EFFECT OF DILUTION RATE AND OF THE NUMBER OF SPERMATOOZOA INSEMINATED ON THE FERTILITY OF RABBITS OVULATED WITH CHORIONIC GONADOTROPHIN

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Summary. Adult does were inseminated with a constant number of spermatozoa in different volumes of diluent or with varying numbers of spermatozoa in a constant volume. The number of spermatozoa required for maximum fertility was 1×10^6 ; 50% of does littered when inseminated with 1 to 2×10^5 cells and inseminations with 2 to 4×10^4 cells were sterile. Even when the number of spermatozoa approached the minimum for fertility, increased dilution rates did not decrease the numbers of does kindling. Some litters were obtained with semen diluted 1 in 10,000 with calcium-free Krebs'-Ringer phosphate.

INTRODUCTION

In studies to determine the minimum number of spermatozoa required for maximum fertility (Walton, 1927; Rowlands, 1944), varying numbers of spermatozoa were inseminated in equal volumes of diluent and thus the effects of spermatozoal number could not be distinguished from those of dilution. Chang (1946a) found that insemination of spermatozoa in concentrated suspension led to a greater percentage of cleaved ova than did the use of the same number of cells in more dilute suspension. Cheng & Casida (1948) also reported a tendency for higher fertility when smaller volumes were used. In all the above experiments, except those of Walton (1927), the results were based on the percentage of ova cleaved at 24 to 48 hr after insemination. In the present study, we have examined the effect of spermatozoal number and rate of dilution on the fertility of does allowed to go to full term.

MATERIALS AND METHODS

A total of 309 adult albino does bred either in the department or at the University central animal house was used. All animals were caged individually from the age of 10 weeks and fed a high quality pelleted diet *ad libidum*, with a weekly supplement of green feed. The animals were housed in a room at a constant temperature of 22° C and were exposed to 12 hr artificial light/day. The average weight of does was 3.3 kg and, unless otherwise stated, they were assigned at random to the various treatment groups in the experiment.

Semen was collected from albino bucks, of similar origin to the does, which had been trained to ejaculate into an artificial vagina. Ejaculates were examined immediately under the low power microscope and only those with good initial motility and density were used. Aliquots of each ejaculate were diluted to the required dilutions and the semen suspension deposited in the vagina with a plastic insemination pipette soon after the dilutions were made. All inseminations were completed within $1\frac{1}{2}$ hr of semen collection. Ovulation in the does was induced by the intravenous injection of 25 i.u. of human chorionic gonadotrophin (Pregnyl, Organon) at the time of insemination. Following insemination, does were caged separately and allowed to go to term.

The diluent used in the experiments was calcium-free Krebs-Ringer phosphate (Ringer diluent): 0.01 M- Na_2HPO_4 , 0.005 M-KCl, 0.001 M- KH_2PO_4 ; 0.001 M- MgSO_4 ; 0.132 M-NaCl (Umbreit, Burris & Stauffer, 1949). In some experiments, 200 mg % fructose was added to the above. The seminal plasma used in the second experiment was obtained by centrifuging normal ejaculates at 500 *g* for 15 min and harvesting the supernatant obtained. This was stored at -20°C until required.

Any diluted ejaculate was partitioned so that it was used with equal numbers of animals from each treatment combination. In experiments 2 and 3 (Tables 2 and 3), only a single ejaculate was used in each experiment. After the experiments, total counts for each ejaculate were made in a haemocytometer and the number of cells inseminated was calculated.

At term, both the number of animals kindling and the number of young/litter were recorded. The quantal response (non-pregnant and pregnant) was scored as 0 or 1 and analysed by standard analyses of variance of the 0, 1 scores. Claringbold (1956) points out that this simple method of analysis is unbiased, but may cause some increase in the variances of estimates. However, the answers obtained for experiment 1 discriminated as well as those from χ^2 analyses, or from analysis of percentage pregnant does after angular transformation.

Analyses of variance are presented in summary form giving only degrees of freedom (D.F.) and variance ratios for each source of variation. The residual variance is given at the base of each variance ratio column. All main effects and their first order interactions were isolated and tested for significance. In the summary form of the tables, however, only those first order interactions which were significant have been presented separately. All non-significant interactions have been combined for presentation.

Mean litter size $\pm$ standard error of the mean is given in the tables. In some instances does destroyed young at birth and no accurate assessment of the number born could be made. In these cases, the values given were calculated using only the remaining data for the group.

RESULTS

In the first experiment aliquots of six ejaculates were diluted with 10 or 40 volumes of Ringer diluent with and without the addition of 200 mg % fructose. For each ejaculate, one doe was inseminated with 0.25, 0.125 or 0.0625 ml of the semen which had been diluted ten times or with 1.0, 0.5 or 0.25 ml of

semen diluted forty times so that, at each dilution rate, 20, 10 or 5×10^6 spermatozoa were inseminated into six does. The does were so chosen for insemination that within each group of six does, there were four nulliparous does and two multiparous does which had been inseminated two to five times previously. The litters obtained and the mean litter size are given in Table 1

TABLE 1
EFFECT OF MODERATE DILUTION ON THE LITTER PRODUCTION OF NULLIPAROUS AND MULTIPAROUS DOES

Diluent	Dilution of semen	No. spermatozoa inseminated					
		20 $\times 10^6$		10 $\times 10^6$		5 $\times 10^6$	
		No. litters (max. 6)	Litter size (Mean $\pm$ S.E.)	No. litters (max. 6)	Litter size (Mean $\pm$ S.E.)	No. litters (max. 6)	Litter size (Mean $\pm$ S.E.)
Ringer	1 : 10	4	4.3 $\pm$ 0.3	5	4.4 $\pm$ 0.7	5	5.0 $\pm$ 0.3
	1 : 40	4	6.0 $\pm$ 1.1	5	4.4 $\pm$ 0.5	3	5.3 $\pm$ 1.4
Ringer fructose	1 : 10	5	5.2 $\pm$ 0.9	4	4.0 $\pm$ 1.8	5	4.2 $\pm$ 1.0
	1 : 40	4	6.3 $\pm$ 1.3	3	4.7 $\pm$ 0.4	4	4.3 $\pm$ 0.5

Dilution of semen	Class of animal					
	Nulliparous		Nulliparous		Multiparous	
	No. litters (max. = 12)	Litter size (Mean $\pm$ S.E.)	No. litters (max. = 12)	Litter size (Mean $\pm$ S.E.)	No. litters (max. = 12)	Litter size (Mean $\pm$ S.E.)
1 : 10	9	5.6 $\pm$ 0.6	11	4.4 $\pm$ 0.6	8	3.6 $\pm$ 0.4
	10	5.0 $\pm$ 0.6	11	5.2 $\pm$ 0.6	2	5.0 $\pm$ 1.0

Summary of the analysis of variance for the fertility data

Source of variation	d.f.	Variance ratios
A. Effect of dilution	1	4.78 *
B. Effect of fructose	1	0.10
C. Effect of sperm number	1	0.10
D. Effect of parity		
Between nulliparous groups	1	0.58
Multiparous <i>v.</i> nulliparous	1	19.42**
First order interactions		
A $\times$ D		
Between nulliparous groups	1	0.00
Multiparous <i>v.</i> nulliparous	1	9.51**
Other	11	1.57
Higher order interactions	16	0.88
Between bucks within doe classes	3	1.45
Residual variance	30†	0.143

Two animals from each class were inseminated for each treatment.

* $P < 0.05$.

** $P < 0.01$.

† 3 d.f. lost for missing plots.

together with the summary of the analysis of variance of the fertility data. Variations in the number of spermatozoa inseminated and the addition of fructose had no effect on fertility. Overall there was a slight effect of dilution which was due entirely to differences in the multiparous group of does. Fertility

TABLE 2

LITTER PRODUCTION OF NULLIPAROUS, UNIPAROUS AND MULTIPAROUS DOES INSEMINATED WITH SEMEN DILUTED WITH RINGER DILUENT OR SEMINAL PLASMA

Diluent	Dilution of semen	No. spermatozoa inseminated		
		6.0 × 10 ⁶ No. litters (max. = 3)	3.0 × 10 ⁶ No. litters (max. = 3)	1.5 × 10 ⁶ No. litters (max. = 3)
Ringer	1 : 40	2	1	3
	1 : 160	2	1	2
Seminal plasma	1 : 40	2	1	3
	1 : 160	3	3	2
Mean litter size ± S.E.		4.6 ± 1.3	6.8 ± 0.9	5.9 ± 1.0

Diluent	Class of animal		
	Nulliparous No. litters (max. = 6)	Uniparous No. litters (max. = 6)	Multiparous No. litters (max. = 6)
Ringer	5	5	1
Seminal plasma	5	4	5
Mean litter size ± S.E.		6.4 ± 1.0	5.6 ± 0.9
		4.5 ± 0.5	

Summary of the analysis of variance for the fertility data

Source of variation	d.f.	Variance ratios
A. Effect of dilution	1	0.16
B. Effect of seminal plasma	1	1.44
C. Effect of sperm number	2	2.08
D. Effect of parity	2	2.08
First order interactions		
B × D		
Nulliparous v. uniparous	1	0.24
Multiparous v. others	1	6.47*
Other	11	1.04
Residual variance	16	0.174

One animal from each of the three classes was inseminated for each treatment.

* $P < 0.05$.

of the multiparous does inseminated with the more concentrated sample was high and similar to that of nulliparous animals. On the other hand the use of semen diluted forty times depressed the number of multiparous does littering

but had no effect on the littering performance of nulliparous does. None of the treatments, however, caused any change in the size of the litters.

A comparison of the effectiveness of Ringer diluent and seminal plasma for diluting semen 1 in 40 or 1 in 160 and inseminating $6, 3$ or 1.5×10^6 spermatozoa, was made in the next experiment. A single pooled ejaculate was used in the experiment and three does were inseminated in each treatment group. The first doe was a 6 to 8 month old virgin (nulliparous), the second a 9 to 10 month old doe, inseminated and littered once (uniparous) and the third was a multiparous doe, approximately 2 years old and inseminated three to six times previously. The results are presented in Table 2. The fertility obtained with

TABLE 3

NUMBER OF LITTERS FROM DOES INSEMINATED WITH DIFFERENT VOLUMES OF SEMEN DILUTED 2000 AND 10,000 TIMES WITH RINGER DILUENT

Dilution of semen	No. spermatozoa inseminated			
	0.20×10^6	0.10×10^6	0.05×10^6	0.025×10^6
1 : 2000	1	1	2	0
1 : 10000	2	1	1	0
Mean litter size $\pm$ s.e.	3.7 ± 1.4	4.5 ± 2.5	2.3 ± 0.7	—

Summary of the analysis of variance for the fertility data

Source of variation	d.f.	Variance ratios
Effect of dilution	1	0.00
Effect of sperm no.		
0.2 to 0.05×10^6	2	0.20
0.025×10^6 v. rest	1	3.03
Dilution $\times$ sperm no.	3	0.40
Within group (error)	32	0.175

A single ejaculate was used and five does were inseminated/group.

1.5×10^6 spermatozoa was as good as that with 6×10^6 and there was no difference between the dilution rates used. As in the previous experiment, the fertility of multiparous does inseminated with semen diluted in Ringer was low. However, if seminal plasma was used as diluent, these does produced as many litters as animals in the other two classes. As in the previous experiment, litter size was little affected by the treatments used.

The effects of high dilution were studied in the next experiment (Table 3). Aliquots of an ejaculate were diluted 1 in 2000 and 1 in 10,000 with Ringer diluent and $0.025, 0.05, 0.10$ and 0.20×10^6 spermatozoa were inseminated into five does at each dilution. The does were selected so that similar animals were allotted to each treatment. It was necessary to use low spermatozoal numbers in the experiment because of the limits to the volume of semen suspension possible to retain in the vagina after insemination. The largest volume

TABLE 4

FERTILITY OF DOES INSEMINATED WITH SEMEN AT VARYING DILUTIONS
IN RINGER DILUENT

Dilution of semen	No. spermatozoa inseminated			
	0.22 × 10 ⁶		0.11 × 10 ⁶	
	No. litters (max. = 10)	Litter size (Mean ± S.E.)	No. litters (max. = 10)	Litter size (Mean ± S.E.)
1 : 200	4	3.0 ± 1.4	5	3.3 ± 1.4
1 : 600	3	5.0 ± 2.1	2	3.5 ± 0.5
1 : 1800	5	2.5 ± 1.0	3	4.0 ± 1.5
1 : 5400	6	3.8 ± 0.8	3	1.0 ± 0.0

Summary of the analysis of variance for the fertility data

Source of variation	d.f.	Variance ratios
Effect of dilution	3	0.50
Effect of sperm number	1	0.91
Ejaculates	9	0.77
First order interactions	39	0.53
Residual variance	27	0.342

Ten does were inseminated for each treatment group.

Aliquots of 0.1 ml undiluted semen from the same ejaculates gave seven litters out of ten with a mean litter size of 4.3 ± 0.8.

TABLE 5

RELATIONSHIP OF OVERALL FERTILITY IN THE PREVIOUS EXPERIMENTS
TO THE NUMBER OF SPERMATOOZOA INSEMINATED AND THE DILUTION
RATE

Experi- ment No.	No. spermatozoa inseminated (× 10 ⁶)	Range of dilution	No. inseminations	Does littering (%)
1	5 to 20	$\frac{1}{10}$ to $\frac{1}{4}$	72	75
2	1 to 5	$\frac{1}{40}$ to $\frac{1}{160}$	36	69
4	0.1 to 0.2	$\frac{1}{200}$ to $\frac{1}{500}$	80	40
3	0.02 to 0.2	$\frac{1}{2000}$ to $\frac{1}{10000}$	40	20

inseminated was 2 ml. With this volume there was virtually no leakage after insemination. Litters were obtained in all groups except those receiving minimum spermatozoal numbers, but the overall fertility was low (20%) and differences due to numbers of spermatozoa inseminated did not test as significant.

The next experiment was planned to investigate dilutions intermediate

TABLE 6

FERTILITY OF DOES INSEMINATED WITH VARYING QUANTITIES OF SEMEN DILUTED 1 IN 200 WITH RINGER DILUENT

	<i>No. spermatozoa inseminated</i>		
	4.0×10^6	0.4×10^6	0.04×10^6
No. litters (max. = 7)	5	6	0
Mean litter size $\pm$ s.e.	2.4 ± 0.7	3.2 ± 0.9	—

Seven animals were inseminated in each group.

TABLE 7

FERTILITY OF DOES INSEMINATED WITH 0.2×10^6 SPERMATOZOA AT VARYING DILUTION IN RINGER DILUENT

	<i>Controls</i>	<i>Dilution rate</i>			
		1 : 30	1 : 180	1 : 1080	1 : 6480
No. litters (max. 12)	9	6	6	9	6
Mean litter size $\pm$ s.e.	5.0 ± 0.6	4.8 ± 1.4	3.0 ± 1.0	5.6 ± 1.5	5.0 ± 0.6
Mean motility score	—	2.4	1.4	0.8	0.6

Summary of the analysis of variance for the fertility data

<i>Source of variation</i>	<i>d.f.</i>	<i>Variance ratio</i>
Effect of treatment		
Control <i>v.</i> dilutions	1	2.52
Between dilutions	3	1.50
Ejaculate differences	3	5.95**
Interactions	12	1.89
Within group (error)	40	0.167

From each ejaculate collected, three does were inseminated at each dilution. Controls were inseminated with 2×10^6 spermatozoa at a 1 : 30 dilution. Motility scores (scale 0 to 4) were made at the completion of insemination.

** $P < 0.01$.

between the moderate dilutions of the first experiments and the high dilution rate used in the previous test (Table 3). Aliquots of ten ejaculates were diluted 1 in 200, 1 in 600, 1 in 1800 and 1 in 5400 in Ringer diluent and 0.22 or 0.11×10^6 spermatozoa inseminated. The results (Table 4) showed no difference in fertility over the range of dilution or between the numbers of spermatozoa used.

Table 5 shows the relationship in the previous experiments between number of spermatozoa inseminated, the degree of dilution, and the fertility of does. Although in nulliparous and uniparous does there was no effect of dilution within any one experiment, there was a fall in overall fertility which could still possibly be related to the degree of dilution.

The results of an experiment designed to test the effects on fertility of large variations in the number of spermatozoa inseminated at constant dilution are presented in Table 6. Seven ejaculates were diluted 1 in 200 with Ringer diluent and 4×10^6 , 4×10^5 or 4×10^4 spermatozoa inseminated. Although there was no drop in fertility with 4×10^5 spermatozoa, no litters were obtained when 4×10^4 spermatozoa were inseminated. The does receiving 4×10^4 spermatozoa were inseminated with a glass micro-pipette (50 μ l capacity, outer diameter 0.7 cm) containing 10 μ l of spermatozoal suspension. When six does were inseminated with 10 μ l of undiluted semen, all animals littered with a mean litter size of 3.7 ± 0.8 .

In the last experiment (Table 7), the effect of up to a 216-fold dilution was tested while the number of spermatozoa/insemination was kept constant by varying the volume from 10 μ l to 2.2 ml. Semen samples from four bucks were initially diluted 1 in 30 to 1:40 in Ringer diluent to give a suspension containing 2×10^5 (S.E. = 0.38×10^5) spermatozoa in 10 μ l. Dilutions of the semen were made at six-fold intervals and at each dilution three does were inseminated for each ejaculate. Overall 75% of the does littered. There was no decrease in fertility when does were inseminated with very dilute semen even though there was a fall with dilution in the motility estimates made at the completion of insemination.

DISCUSSION

In making any study where there is either a constant number of spermatozoa inseminated at changing dilution rate or vice versa, upper and lower limits are set by the volume that it is possible to inseminate. It can be expected that, with a volume above 2 ml, leakage from the vagina will occur and errors may arise (see Walton, 1927). In the final experiments reported here, it was possible, by using a micro-pipette, to inseminate successfully with a 10 μ l semen sample. In the other experiments where a standard insemination pipette was used, a 50 μ l sample was the smallest that could be delivered with accuracy.

Where both dilution rate and spermatozoal number were varied at the same time, it was not possible within any one experiment to study both extremes of dilution and spermatozoal number. In the final experiments where 100- to 200-fold changes in dilution and spermatozoal number were examined separately the number of spermatozoa inseminated was important to fertility. Dilution, however, did not seem to be important because, even at spermatozoal numbers approaching the median effective dose (1 to 2×10^5 spermatozoa), a 200-fold increase in dilution had no significant effect on fertility. Chang (1946a), however, found that a ten-fold increase in dilution when 3 to 4×10^4 spermatozoa were inseminated greatly depressed their fertilizing capacity. Cheng & Casida (1948) reported a decrease in the percentage of ova cleaved when 2×10^4 spermatozoa were inseminated and dilution was increased eight-fold. The differences between these and the present results may arise from the

different criteria used to measure fertility. Chang (1946a) and Cheng & Casida (1948) both used the percentage of ova cleaved soon after insemination and the effects of dilution were studied with the minimum numbers of spermatozoa that would cause cleavage. Larger numbers of spermatozoa were required to obtain reasonable fertility in the present experiments and these numbers may have been too great to show the dilution effect reported by Chang (1946a) and Cheng & Casida (1948). However, insemination with 4×10^4 spermatozoa produced no litters in our tests and possibly ova cleaved after insemination with very low numbers of spermatozoa do not undergo normal development.

There are wide variations in the recommended minimum numbers of rabbit spermatozoa required for maximum fertility. Using cleavage of ova as a criterion Cheng & Casida (1948) reported that 9×10^4 spermatozoa give maximum fertility, a number reported by Rowlands (1944) to be incapable of fertilization. On the other hand Chang (1946b), studying litter production, found that 4×10^5 were required but Walton (1927) reported that 1×10^6 or more were needed. In the present studies, 1×10^6 spermatozoa also gave satisfactory results, but below 2×10^5 spermatozoa fertility was depressed and inseminations of 4×10^4 or less were sterile. It should be noted, however, that within any one experiment ten-fold variations in the numbers of spermatozoa can occur without affecting fertility.

Chang (1947, 1949) noted that seminal plasma increased the number of ova cleaved 25 hr after insemination. Its addition did not increase the number of litters produced in the present trials except in older does. It is possible that the low littering performance in old animals is due to deficiencies in the transport of spermatozoa to the site of fertilization, and oxytocic factors in semen (Hawker, Roberts & Walmsley, 1960) may explain the beneficial effect of seminal plasma on fertility. Since the fertility of multiparous does was normal when concentrated semen or semen mixed with seminal plasma was used, their low fertility when inseminated with dilute semen was not due to the production of circulating antigonadotrophins following a series of gonadotrophin injections (Adams, 1961).

The observation that spermatozoa are fertile even at a dilution at which deleterious effects on motility begin to show (Cheng & Casida, 1948; White, 1954), suggests that dilution of constituents of seminal plasma is unimportant in transport or fertilization. The observation that spermatozoa washed free of all seminal plasma (R. G. Wales & T. O'Shea, unpublished data) are still fertile suggests that endogenous substrates or those present in fluids of the female reproductive tract are sufficient to maintain the cell from insemination until fertilization. The mean litter size between groups of does used in these experiments varied considerably. However, the variability in litter size within the groups was also quite large, as shown by the large standard errors of the means. In most cases, differences in the litter size between groups of does in any experiment did not test as significant and the variations probably reflect normal differences that can be expected. A mean litter size of 4.6 (range = 1 to 11, standard deviation = 2.23) was obtained for another eighty-five litters from does of the same strain artificially inseminated after ovulation with chorionic gonadotrophin.

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REFERENCES

- ADAMS, C. E. (1961) Artificial insemination in the rabbit. *J. Reprod. Fertil.* **2**, 521.
- CHANG, M. C. (1946a) Effect of dilution on fertilizing capacity of rabbit spermatozoa. *Science*, **104**, 361.
- CHANG, M. C. (1946b) Fertilizing capacity of spermatozoa following cold treatment of the scrotal testis of rabbits. *J. exp. Biol.* **22**, 95.
- CHANG, M. C. (1947) Effects of testis hyaluronidase and seminal fluids on the fertilizing capacity of rabbit spermatozoa. *Proc. Soc. exp. Biol., N.Y.* **66**, 51.
- CHANG, M. C. (1949) Effects of heterologous seminal plasma and sperm cells on fertilizing capacity of rabbit spermatozoa. *Proc. Soc. exp. Biol., N.Y.* **70**, 32.
- CHENG, P. & CASIDA, L. E. (1948) Fertility in rabbit as affected by the dilution of semen and the number of spermatozoa. *Proc. Soc. exp. Biol., N.Y.* **69**, 36.
- CLARINGBOLD, P. J. (1956) The within-animal bioassay with quantal responses. *J. roy. statist. Soc.* **18**, 133.
- HAWKER, R. W., ROBERTS, V. S. & WALMSLEY, C. F. (1960) Some properties of an oxytocic substance in extracts of semen. *Endocrinology*, **67**, 187.
- ROWLANDS, I. W. (1944) Capacity of hyaluronidase to increase the fertilizing power of sperm. *Nature, Lond.* **154**, 332.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. R. (1949) *Manometric techniques and tissue metabolism*. Burgess, Minneapolis.
- WALTON, A. (1927) The relation between density of sperm-suspensions and fertility as determined by artificial insemination of rabbits. *Proc. roy. Soc. B*, **101**, 303.
- WHITE, I. G. (1954) The effect of some seminal constituents and related substances on diluted mammalian spermatozoa. *Aust. J. biol. Sci.* **7**, 379.