FERTILIZATION OF RABBIT EGGS *IN VITRO* WITHOUT SUPPLEMENTAL CO₂ IN THE ATMOSPHERE

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To achieve fertilization of mammalian eggs *in vitro*, the gametes are often mixed and incubated in a CO₂-enriched atmosphere, e.g. rabbit (Brackett & Williams, 1968), mouse (Cross & Brinster, 1970), human (Edwards, Steptoe & Purdy, 1970). Since the various media are usually buffered with relatively high levels of bicarbonate, the CO₂ is needed to maintain a pH which will allow sperm penetration. Bicarbonate has a stimulatory effect on sperm metabolism (Murdoch & White, 1971) and may facilitate capacitation of hamster spermatozoa *in vitro* (Bavister, 1969). Furthermore, replacement of bicarbonate with tris or phosphate in a defined medium for ovum culture resulted in cessation of development (Brinster, 1969).

Using the technique of Brackett & Williams (1968) in which eggs and spermatozoa are cultured for 4 hr in an atmosphere of 5% CO₂:95% air, it has been possible to achieve a high rate of fertilization of rabbit eggs *in vitro* (Fraser, Dandekar & Vaidya, 1971). In some culture systems, the medium and overlying paraffin oil are open to the incubator atmosphere but in this system, the culture dishes containing the gametes are filled with CO₂-equilibrated paraffin oil and lids with ground glass seals are added. This would seem effectively to seal off the contents of the dishes from the atmosphere in the incubator, although Brackett (1969) reported that CO₂ in the incubator improved results. Experiments were carried out to determine whether supplemental CO₂ in the atmosphere was indeed beneficial to the fertilization process.

Virgin New Zealand white females were induced to superovulate with FSH/LH (Armour) and unfertilized eggs were recovered 12 or 13 hr after the injection of LH; the earlier timing was used when subsequent transfer of embryos to foster mothers was planned. Capacitated spermatozoa were recovered from does mated with three fertile males 16 to 18 hr previously. Cumulus and corona cells were removed from the eggs by the method described by Fraser *et al.* (1971). The fertilization medium (Brackett & Williams, 1968) was gassed, as usual, with 5% CO₂:95% air for 10 min to achieve a pH of 7.8 to 7.9, and the paraffin oil was equilibrated with the same gas mixture.

The sperm suspensions were pooled and divided into two lots, each of which received half of the corona-devoid eggs. Following addition of paraffin oil and lids, one dish was incubated in an atmosphere of 5% CO₂:95% air and the

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other in air alone. Under both sets of conditions, the pH of the medium remained 7.8 to 7.9 for a period of at least 24 hr. After 4 hr, the eggs were transferred to serum-containing culture medium (Fraser *et al.*, 1971) and cultured for 20 hr in air according to the standard procedure. The dishes were then examined for two- to four-cell embryos. The cleavage results are shown in Table 1. There was no significant difference between the two groups (χ^2 , $P > 0.05$), although the overall percentage of eggs fertilized in the CO₂-enriched atmosphere was slightly higher.

Most of the embryos were cultured for another 20 hr to allow examination of chromosome complements (Fraser *et al.*, 1971). Examination of the metaphase figures indicated that the majority of the embryos in each group were diploid, but three out of fifty-six (6%) from the group without CO₂ and one out of thirty-three (3%) from the group with CO₂ were triploid. Although initial experiments with this system yielded diploid embryos (Fraser *et al.*, 1971), the sample size of the embryos was relatively small. With the examination of more

Table 1. Fertilization of rabbit eggs *in vitro* with and without supplemental CO₂ in the incubator

Experiment No.	With CO ₂	Without CO ₂
1	42/42*	16/16
2	7/7	16/16
3	43/43	24/27
4	28/30	39/41
5	15/19	13/18
Total	135/141, 96%	108/118, 92%

* Expressed as no. of eggs cleaved/total no. of eggs.

embryos, the incidence of triploidy has been found to be approximately 5%. This may possibly reflect ageing in the eggs before mixing with spermatozoa. Experiments involving ageing of eggs *in vitro* before fertilization *in vitro* have resulted in a significant level of triploidy (Fraser & Dandekar, 1973) and ageing *in vivo* can lead to similar chromosomal anomalies (Austin, 1967). Furthermore, although eggs recovered 13 hr after the injection of LH showed a high rate of fertilization, the rate of implantation and fetal development after transfer of embryos to foster mothers was much lower than when eggs recovered 12 hr after LH were used (L. R. Fraser and P. V. Dandekar, in preparation). The triploidy might also be due to submergence of the second meiotic spindle during the agitation to remove corona cells. It has been noted that the two polar bodies, while generally close to one another in embryos fertilized *in vivo*, are often separated in embryos fertilized *in vitro*. This may indicate lateral movement of either the first polar body or of the spindle, and perhaps the spindle occasionally moves further into the cytoplasm rather than along the cortical region. This could result in retention of the second polar body and a triploid embryo.

Some two- to four-cell embryos were transferred to the oviducts of pseudo-pregnant recipient Dutch belted females (18 hr after mating with a vasectomized male). Of sixteen embryos transferred to two females, six implants were

observed by laparotomy on Day 10 *post coitum*, and four young were delivered on Day 32 *post coitum*. This is consistent with results obtained after transplantation of embryos fertilized with supplemental CO₂ in the incubator (L. R. Fraser and P. V. Dandekar, in preparation).

From these results, it seems clear that this particular culture system alone can maintain an environment which will support fertilization *in vitro*: supplemental CO₂ is not necessary at the time of sperm penetration in order to allow normal development to proceed. The fact that the pH of the medium inside the culture dishes remains constant indicates that little or no loss of CO₂ to the external atmosphere occurs. This finding thus simplifies the study of fertilization *in vitro* by alleviating the need for a gassing incubator. Since a recent study has reported the successful culture of one-cell rabbit eggs into expanding blastocysts using a complex medium but no CO₂ (Ogawa, Satoh & Hashimoto, 1971), it should be possible to study development of rabbit eggs *in vitro* from the unfertilized stage up to the stage of implantation, using a relatively simple apparatus.

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