SPERM NUMBERS AND FERTILIZATION IN THE RABBIT

J. W. OVERSTREET

A.R.C. Unit of Reproductive Physiology and Biochemistry,
University of Cambridge*

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Summary. Groups of does were tubally inseminated with concentrations of 500 to 10,000 motile capacitated spermatozoa in 10 μ l of serum acidic saline about the time of ovulation, or with 1000 to 10,000 motile untreated spermatozoa 10 hr before the expected time of ovulation. The minimum number of spermatozoa required for fertilization was in the 500 to 1500 range. Fertilization levels >90% were not reached until 10,000 motile capacitated spermatozoa were inseminated at the time of ovulation, while 10,000 untreated spermatozoa deposited 10 hr earlier, fertilized less than 70% of the eggs.

A higher level of fertilization was consistently observed when the spermatozoa were inseminated in 10- μ l volumes than when the same number of spermatozoa was deposited in 100 μ l. There was little difference in the level of fertilization irrespective of whether tubal insemination of capacitated spermatozoa took place about the time of, or 4 hr before, ovulation.

INTRODUCTION

At the time of ovulation, it has been estimated that 2000 to 5000 spermatozoa are present in the entire Fallopian tubes of the naturally mated rabbit (Austin, 1948; Chang, 1951a; Braden, 1953). Braden (1953) suggested that the cervix, utero-tubal junction and isthmus of the Fallopian tube act as barriers to sperm ascent, thereby restricting massive sperm migration from the vagina so that less than 500 spermatozoa may reach the site of fertilization.

In previous investigations involving the deposition of spermatozoa in the Fallopian tubes, the numbers used have generally been in excess of postulated physiological levels, and fertilization levels observed under these conditions have seldom exceeded 70% to 80% (Chang, 1951b, 1957, 1958; Noyes, Walton & Adams, 1958; Adams & Chang, 1962; Dukelow, Chernoff & Williams, 1967). Tubal insemination of up to 2×10^6 capacitated spermatozoa may still result in less than 50% fertilization of recovered eggs (Hamner & Sojka, 1967; Hamner, Jones & Sojka, 1968). Austin (1948) deposited 2×10^6 epididymal spermatozoa into the tubes 5 to 6 hr before ovulation and recovered 78% of the eggs fertilized, but only 5% fertilization was recorded when less than 1000 motile epididymal spermatozoa were used. Since the discovery of the capacitation

* Postal address: 307 Huntingdon Road, Cambridge CB3 0JQ.

phenomenon (Austin, 1951; Chang, 1951b), no study has been made of the effect on fertilization of regulating the number of spermatozoa deposited in the Fallopian tube. The present series of experiments was undertaken to compare the performance of capacitated and untreated sperm samples with reference to their fertilizing ability.

MATERIALS AND METHODS

Animals and treatment

Eighty-five female rabbits of mixed breeds were used. All does were caged separately and were left unmated for a period of 21 days before being used in an experiment. Forty does were treated with a series of six subcutaneous injections of 2 mg of a saline suspension of acetone-extracted horse anterior pituitaries at 12-hr intervals to induce superovulation. These animals were assigned at random to experimental groups, which contained a further twenty-five does. An additional twenty oestrous does were used to provide uterine-incubated spermatozoa. Ovulation was induced by the intravenous injection of 25 i.u. HCG (Lutormone, Burroughs Wellcome) either at the time of tubal insemination when untreated spermatozoa were deposited, or 10 to 13 hr before tubal insemination in experiments involving capacitated spermatozoa. Henceforth, it will be assumed that ovulation occurs 10 hr after injection of HCG.

Sperm recovery

Semen was collected with an artificial vagina from six crossbred bucks of known high fertility which had not been used for at least 3 days before collection. Pooled semen samples from at least three bucks were diluted to concentrations of 1000, 2000, 3500, 5000 and 10,000 motile cells/10 μ l with acidic saline (Hammond, 1949), composed of NaCl, 880 mg/100 ml; KCl, 30 mg/100 ml; CaCl₂, 25 mg/100 ml; MgCl₂, 5 mg/100 ml; NaH₂PO₄, 10 mg/100 ml in distilled water, to which 5% rabbit serum and 0.25% glucose had been added. These samples will be referred to as untreated spermatozoa. Spermatozoa were recovered *in vivo* or at autopsy from the uterine horns of does 12 hr after mating or uterine insemination (0.1 ml ejaculated semen) by flushing the uterus with 5% serum acidic saline; these spermatozoa were presumed to have undergone at least partial capacitation and are referred to in this paper as capacitated spermatozoa. The capacitated sperm suspensions were brought to concentrations of 500, 1500, 2000, 2500, 5000 or 10,000 motile cells/10 μ l of fluid by centrifugation or dilution with 5% serum acidic saline. Final concentrations of motile spermatozoa were determined by multiple counts in a haemocytometer and checked in random samples by the nigrosin-eosin staining technique (Beatty, 1957).

Tubal insemination

Capacitated sperm samples were deposited in 10- μ l suspensions to a depth of 2 to 4 cm into the tubal ostium of thirty-four does. Twenty-four does were similarly inseminated with concentrations of untreated spermatozoa. To determine the effect of inseminate volume on fertilization, 5000 motile capaci-

tated spermatozoa in 100 μ l of fluid were deposited into the tubes of two additional animals as well as the contralateral tube of four does inseminated with the same number of spermatozoa in 10 μ l. A fourth group of five animals was tubally inseminated with 10,000 motile capacitated spermatozoa in 10 μ l of fluid 4 hr before ovulation to examine the effect on fertilization of the timing of sperm deposition relative to ovulation.

Egg recovery and examination

Eggs were recovered from does either *in vivo* or at autopsy by flushing 2 ml of physiological saline through the tubes 24 to 48 hr after tubal insemination. The eggs were examined microscopically at a magnification of $\times 200$ for evidence of pronuclei or symmetrical cleavage and for sperm penetration. It was frequently impossible to determine with certainty whether a particular spermatozoon lay within the zona pellucida or only in contact with its surface. In these cases, the spermatozoon was counted as being within the zona pellucida. The total number of spermatozoa counted within both the perivitelline space and the zona pellucida is hereafter referred to as the extra spermatozoa within the egg.

RESULTS

Fertilization following tubal insemination of capacitated spermatozoa

Details of the proportion of eggs fertilized (fertilization level) relative to the concentration of capacitated spermatozoa deposited in the tubes, as well as sperm numbers in the eggs, are presented in Table 1. The variation in fertiliza-

TABLE 1

FERTILIZATION FOLLOWING DEPOSITION OF 500 TO 10,000 MOTILE CAPACITATED SPERMATOZOA IN 10 μ l FLUID INTO THE FALLOPIAN TUBE 10 TO 13 HR AFTER HCG INJECTION

No. of spermatozoa	No. of Fallopian tubes	No. of Fallopian tubes with fertilized eggs	No. of ovulations	No. of eggs recovered	No. of eggs fertilized	Proportion of eggs fertilized (%)	Mean no. of sperm./fertilized egg*
500	17	6	147	145	24	16.6	1.25
1500	12	8	99	88	29	33.0	1.30
2000	9	7	134	129	88	68.2	1.68
3500	5	5	57	48	35	72.9	2.51
5000	11	9	130	124	76	61.3	2.72
10,000	10	10	86	85	78	91.8	6.34

* Includes the fertilizing spermatozoon and all spermatozoa counted in the perivitelline space or in contact with the zona pellucida.

tion level within each group is depicted in Text-fig. 1 and the distribution of extra spermatozoa in Text-fig. 2. Insemination of 500 motile capacitated spermatozoa in 10 μ l of fluid resulted in 16.6% fertilization, with 35% of the tubes yielding fertilized eggs. Following the deposition of 10,000 motile spermatozoa in 10 μ l, fertilization occurred in every one of the Fallopian tubes examined and 91.8% of the eggs were fertilized; in 50% of the tubes in this

TABLE 2

FERTILIZATION FOLLOWING DEPOSITION OF 1000 TO 10,000 MOTILE UNTREATED SPERMATOOZOA IN 10 μ L FLUID INTO THE FALLOPIAN TUBE AT THE TIME OF HCG INJECTION

TABLE 2

FERTILIZATION FOLLOWING DEPOSITION OF 1000 TO 10,000 MOTILE UNTREATED SPERMATOTAZOA IN 10 μ L FLUID INTO THE FALLOPIAN TUBE AT THE TIME OF HCG INJECTION

<i>No. of spermatozoa</i>	<i>No. of Fallopian tubes</i>	<i>No. of Fallopian tubes with fertilized eggs</i>	<i>No. of ovulations</i>	<i>No. of eggs recovered</i>	<i>No. of eggs fertilized</i>	<i>Proportion of eggs fertilized (%)</i>	<i>Mean no. of sperm./fertilized egg*</i>
1000	9	4	55	46	5	10.9	1.0
2000	9	7	58	46	17	37.0	1.65
3500	5	5	74	60	41	68.3	1.71
5000	10	8	88	63	31	49.2	1.90
10,000	13	12	69	57	39	68.4	2.95

Fertilization following tubal insemination of untreated spermatozoa

The proportion of eggs fertilized following the tubal insemination of untreated spermatozoa 10 hr before ovulation is presented in Table 2 and the distribution of fertilized eggs and extra spermatozoa in Text-figs. 3 and 4. With 1000 motile

TEXT-FIG. 3. Distribution of fertilized eggs recovered following tubal insemination of untreated spermatozoa at the time of HCG injection. (Each dot represents one Fallopian tube.)

spermatozoa, fertilized eggs were recovered from 44% of the tubes and 10.9% of all eggs recovered were fertilized. In general, increasing the concentration of spermatozoa resulted in higher fertilization levels. Variation in fertility within each experimental group was wider than that observed when capacitated

* Data from Adams (1955).

spermatozoa were inseminated. Thus, Fallopian tubes yielding no fertilized eggs were observed at every concentration as were tubes with maximal fertilization. A comparison of the number of spermatozoa present in eggs following deposition of capacitated or untreated sperm samples is shown in Text-fig. 5.

Fertilization in relation to inseminate volume and time of insemination

The results obtained when 5000 motile, capacitated spermatozoa were deposited either in 10 μ l or 100 μ l appears in Table 3. A higher level of fertilization was consistently observed when the same number of spermatozoa was

TABLE 3

EFFECT OF INSEMINATE VOLUME ON FERTILIZATION FOLLOWING THE DEPOSITION OF 5000 CAPACITATED SPERMATOZOA INTO THE FALLOPIAN TUBES

<i>Inseminate volume (μl)</i>	<i>No. of Fallopian tubes</i>	<i>No. of ovulations</i>	<i>No. of eggs recovered</i>	<i>No. of eggs fertilized</i>	<i>Proportion of eggs fertilized (%)</i>	<i>Mean no. sperm./fertilized egg</i>
10	11	130	124	76	61.3	2.67
100	8	158	135	53	39.2	1.11

deposited in the smaller volume. There was a tendency for fewer spermatozoa to be present in eggs recovered from does receiving the more dilute suspension of spermatozoa. Table 4 shows there was little difference in fertilization level in does inseminated about the time of, or 4 hr before, ovulation. Somewhat fewer spermatozoa were observed in eggs fertilized with spermatozoa deposited 4 hr before ovulation.

TABLE 4

EFFECT ON FERTILIZATION OF THE TIMING OF TUBAL INSEMINATION RELATIVE TO OVULATION: 10,000 MOTILE CAPACITATED SPERMATOZOA IN 10 μ l INSEMINATED

<i>Time of insemination (hr after HCG)</i>	<i>No. of Fallopian tubes</i>	<i>No. of ovulations</i>	<i>No. of eggs recovered</i>	<i>No. of eggs fertilized</i>	<i>Proportion of eggs fertilized (%)</i>	<i>Mean no. sperm./fertilized egg</i>
6	10	65	53	44	83.0	4.34
10	10	86	85	78	91.8	6.34

DISCUSSION

The results obtained in the present study indicate that the minimal number of spermatozoa required for fertilization in the tube is in the 500 to 1500 range. It seems unlikely that fertilization can occur when less than 500 spermatozoa are deposited, while the presence of fewer than 2000 spermatozoa generally limited fertilization to less than 50%. It was necessary to increase twenty-fold the minimal concentration of capacitated spermatozoa in order to raise fertilization levels to >90%. These observations are in agreement with pre-

viously reported results obtained under comparable conditions (Chang, 1957; Noyes, Walton & Adams, 1958; Dukelow, Chernoff & Williams, 1967). In contrast, when 10,000 motile, untreated spermatozoa were deposited 10 hr before ovulation, less than 70% of the eggs were fertilized. Similarly, in previous studies the deposition of untreated spermatozoa, even in numbers greater than 1×10^6 , has generally resulted in less than 80% of eggs being fertilized (Austin, 1948; Chang, 1951b, 1958; Adams & Chang, 1962; Hamner & Sojka, 1967).

By comparison with capacitated spermatozoa, fertilization levels and sperm counts were lower in all groups receiving untreated spermatozoa. This may have been due to the migration or dispersal of spermatozoa away from the site of fertilization since the untreated spermatozoa were inseminated 10 hr earlier. However, the wide variation within each group suggests that other variables should be considered. Aliquots from an ejaculate are random samples of an unselected population. Spermatozoa designated capacitated, although not necessarily fully capacitated, have survived 12 hr in the uterine environment. The greater variability in fertilization resulting from the insemination of comparable numbers of untreated spermatozoa may reflect variable proportions of spermatozoa in each sample capable of capacitation and subsequent fertilization. The variable fertilization levels also observed in groups inseminated with uterine spermatozoa suggest the utero-tubal junction, the ampulla and possibly the mechanism of tubal transport also have a selective effect on sperm quality. However, at least some part of the variation arises from limitations in the technique of sampling and handling small numbers of spermatozoa.

It is of interest to consider the findings of previous studies in the light of the present investigation. Braden (1953) estimated that less than 150 spermatozoa reach the site of fertilization, representing only one of every 10,000 to 100,000 spermatozoa ejaculated into the vagina. Yet Chang (1946a, b) and Wales, Martin & O'Shea (1964) reported fertilization when less than 100,000 spermatozoa were deposited in the vagina and maximal levels with 1×10^6 spermatozoa. Chang (1951a) found no difference in sperm numbers in the tubes irrespective of whether 20×10^6 or 200×10^6 spermatozoa were deposited into the vagina. All of these studies are based on examination of the tract at single points in time and as such may not necessarily reflect the sperm numbers present over a period of time. Braden (1953) observed a gradual build up in sperm numbers after coitus in the various segments of the female tract to reach a state of equilibrium at 6 hr *p.c.*, after which sperm concentrations in the tube changed little. Since these levels were maintained over the 28-hr period of observation, sperm movement into the tubes must have continued to offset any losses due to absorption, phagocytosis, or escape from the fimbrial ostium. Viewed over a period of time, therefore, the number of spermatozoa entering the tubes may be far greater than previous studies have suggested.

In a separate series (unpublished observations) involving ninety-five eggs examined 27 hr *p.c.*, the number of spermatozoa ranged from less than 5 to 500 per egg. In the present experiments, even the largest numbers of spermatozoa deposited never resulted in more than fifty spermatozoa in any egg. Austin & Braden (1952) and Braden & Austin (1954) suggested an important function of the female tract was to control sperm numbers at the site of fertilization and

thus decrease the risk of polyspermy. Adams & Chang (1962) concluded female tract restriction must be important for other reasons, since they recorded no abnormality in the number of spermatozoa in eggs even when numbers 8000 times greater than the supposed physiological levels were deposited in the tubes. By contrast, Bedford (1966) reported that the number of spermatozoa in the perivitelline space and zona pellucida of eggs recovered following the deposition of epididymal spermatozoa was often too large to allow accurate counts. Braden, Austin & David (1954) recorded an increase in the rate of penetration of the rabbit egg over the first 6 hr after ovulation. In the present study, when low sperm numbers were inseminated, the spermatozoa were distributed evenly in the eggs in such a manner as to suggest that a selectivity to sperm penetration may be exercised by the egg in the early post-ovulatory period. Dziuk (personal communication) considers a similar phenomenon may exist in the pig and has offered an explanation based on the position of the egg in the tube, with penetrability increasing as the egg is transported through the tube. The observation by Orgebin-Crist (1967) that fertilization is delayed following insemination of epididymal spermatozoa suggests that the high incidence of penetration observed by Bedford may have been due to late sperm penetration, and that delayed sperm capacitation and transport could facilitate the penetration of hundreds of spermatozoa under natural conditions.

No significant decrease in fertilization level was observed when capacitated spermatozoa were inseminated into the tubes 4 hr before ovulation. This is counter to the observations by Dukelow & Williams (1967) who reported a detrimental effect of the Fallopian tube on sperm survival and subsequent fertility.

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