PENETRATION OF MOUSE EGGS *IN VITRO*: OPTIMAL SPERM CONCENTRATION AND MINIMAL NUMBER OF SPERMATOZOA

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It is well known that a large number of the spermatozoa in the ejaculate reach the uterus but only a few are found in the oviducts of mammalian species after mating (see Bishop, 1961). This suggests that a small number of spermatozoa competent to fertilize may be selected during their passage through the female tract. In a study of the optimal sperm concentration and the minimal number of spermatozoa required to fertilize a rat egg *in vitro*, Niwa & Chang (1974a) have shown that the optimal sperm concentration was about 0.5 to 1.5×10^6 spermatozoa/ml and that the minimal number of spermatozoa required to fertilize a rat egg was about 3000 to 6000 spermatozoa. The present experiment was designed to determine the optimal sperm concentration, the minimal number of spermatozoa required to fertilize a mouse egg *in vitro*, and whether the number of eggs present in each preparation affects the fertilization rate.

The ducts of the cauda epididymidis of a mature Swiss albino mouse were cut with a sharp knife. A small drop of the sperm mass was placed by means of a glass needle into 0.5 ml of a chemically defined medium (Toyoda, Yokoyama & Hosi, 1971) in a Falcon plastic dish covered with light paraffin oil which had previously been equilibrated with 5% CO₂ in air (original suspension). After 5 to 10 min, 0.04 ml of the original suspension was placed in 0.4 ml of the same medium covered with paraffin oil (Suspension 1). In the same way, this sperm suspension was diluted three times (Suspensions 2 to 4), and each suspension was preincubated for 1 hr in an incubator at 37°C saturated with 5% CO₂ in air.

Mature female Swiss albino mice weighing 23 to 30 g were injected intraperitoneally with 5 i.u. PMSG and 5 i.u. HCG 44 to 48 hr later. They were killed 15 to 18 hr after the HCG injection and their oviducts were placed under oil in the same dish containing a droplet of sperm suspension. The eggs in cumulus clot were dissected from the ampullary portion of the oviducts with sharp needles; various numbers of eggs were introduced into each sperm suspension, and oviducts and debris were removed from the dishes. After incubation for 3 to 5 hr, the eggs were removed, mounted *in toto*, stained with 0.25% lacmoid (Chang, 1952), and examined for evidence of sperm penetration and fertilization.

The numbers of spermatozoa in the original suspension and in Suspension 1 were counted by means of a haemocytometer at the end of an experiment but the numbers of spermatozoa in Suspensions 2 to 4 were calculated according to their dilutions and the number found in Suspension 1.

Table 1. Optimal sperm concentration and minimal number of spermatozoa for fertilization *in vitro* of mouse eggs

Sus-pensions	Range of sperm concentration ($\times 10^4/\text{ml}$)	Total no. of sperm. ($\times 10^3/0.4$ to 0.44 ml)	No. of eggs introduced	No. of tests	Sperm no./egg	No. of eggs penetrated/no. of eggs examined (%)	Total
Original	100 to 860	400 to 3,612	2 to 40	7 to 5	306,000 to 1,170,000	12/14 (86) to 31/111 (28)	69/197 (35)
1	10 to 63	40 to 235	2 to 40	6 to 8	24,000 to 11,700	8/13 (62) to 154/177 (87)	212/273 (78)
2	0.52 to 4.5	2.1 to 19.2	2 to 31	6 to 5	2,200 to 840	9/12 (75) to 68/114 (60)	120/217 (55)
3	0.08 to 0.43	0.32 to 1.89	2 to 20	6 to 5	200 to 84	3/12 (25) to 20/87 (23)	57/195 (29)
4	0.007 to 0.048	0.03 to 0.192	2 to 22	6 to 3	18 to 9	2/12 (17) to 2/61 (3)	7/132 (5)

Eggs were examined 3 to 5 hr after exposure to epididymal spermatozoa preincubated for 1 hr.

It can be seen from Table 1 that the optimal sperm concentration for fertilization *in vitro* of mouse eggs was about 10 to 63×10^4 spermatozoa/ml and that the penetration rates decreased when sperm concentration was decreased or increased, although 5% of the eggs were penetrated in a suspension of 70 to 480 spermatozoa/ml (Suspension 4). It appears that at least 100 spermatozoa were required to fertilize each egg in order to obtain a penetration rate above 60% (Suspension 2), although 2 to 84 spermatozoa could fertilize from 3 to 23% of the eggs (Suspensions 3 and 4). There was no striking difference in the penetration rates whether two or twenty eggs were introduced into a sperm suspension ranging from 0.08 to 63×10^4 spermatozoa/ml (Suspensions 1, 2 and 3). But the penetration rates were decreased when a large number of eggs were introduced in a low (Suspension 4) or a high sperm concentration (original suspension).

Milovanov & Sokolovskaya (1945) stated that "both insufficiency and superabundance of spermatozoa have an adverse effect on fertilization". Although capacitation of spermatozoa and fertilization of eggs can be achieved in a relatively high concentration of spermatozoa for the mouse (Iwamatsu & Chang, 1970; Toyoda *et al.*, 1971), it is clear that an optimal sperm concentration is better for the capacitation of spermatozoa and fertilization of eggs in the mouse (Fukuda, Okada & Toyoda, 1972), golden hamster (Talbot, Franklin & Fussell, 1974), and rat (Niwa & Chang, 1974a, b). In confirmation of these studies, the present experiment has shown that the optimal sperm concentration for fertilization of mouse eggs *in vitro* was about 0.1 to 0.63×10^6

spermatozoa/ml. Whether such an optimal concentration is for the maintenance of the physiological integrity of spermatozoa, for the capacitation of spermatozoa, or for the penetration of eggs has still to be determined.

The minimal number of spermatozoa required to fertilize a rat egg *in vitro* was estimated to be 3000 to 6000 (Niwa & Chang, 1974a), whereas in this study, about 100 to 840 spermatozoa were required to fertilize a majority of the mouse eggs in Suspension 2. The discrepancy may be due to a species difference because mouse spermatozoa appear to withstand manipulation better than rat spermatozoa and the capacitation of mouse spermatozoa *in vitro* took a shorter time than that of rat spermatozoa (Toyoda *et al.*, 1971; Niwa & Chang, 1974b). Although there was an indication that the fertilization rate of rat eggs was higher in the presence of a larger rather than a smaller number of eggs (Niwa & Chang, 1974a), this was not confirmed in the present study on mouse eggs. In addition to the probability of successful collision between gametes (Rothschild & Swann, 1951), their physiological integrity under certain conditions also seems to be involved.

It has been suggested that due to certain "inexact chiasma formation" during spermatogenesis, only a small proportion of spermatozoa are competent (Cohen, 1969) and the probable selection of a small population of spermatozoa by the female tract of the rabbit has been reported (Cohen & McNaughton, 1974). Inspection of the raw data (Table 2) revealed that, in the presence of 30 to 84 spermatozoa, or 2 to 42 spermatozoa for each egg, 6 (12.8%) of 47 eggs

Table 2. Detailed results following the exposure of various numbers of mouse eggs to minimal numbers of spermatozoa

	Sperm conc. (10 ⁴ /ml)	Total no. of sperm. ($\times 10^3$ / 0.4 to 0.44 ml)	No. of eggs introduced	Sperm no./egg	No. of eggs penetrated/ no. of eggs examined (%)
	0.009	0.036	2	18	0/2
	0.013	0.057	2	29	0/2
	0.013	0.057	2	29	1/2 (50)
	0.021	0.084	2	42	1/2 (50)
	0.021	0.084	2	42	0/2
	0.023	0.101	2	51	0/2
Range and Total	0.009 to 0.023	0.036 to 0.101	12	18 to 51	2/12 (17)
	0.037	0.148	5	29	0/4
	0.008	0.032	6	5	1/6 (17)
	0.023	0.101	5	20	0/5
	0.023	0.101	5	20	0/4
	0.023	0.101	5	20	0/5
Range and Total	0.008 to 0.037	0.032 to 0.148	26	5 to 29	1/24 (4)
	0.010	0.044	12	4	1/12 (8)
	0.038	0.167	10	17	0/10
	0.007	0.030	10	3	1/6 (17)
	0.007	0.030	10	3	0/7
Range and Total	0.007 to 0.038	0.030 to 0.167	42	3 to 17	2/35 (6)
	0.008	0.035	20	2	0/20
	0.048	0.192	22	9	1/22 (5)
	0.007	0.030	20	2	1/19 (5)
Range and Total	0.007 to 0.048	0.03 to 0.192	62	2 to 9	2/61 (3)

were penetrated in six tests in which penetration had occurred. It is possible that all spermatozoa are at least potentially competent to fertilize. Whether such a fertilized egg can develop normally still requires further investigation. Nevertheless, it appears that the selection of spermatozoa during their passage through the female tract is not necessarily for their fertilizing capacity but for their physiological function as a whole.

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