since the performance of the two sperm populations (Group III) was equal throughout the entire reproductive tract. Furthermore, these populations generally achieved normal fertilization rates as judged by the number of ova containing spermatozoa in the zona (data not shown).

Sperm numbers were determined at 18 h *post coitum* only because the paucity of spermatozoa before that precluded the collection of adequate data. It is possible that 'selected' spermatozoa reached the ampulla and fertilized all the ova at some time before sperm evaluation (i.e. before 18–20 h *post coitum*) since the fertilizing spermatozoa were not identified. However, the absence of any unusual deviations in the sperm ratios throughout the reproductive tract or associated with ova in any animal studied suggests that this is unlikely. The results of the embryo transfer experiments are also against this supposition. Hence, the present study does not provide support for the existence of an elite subpopulation of spermatozoa in the rabbit ejaculate, or for the idea that any specific population is selected by the process of sperm transport in the female.

This work was supported by grants from the National Institutes of Health (HD03820, HD-05797), an NIH postdoctoral fellowship awarded to R.G.F. (1F32HD05795), and the Andrew W. Mellon Foundation.

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Received 11 April 1983