Development of fertilized embryos transferred to oviducts of immature mice

V. E. Papaioannou and K. M. Ebert*

*Departments of Pathology and * Anatomy and Cellular Biology, Tufts University School of Medicine and Veterinary Medicine, 136 Harrison Avenue, Boston, MA 02111, U.S.A.*

Summary. The in-vitro culture of fertilized 1-cell mouse embryos to the blastocyst stage is associated with subsequent decreased viability. In this study, 1-cell embryos were cultured for 3 days in the reproductive tract of immature female mice as an alternative to in-vitro culture. Embryos that spent 3 days in immature females were developmentally more advanced, had higher cell numbers and better viability, as measured by development to mid-gestation, after transfer to pseudopregnant recipient females than did embryos maintained for the same period in culture. Embryos that developed in immature females had lower cell numbers but comparable rates of development and subsequent viability when compared with embryos transferred to synchronous pseudopregnant females for the same preimplantation period. The immature mouse oviduct is therefore a suitable alternative environment to in-vitro culture or a pseudopregnant host for complete preimplantation development and has the additional advantage that synchrony between embryo and temporary host is not necessary. This method will allow for evaluation of manipulation procedures while maintaining viability before the embryos are finally committed to a foster mother for development to term.

Introduction

Culture of mouse 1-cell embryos to the blastocyst stage has been reported with varied degrees of success, although in-vitro development is delayed compared to normal development within the reproductive tract of pregnant females (Bowman & McLaren, 1970a; Streffer, Van Benningen, Molls, Zamboglon & Schulz, 1980). Cross & Brinster (1973) reported 33.3% blastocyst development after 4 days of culture while Kaufman & Sachs (1976) and Tarkowski (1977) reported 48% and 67.5% respectively. Different culture medium (Cross & Brinster, 1973) and gas environments (Quinn & Harlow, 1978) as well as the strain of mice used appear to be variables that affect the success rate from one study to another.

Complete assessment of a culture system is only possible when the blastocysts are transferred to pseudopregnant females and the number of live fetuses or offspring is determined. Bowman & McLaren (1970b) showed that there was limited viability of cultured blastocysts after transfer. Harlow & Quinn (1979) improved the culture conditions, as indicated by an increase in live fetuses after transfer of in-vitro developed blastocysts, but with only 30% successful fetal development. Currently used culture conditions are still suboptimal and complicate experiments designed to study development of the preimplantation embryo after manipulation of the zygote (e.g. gene injection, nuclear transfer, sperm injection).

Beyer & Zeilmaker (1973) found that the mouse zygote could develop to the morula stage in the oviduct of prepubertal mice, demonstrating that the environment within the oviduct of a pregnant female was not necessary for the early stages of embryonic development. The relatively high success rate of continued development after transfer to pseudopregnant females (54% live offspring) suggested that this environment may be better than in-vitro culture systems.

Materials and Methods

Mice

Random-bred CD-1 mice were used throughout. To obtain zygotes, mature females were selected for oestrus by vaginal appearance and placed with males overnight (lights off 18:00–06:00 h). Mating was confirmed by the presence of a vaginal plug the next morning. To provide immature female recipients, pregnant females were housed singly or in pairs, allowed to litter, and male offspring were removed shortly after birth. Immature females with an imperforate vagina were used between 18 and 28 days of age. Pseudopregnant recipients for oviduct transfers and viability testing of embryos by uterine transfers were mature females in the 1st or 3rd day of pseudopregnancy respectively after mating with a proven sterile vasectomized male.

Embryo collection, transfer and culture

Oviducts were removed from pregnant females between 11:00 and 15:30 h on the day of mating and fertilized eggs were collected in modified Brinster's medium (PLG) with Hepes buffer (Ebert & Brinster, 1983) by tearing open the oviduct at the site of swelling caused by the cumulus mass. Cumulus cells were removed by brief exposure to hyaluronidase (Sigma) at 60 units/ml. Hyaluronidase was kept as a frozen stock of 300 units/ml in PBS with 1% polyvinylpyrrolidone and diluted to the final concentration in medium PLG with Hepes buffer. Embryos were washed in PLG medium and then transferred to oviducts of immature females, transferred to oviducts of pseudopregnant females or placed in equilibrated drops of PLG medium under liquid paraffin oil for culture at 37°C in 5% CO₂ in air. In one experiment, embryos were cultured in 5% O₂, 5% CO₂ and 90% N₂ (reduced oxygen).

Embryo transfers were carried out under tribromoethanol anaesthesia. A small incision was made in the skin at the level of the lumbar vertebrae, either mid-dorsally or on the flank, and the ovary was localized by sliding the skin over the peritoneum. A second incision was made in the peritoneum, the ovary and oviduct were pulled through both incisions by means of the ovarian fat pad, and stabilized by placing the utero-tubal junction in a V-shaped notch on a small piece of card. A single tear was made in the ovarian bursa using fine watchmakers forceps and a tightly rolled corner of tissue was then used to blot any blood and to probe for the ostium. Five to 10 zygotes were placed through the ostium into one oviduct of each immature or synchronous pseudopregnant female with a mouth-controlled, glass micropipette containing marker air bubbles that could be seen in the oviduct after transfer. The oviduct and ovary were then gently replaced into the peritoneal cavity and the skin incision was closed with a single wound clip.

After 3 days, the females were killed and transferred embryos were collected by flushing the oviducts and uteri with modified PB-1 medium (Papaioannou & West, 1981). Recovered embryos were scored for developmental stage and normality. Some were used to determine cell numbers and others were retransferred to the uteri of mature, pseudopregnant recipients (4–10 per recipient) to test their viability. These recipients were in the 3rd day of pseudopregnancy and were therefore 1 day asynchronous with the embryos (McLaren & Michie, 1956). These secondary recipients were killed at 9.5–12.5 days of gestation and any resulting fetuses were examined and scored for developmental stage and normality.

Cultured embryos were scored for developmental stage after 3 days (69–75 h) of culture. Some were used to determine cell numbers, others were transferred to pseudopregnant recipients to determine their viability, while others were maintained in culture for an additional day before transfer to pseudopregnant recipients. Cell number was estimated from nuclear counts in embryo squashes by the method of Ebert, Hammer & Papaioannou (1985) after a 30-min incubation in the DNA dye Hoechst 33258 (Polysciences Inc., Warrington, PA 18976). Synchronous control embryos for cell counts were flushed from the uteri of pregnant females at 3.5 days *post coitum* and stained immediately (Papaioannou, 1986).

Results

Table 1 summarizes the developmental stages of the transferred and cultured embryos. Of 144 embryos transferred to the oviducts of 12 pseudopregnant recipients, at least 90 were recovered 3 days later as morulae or blastocysts. Three abnormal morulae contained 5–18 cells plus fragments. The majority (79/90) were recovered from the uterus and all were within the zona pellucida. In addition, 80 degenerating or fragmenting 1–3-cell eggs were recovered, at least half and probably more of which were unfertilized host oocytes; because of the impossibility of determining their origin as host or transferred eggs, these are not included in Table 1. Of 122 zygotes transferred to 15 immature recipient females, 91 embryos (75%) were recovered 3 days later. Of the 15 females, 3 had a perforate vagina at the time of death but were not in oestrus and recovery from these 3 females was over 95%. Most embryos (81/91) were recovered from the oviducts but in 3 females embryos were recovered from the oviduct and uterus and in one female embryos were found only in the uterus. All embryos recovered were within the zona pellucida.

After 3 days, embryos in the immature and pseudopregnant females were more advanced morphologically than after culture in either gas condition; most embryos developed to the blastocyst stage after 3 days *in vivo* whereas none developed further than the morula stage after 3 days in

Table 1. Developmental stage of embryos maintained for 3 days *in vivo* in the oviducts of pseudopregnant or immature females, or for 3 days *in vitro* under two gas conditions

		Developmental stage				
		Normal		Abnormal		
		Blastocyst	Morula	Morula	1-8 cell	Fragmenting
	Total no.					
<i>In oviduct</i>						
Pseudopregnant female	90 (144)*	76	11	3	—†	—†
Immature female	91 (122)*	63	23	1	1	3
<i>In vitro</i>						
5% CO ₂ in air	231	0	183	1	42	5
5% CO ₂ , 5% O ₂ , 90% N ₂	58	0	54	0	2	2

* Number in parentheses is the total number of embryos transferred.

† Numbers are not included since these could not be distinguished from unfertilized host oocytes (see text).

Table 2. Midgestation viability of embryos transferred to the uteri of recipients after 3 days in the oviduct of pseudopregnant or immature females, or after 3 or 4 days *in vitro*

	Implantations/ no. transferred	Normal fetuses (%)	Abnormal fetuses (%)	Trophoblast only or empty decidua (%)
<i>In oviduct</i>				
Pseudopregnant female	19/29*	18 (95)	0 (0)	1 (5)
Immature female	27/35†	23 (85)	1 (4)	3 (11)
<i>In vitro</i>				
3 days	37/61‡	13¶ (35)	1 (3)	23 (62)
4 days	9/20§	1 (11)	1 (11)	7 (78)

* Another recipient with 4 embryos failed to become pregnant.

† Another 2 recipients with 19 embryos failed to become pregnant.

‡ Another 2 recipients with 14 embryos failed to become pregnant.

§ Another recipient with 5 embryos failed to become pregnant.

¶ Of these, 6 were retarded for their age but were morphologically normal.

culture. In addition, amongst embryos recovered from immature females there were fewer abnormal embryos compared with cultured embryos. With an additional day of culture, 29/39 and 34/37 of the morulae with a normal appearance became blastocysts in 5% CO₂ in air or in reduced oxygen, respectively. From these figures, the estimated overall rates of blastocyst formation after 4 days of culture were 59% in 5% CO₂ in air and 86% in reduced oxygen.

Table 2 presents the results of viability testing of morphologically normal morulae and blastocysts after recovery following 3 days in the pseudopregnant or immature female and after 3 or 4 days of culture. Considering only pregnant females, the rate of implantation of normal embryos recovered from immature females (77%) was higher than for any other group (66% from pseudopregnant females, 61% after 3 days of culture and 45% after 4 days of culture), but the more striking difference was apparent when the normality of development was considered. Only 14/46 (30%) of implanted cultured embryos developed normally to mid-gestation whereas 18/19 (95%) and 23/27 (85%) of embryos recovered from pseudopregnant and immature females respectively developed normally. There were a few abnormal fetuses but the most common type of developmental abnormality was a decidual swelling devoid of embryonic material or containing only a few trophoblastic giant cells.

Table 3. Cell numbers from morphologically normal embryos developing entirely *in vivo* to 3·5 days *post coitum*, recovered after 3 days in the oviduct of pseudopregnant or immature females, or after 3 days *in vitro* under two gas conditions

	Morphology	No.	Mean $\pm$ s.e.m. cell no.*
<i>In vivo</i>			
	Blastocysts	31	54·0 $\pm$ 3·4
In oviduct of pseudopregnant female	Blastocysts and morulae	45	40·5 $\pm$ 3·7
In oviduct of immature female	Blastocysts and morulae	28	33·2 $\pm$ 2·7
<i>In vitro</i>			
5% CO ₂ in air	Morulae	15	16·8 $\pm$ 2·3
5% CO ₂ , 5% O ₂ , 90% N ₂	Morulae	15	14·1 $\pm$ 2·3

* Using *t* tests for pairwise comparisons, the only results that are not significantly different are the two *in-vitro* cell counts.

As shown by nuclear counts (Table 3), embryos grown for 3 days in the reproductive tract of immature females contained significantly more cells than did morphologically normal embryos grown *in vitro* for the same period of time (*t* test, $t=7\cdot5$ and $t=8\cdot7$, df 41, $P < 0\cdot001$), but significantly fewer cells than in embryos developing entirely *in vivo* (Papaioannou, 1986) ($t = 8\cdot0$, df 57, $P < 0\cdot001$) or in pseudopregnant females ($t=2\cdot55$, df 71, $P < 0\cdot02$), although embryos grown in pseudopregnant females also had lower cell numbers than did embryos grown entirely *in vivo* ($t=4\cdot4$, df 74, $P < 0\cdot001$).

Discussion

The development of a blastocyst from a zygote *in vitro* does not guarantee that the embryo will produce a normal, viable fetus upon transfer to a recipient female. For example, parthenogenetic mouse embryos can develop into blastocysts of normal appearance but the development of a parthenogenetic adult mouse has not been achieved (Kaufman, Barton & Surani, 1977). Additionally, transfer of blastocysts of normal appearance that develop from the zygote *in vitro* results in only a small proportion (<30%) of the embryos developing into normal fetuses (Harlow & Quinn, 1979; present study).

This low percentage of normal fetal development interferes with the evaluation and interpretation of experiments involving manipulation of the fertilized ovum. To avoid the loss of viability of embryos in culture, manipulated zygotes are routinely transferred immediately after manipulation to the oviducts of pseudopregnant females even though this eliminates the possibility of assessing preimplantation development directly. An alternative surrogate environment, and one that does not require synchrony with the embryos, is the immature mouse oviduct as previously shown by Beyer & Zeilmaker (1973). The present experiments confirm that the immature mouse oviduct will support the complete development of the 1-cell embryo to the blastocyst. More importantly, the rates of implantation (77% vs 57%) and normal development amongst implantations (85% vs 30%) are much greater for embryos that develop within the immature oviduct than for those cultured *in vitro*. It appears that the immature oviduct is not only capable of supporting embryo development but that it is superior to in-vitro culture and compares favourably with embryos that develop in pseudopregnant females.

In evaluating an alternative to in-vitro culture for preimplantation development, any improvement in viability must more than compensate for any technical losses introduced before the method can be considered superior. Thus, in the present study, the improved development of embryos in an immature or pseudopregnant oviduct must compensate for losses of embryos due to transfer or recovery techniques since loss of embryos in culture due to causes other than developmental failure is rare. Amongst cultured embryos, 82% were morphologically normal after 3 days but only 17% of these implanted and developed normally in recipients. Therefore, the overall success of cultured embryos to mid-gestation was only 14%. For embryos transferred to pseudopregnant or immature females, recovery of normal embryos was 60% and 70% respectively of the total number transferred. Of these, 62% and 65% implanted and developed normally. These results give overall developmental success rates of 37% for embryos that developed for 3 days in pseudopregnant females and 46% for embryos that developed for 3 days in immature females. Therefore, not only is the immature oviduct a superior environment to in-vitro culture for preimplantation development but the overall success of the technique is superior and compares favourably with growth in a pseudopregnant reproductive tract, with the additional advantage that synchrony of embryo and temporary host is not required.

The present study does not address the question of why the immature oviduct is a better environment for complete development of the fertilized embryo than in-vitro culture conditions. The low cell number seen in cultured embryos is not as marked in embryos kept in oviducts of immature females but the number of cells in the latter embryos is still significantly lower than for embryos recovered directly from pregnant females or from pseudopregnant females at the same age. This may suggest that the hormones of pregnancy are important in producing an optimal environment for maintaining a normal rate of cell division or cell survival in the preimplantation embryo. The fact that embryos transferred and recovered from pseudopregnant females have significantly lower cell numbers than those recovered directly from pregnant females also indicates that removal from the reproductive tract for even a brief period of time can decrease cell division rate or increase cell death. In addition, the fact that zygotes develop only to the morula stage after 3 days *in vitro* while the majority of those transferred to the immature oviduct, like those in the pseudopregnant female, reach the blastocyst stage suggests that there is less retardation of the developmental programme within the immature oviduct compared with culture. This cannot entirely be attributed to effects on cell number, since blastulation is independent of cell number (Smith & McLaren, 1977).

The outbred strain used in this study is not subject to a block in development in culture at the 2-cell stage as are some other outbred strains (Goddard & Pratt, 1983). At present we have no information on the development in the immature oviduct of zygotes from strains that do exhibit the 2-cell block. It is possible that these zygotes will block at the 2-cell stage even within the oviduct of immature females. On the other hand, it is equally possible that the immature oviduct might provide a means of circumventing the 2-cell block seen in cultured embryos of certain strains.

The immature oviduct is therefore a convenient and suitable alternative environment for the early development and maintenance of subsequent viability of the mouse embryo and can be used effectively for studies of early embryo development after any manipulation of the fertilized egg.

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