FERTILIZATION AND DEVELOPMENT *IN VIVO* OF RABBIT OOCYTES CULTIVATED *IN VITRO*

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Pincus & Enzmann cultivated rabbit ovarian oocytes *in vitro* as early as 1935. In a study of the development of cultivated oocytes transferred to the oviducts of mated females, Chang (1955a) obtained 81% cleavage. In further experiments, Chang (1955b) evaluated embryonic development after the transplantation of cultivated oocytes and observed positive results with the oocytes isolated for cultivation from the ovaries 6 hr or more after injecting sheep pituitary extract. Thibault & Gérard (1970) fertilized cultivated oocytes *in vitro* and described the formation of anomalies in the development of the male pronuclei. The aim of the present study was to determine whether oocytes collected shortly after mating followed by an injection of HCG and then cultivated *in vitro* would be capable, after fertilization in the oviduct, of normal development in the initial stages, or whether anomalies would again be found in the formation of the male pronuclei.

Twenty-three sexually mature female rabbits of different breeds were used. One group consisted of eleven does in oestrus. In the second group of does, pseudopregnancy was induced with 100 i.u. HCG (Praedyn, Spofa) and superovulation with FSH (Foliotropin, Spofa) according to the method described by Kennelly & Foote (1965). On the day following the last day's treatment of the second group, all the does in both groups were mated with a fertile male and received an intravenous injection of 100 i.u. HCG. Within 30 min after mating and HCG injection, they were subjected, under ether anaesthesia, to unilateral ovariectomy performed through an incision in the linea alba. The ovary with the greater number of large follicles was always removed and oocytes were isolated by perforating the wall of large clear-walled follicles with a dissecting needle under a stereomicroscope. A few oocytes served as controls for the stage of nuclear development and the others were cultivated with cumulus cells, as drops, in 0.1 ml medium under paraffin oil, at 38°C in 5% CO₂ in air (Motlík, 1972). The composition of the culture medium (pH 7.8) was as follows: isotonic TC 199 solution (USOL, Prague), 72 ml; 1.45% NaHCO₃ + 0.002% phenol red, 18 ml; 5.5% glucose solution, 10 ml; sodium pyruvate, 0.004 g; freeze-dried calf serum growth proteins (USOL, Prague), 30 mg/ml; penicillin, 50 i.u./ml; streptomycin, 50 i.u./ml.

According to the results of previous experiments (J. Motlík and J. Fulka, unpublished results), more than 90% of the oocytes, under the above conditions,

mature in 9½ to 10 hr to the metaphase II stage, with the first polar body. After cultivation for 9½ to 10 hr, the oocytes were transplanted into the ampullae of the oviducts on the ovariectomized side. The oocytes of the other ovary served, after ovulation, as the controls for fertilization and the first stages of development. The females were killed 8 to 10 hr after transplantation and the eggs were flushed from both oviducts, fixed in acetalccohol, stained with orcein and examined in a phase-contrast microscope. Eggs clearly showing two polar bodies and two pronuclei, with a tail near one of them, were evaluated as normally fertilized.

The results given in Table 1 show that penetration of spermatozoa into the cytoplasm occurred in 80.4% of cultivated oocytes from untreated females and in 80.8% following superovulation. Development of both pronuclei (Pl. 1, Fig. 1) was normal in 60.8% of the eggs in the former group and in 51.8% in the latter. There were no significant differences between the two groups in the proportion of eggs with abnormal development of the male pronucleus (17.6% and 22.7%). The frequency of polyspermic eggs was somewhat higher following superovulation. Anomalies that do not occur under normal maturation conditions are interesting from consideration of the suitability of the cultivation technique. Transformation of the sperm head into a male pronucleus was limited to simple swelling or to distribution into one or several clearly staining chromatin structures. The latter were either localized at random in the cytoplasm (Pl. 1, Fig. 2) or, more often, near the normally developing female pronucleus (Pl. 1, Fig. 3). The presence of a tail in the vicinity, allowing definite identification of the male chromatin, was characteristic for these structures (Pl. 1, Fig. 2 and inset in Fig. 3).

Similar anomalies have already been described in cold-activated rabbit ova (Chalmel, 1962). Iwamatsu & Chang (1972) observed inability of the sperm head to transform to a male pronucleus in the egg cytoplasm of mouse oocytes penetrated by spermatozoa before reaching metaphase I. An incidence of similar post-fertilization anomalies was also very common in pig eggs cultivated from the germinal vesicle stage (Motlík & Fulka, 1974). Thibault & Gérard (1970) observed them almost solely in rabbit oocytes isolated for cultivation from large preovulatory follicles and then fertilized *in vitro*. Later, Thibault (1972) found that if the interval between HCG administration and isolation of the oocytes for cultivation was prolonged to 7 hr, the incidence of anomalies

EXPLANATION OF PLATE 1

All figures are phase-contrast photomicrographs.

FIG. 1. Cultivated rabbit eggs obtained 10 hr after transplantation. Both pronuclei are normally developed. $\times 500$.

FIG. 2. Rabbit egg obtained 8 hr after transplantation. Female pronucleus (FP) is apparently normal, transformation of sperm head is arrested. Sperm tail (arrow) is clearly visible. $\times 800$.

FIG. 3. Rabbit egg obtained 9 hr after transplantation. Female pronucleus with adjacent sperm head (arrow) and polar bodies (PBS) are visible. $\times 600$.

Inset: the same egg with sperm tail (arrow) in the focal plane. $\times 1000$.

FIG. 4. Control oocyte at the late dictyate stage at the beginning of the cultivation. $\times 500$.

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Table 1. Fertilization of rabbit oocytes cultivated in vitro

	No. of females	Cultivated oocytes					Ovulated oocytes		
		No. of oocytes transferred	No. of eggs recovered	Fertilized			No. of eggs recovered	Fertilized	
				No. of eggs with male and female pronuclei (%)	No. of eggs with arrested development of male pronuclei (%)	No. of eggs showing polyspermy (%)		No. of eggs with male and female pronuclei (%)	No. of eggs showing polyspermy (%)
Normal oestrous rabbits Rabbits subjected to superovulation	11	69	51	31 (60.8)	9 (17.6)	1 (2.0)	23	23 (100.0)	
	12	140	110	57 (51.8)	25 (22.7)	7 (6.4)	53	47 (88.7)	1 (1.9)

decreased significantly. This observation is in agreement with an earlier finding by Chang (1955b). Thibault (1972) assumed that synthesis of a substance responsible for development of the male pronucleus, which he termed the 'male pronucleus growth factor' (MPGF), did not occur under *in-vitro* conditions. At 6 hr or more after the administration of HCG or mating, when the nucleus of naturally maturing oocytes reaches metaphase I, a sufficient amount of MPGF does accumulate in the cytoplasm and ensures the development of the male pronucleus. Plate 1, Fig. 4 shows the nuclear stage usual in oocytes at the beginning of cultivation, when the nucleolus is indiscernible, but a distinct nuclear membrane encloses the bivalents characteristic for a late dictyate stage. The interval between mating and hormone injection and fixation of oocytes in acetalcohol did not exceed 30 min. Oocytes obtained from large follicles of untreated rabbits were also at the same stage of the development. Thibault & Gérard (1970) and Thibault (1972) cultivated oocytes from the same stage of nuclear development and obtained almost 100% anomalous forms of the male pronuclei but in our experiments two-thirds of the eggs developed normally. These differences could be caused by the composition of the culture medium. Alternatively, after transplantation of cultivated oocytes into the oviduct, complementary maturation could occur and the MPGF in the cytoplasm is then capable of ensuring normal development of both pronuclei.

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