

Fertility in Rabbit as Affected by the Dilution of Semen and the Number of Spermatozoa.*

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Walton¹ first demonstrated in the rabbit that fertility is influenced by the number of spermatozoa introduced into the vagina; maximum fertility was obtained when the number of spermatozoa was more than 1,000,000. The semen was diluted in 0.9% sodium chloride solution and 3 cc of semen suspension was used per insemination. Rowlands² used Baker's solution to dilute the semen and 2 cc of such semen suspension was deposited per insemination. He found the same number of spermatozoa necessary for maximum fertility as Walton did and sterility was reached when the number was dropped to 100,000. Chang³ also used 0.9% sodium chloride solution as the diluent of rabbit semen with only 1 cc per insemination, and obtained a much lower figure on the number of spermatozoa for both maximum and minimum fertility (for maximum fertility: 330,000-420,000; for minimum fertility: 40,000). Chang⁴ also tested the effect of dilution on the fertilizing capacity of rabbit sperm by inseminating the same number of spermatozoa suspended in various volumes of physiological saline (1.0 cc, 0.4 cc and 0.1 cc). The advantage appeared to be with the least volume.

The present studies attempt to determine the minimal number of sperm for fertility from insemination into the vagina and into the uterine horns with highly diluted semen. Further, an attempt was made to determine the effects of high dilution with physiological

saline and Krebs solutions upon the motility of spermatozoa. Also the effect of high dilution with Krebs, and its consequent effect upon motility, upon the fertilizing capacity of the sperm was determined.

Experiment I. Fertility in relation to number of spermatozoa.

Material and methods. Thirty-six female rabbits were used in this study. All animals were in heat and mated to vasectomized bucks to induce ovulation. Semen samples were collected from 2 or 3 normal males with an artificial vagina⁵ and pooled together. The number of spermatozoa was determined from the average counts of 2 independent hemocytometer samples. Four hundred hemocytometer squares were counted in each case and no pairs of counts were accepted which showed differences of statistical significance between them. The initial motility of spermatozoa was estimated in ranks or grades. The semen was then diluted with Krebs solution, modified according to Lardy and Phillips⁶ until the desired number of spermatozoa per unit of volume was obtained and then kept in a warm water bath at approximately 37°C for the short time until it was used.

Four females were used in each trial with 2 in each group. In the "vaginal group" 0.2 cc of semen suspension was deposited into the anterior portion of the vagina by an inseminating tube; in the "uterine group," a laparotomy was made under light anesthesia and 0.1 cc of the same semen suspension was injected into the tubal ends of each of the uterine horns with a $\frac{1}{4}$ cc tuberculin hypodermic syringe. Animals in both groups,

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¹ Walton, A., *Roy. Soc. London Proc.*, 1927, **101B**, 303.

² Rowlands, I. W., *Nature*, 1944, **154**, 332.

³ Chang, M. C., (In "The Problem of Fertility," 1946. Edited by E. T. Engle, Princeton Univ. Press, p. 169), 1946.

⁴ Chang, M. C., *Science*, 1946, **104**, 361.

⁵ Macirone, C., and Walton A., *J. Agr. Sci.*, 1938, **28**, 122.

⁶ Lardy, H. A., and Phillips, P. H., *Amer. J. Physiol.*, 1942, **138**, 741.

TABLE I.
Fertility in Relation to Number of Spermatozoa.

No. sperm. per insem. × 1000	Dilution rate	Vaginal group			Uterine group		
		No. eggs	% fert.	Avg % fert.	No. eggs	% fert.	Avg % fert.
92	1/1000	10	100	100	8	100	100
		7	100		0	—	
61	1/1000	7	71	36	5	0	0
		5	0		6	0	
46	1/1000	11	100	100	7	100	100
		8	100		0	—	
32	1/2500	8	50	50	10	70	35
		0	—		10	0	
24	1/2000	13	100	81	12	100	100
		8	62		5	100	
17	1/3500	9	0	0	5	0	0
		9	0		9	0	
17	1/4500	9	0	0	8	0	14
		16	0		11	27	
16	1/3500	9	11	20	12	0	15
		10	30		10	30	
16	1/5000	8	62	62	6	0	0
		0	—		7	0	
Group fertility (unweighted avg)				50	40		

therefore, received approximately equal amounts of suspension containing approximately equal numbers of spermatozoa.

The insemination was made about 1½ to 3½ hours after the sterile mating (only sperm free seminal fluid deposited in vagina by the vasectomized male). Autopsy was made 25 to 30 hours after insemination; the oviducts were flushed, and fertilization of the ova was determined microscopically from the presence of 2 to 8 equal blastomeres.

Results. The findings of the present study show that partial fertility can result from a very small number of spermatozoa per insemination, as few as 16,000 (Table I). This was true in both vaginal and uterine inseminations. Maximum fertility was obtained with a number of spermatozoa around 90,000, and fertility was affected greatly when the number was cut below 20,000. A negative relationship was found between the dilution rates and the fertilizing capacity of the spermatozoa but the effect of a small number of sperm was confounded with the effect of high dilution. The effects of dilution rate upon motility were observed incidentally but the effect of this factor upon fertility also was confounded with number and concentration. The average fertility resulting from vaginal insemination was higher than that

from uterine insemination (50% vs 40%). However, statistical analysis of the data from the 2 groups failed to show a significant difference between them ("t" test: $P > .05$). If the vaginal group could have been inseminated at laparotomy similar to the uterine group, the data could be considered more critical in comparing the effects of the two placements upon fertility.

Experiment II. Motility of spermatozoa as affected by high dilutions with different diluents. The above data show that the number of spermatozoa necessary either for maximum or for partial fertility is less than that previously reported. The discrepancy between different results may be due to the difference in diluents. The effects of high dilution upon motility of spermatozoa in a series of dilutions with both 0.9% sodium chloride and Krebs solutions were investigated next.

Material and methods. Twelve semen samples from 9 males were collected at different times and diluted in 8 successive dilutions with both 0.9% sodium chloride and Krebs solutions from 1:100 to 1:12800. Semen was introduced into containers of adequate sizes by a 0.01 cc graduated pipette; the diluent was then added slowly, and shaken gently in order to obtain an even dispersion

TABLE II.
Motility and Highest Dilution Showing Motility of Rabbit Spermatozoa in Two Diluents.

Sample No.	Avg % motility in 8 dilutions (from 1:100 to 1:12800)		Highest dilution showing motility	
	0.9% NaCl	Krebs	0.9% NaCl	Krebs
1	29.4	36.2	3200	12800
2	21.2	36.2	800	3200
3	13.1	24.1	400	12800
4	6.4	20.6	400	6400
5	19.4	32.0	800	6400
6	6.3	10.4	200	3200
7	13.4	20.6	800	3200
8	1.4	5.1	200	400
9	30.1	38.7	3200	6400
10	8.1	13.9	400	1600
11	23.1	43.9	1600	3200
12	0.9	6.9	400	800
Avg	14.4	24.1	1033	5033

of spermatozoa. All semen suspensions were kept in the warm water bath for 30 minutes and the percentage of spermatozoa showing progressive motion then estimated.

Results. Almost without exception, the average motility in the 8 dilutions and also the highest dilution rate supporting motility was higher for Krebs solution than for the 0.9% sodium chloride solution (Table II), and a trend was shown in both diluents that

TABLE III.
Progressive Motility of Rabbit Spermatozoa in Dilutions from 1:100 to 1:12800.

Dilution	Avg percentage motility in	
	0.9% NaCl	Krebs
1: 100	48.3	57.9
1: 200	37.7	47.5
1: 400	18.2	35.5
1: 800	8.0	23.8
1: 1600	2.5	17.4
1: 3200	0.5	8.6
1: 6400	0	1.2
1:12800	0	0.6
Avg	14.4	24.1

the higher the dilution rate the lower the motility (Table III). Statistical analyses showed that the motility of spermatozoa varied significantly from one dilution to another, and the average difference between the diluters was also highly significant ("F" test, $P < 0.01$).

Experiment III. Fertility as affected by rates of dilution and motility of spermatozoa.

The low motility observed in the high dilutions causes one to recall the results in Experiment I, in which the rates of dilution were from 1/1000 up to 1/5000. Considering the fact that the average highest dilution showing motility was 1:1033 in 0.9% sodium chloride solution and 1:5033 in Krebs solution, it was suspected that the motility of the spermatozoa is one of the determining factors in fertilization and the difference in solutions accounts for the discrepancy between present results and those of former workers. The effect of dilution upon the fertilizing capacity of the spermatozoa was therefore studied next.

Material and methods. The same small number of spermatozoa, viz., 20,000 all from the same sample of semen, were suspended in 0.1 cc, 0.2 cc, 0.4 cc and 0.8 cc of Krebs solution. Four animals were used for each trial, each animal received a different volume of suspension; altogether there were 7 trials in this study. The same procedure as mentioned in Experiment I was followed except the semen suspension was deposited in the vagina in all animals. The motility of spermatozoa was estimated on each semen suspension before the insemination.

Results. The fertility data are based on 22 animals; the remaining 6 failed to ovulate. The average fertility in each of the 4 different volume groups was: 0.1 cc, 60.0%; 0.2 cc, 60.5%; 0.4 cc, 37.2%, and 0.8 cc, 38.2% (Table IV) for a range of 23.3%. The

TABLE IV.
Fertility of Rabbits in Relation to Dilution and Motility of Spermatozoa.

Motility rank*	200,000/cc 0.1 cc/insem.		100,000/cc 0.2 cc/insem.		50,000/cc 0.4 cc/insem.		25,000/cc 0.8 cc/insem.		Total No. eggs	Avg % fertility
	No. eggs	% fert.	No. eggs	% fert.	No. eggs	% fert.	No. eggs	% fert.		
1					6	0	8 11 7	0 91 0	32	22.7
2					8 7	50 0	10	100	25	50.0
3			7 8 9	100 25 0	6	100	5	0	35	45.0
4	9 11	100 0	8	38	11 9	73 0			48	42.2
5	7 9 8	100 0 100	7 8	100 100					39	80.0
Total No. eggs	44		47		47		41		179	
Avg % fertility		60.0		60.5		37.2		38.2		

* 1, no motion at all; 2, oscillatory motion; 3, motility from 1% to 25%; 4, motility from 25% to 50%; and 5, motility from 50% to 75%.

different motility groups, however, showed a range of 57.3% from 22.7 for grade 1 motility up to 80.0 for grade 5.

A significant correlation was found between motility and fertility, 0.43, but an insignificant correlation was shown between sperm concentration and fertility, 0.22. The motility of the spermatozoa and the sperm concentration showed a rather high correlation, 0.78. The partial correlation between fertility and motility with concentration held constant was 0.42 whereas between fertility and concentration with motility held constant it was -0.17 and insignificant. Under the conditions of high dilutions, the motility of the spermatozoa appears to have some value for predicting fertility. It also appears that much of the deleterious effect of high dilution upon fertility of sperm is brought about through its inhibitory action upon motility.

Summary. Female rabbits were inseminated with semen diluted with Krebs solution at rates of 1/1000 up to 1/5000 and 0.2 cc of suspension were used. The number of

spermatozoa effective for partial fertility was 16,000, and for maximum fertility was around 90,000. The group percentage of fertility resulting from vaginal insemination was slightly higher than from uterine insemination (50% vs 40%); this difference was not of statistical significance.

When semen samples were diluted from 1:100 up to 1:12800, the motility of the spermatozoa decreased significantly as the rates of dilution increased and the average motility shown in Krebs solution and the maximal dilution that would support motility was significantly higher than in 0.9% sodium chloride solution.

When females were inseminated with a small number of spermatozoa (20,000) suspended in different volumes of Krebs solution, the average percentages of fertility were higher in the smaller volumes. The motility of the spermatozoa, however, appeared to have a greater effect on the fertility than the dilution.