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Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro*

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The morphology, cleavage rate and viability of preimplantation embryos from random bred Swiss mice were assessed after culture in different incubation volumes and embryo densities. Decreasing the incubation volume, from 320 to 20 μl, significantly increased blastocyst cell number ($P < 0.01$) and embryo development after transfer ($P < 0.01$). Increasing the number of embryos incubated per drop from 1 to 16 significantly increased the number of two-cell embryos reaching the blastocyst stage in 5 or 320 μl. Culturing embryos in groups significantly increased blastocyst cell numbers in all volumes employed and elevated embryo viability. Such observations are consistent with the hypothesis that the preimplantation mammalian embryo produces a factor(s) which can stimulate its own development. The results of this study have implications for clinical in-vitro fertilization, where embryos are routinely cultured individually in relatively large volumes.

Key words: cleavage/culture/embryo/viability/volume

Introduction

The cleavage rate and viability of the mammalian preimplantation embryo is greatly reduced by culture *in vitro* (Bowman and McLaren, 1970; Harlow and Quinn, 1982; Streffer *et al.*, 1980), indicating that present culture systems are far from optimal. The formulations of conventional embryo culture media do not reflect either the ionic (Borland *et al.*, 1977, 1980) or the metabolite (Gardner and Leese, 1990) composition of luminal fluid within the female reproductive tract. Furthermore, the physical conditions of the oviduct and uterus are not mimicked, and so during culture any paracrine factor(s) secreted or synthesized by the female tract will be absent from the in-vitro system. In addition, embryos are often cultured in relatively large volumes of medium (≤ 1 ml) compared to the sub-microlitre amounts of fluid present in the lumen of the reproductive tract. Therefore, any autocrine factor(s) produced by the embryo will be greatly diluted during culture *in vitro*. Thus, it is conceivable that irrespective of the suitability of the culture medium, physical factors, such as incubation volume, may become a limiting parameter in the culture system. Previous studies (Wiley *et al.*, 1986; Paria and Dey, 1990) have shown that embryo development *in vitro* is enhanced by increasing the embryo:volume ratio.

The aim of this study was to determine whether incubation volume and embryo density in culture had an effect on mouse embryo morphology, cleavage rate and subsequent viability. Determining the viability of an embryo is the only true method of assessing the suitability of culture conditions *in vitro*. If the preimplantation mouse embryo produces a factor(s) which is integral to development, then the cleavage rate and viability should increase when the incubation volume is decreased, and when embryos are cultured in groups.

Materials and methods

Embryo culture

Embryos were obtained from 6- to 8-week-old random-bred Swiss female mice. Females were superovulated with 10 IU pregnant mares' serum (Folligon, Intervet), administered i.p. followed 48 h later by 5 IU human chorionic gonadotrophin (HCG) (Chorulon, Intervet). Females were placed with males of the same strain immediately following the second injection. The presence of a vaginal plug the following morning (day 1) indicated that mating had taken place.

Two-cell embryos were flushed from the oviduct 46 h post-HCG (day 2) and collected in a HEPES-buffered mouse tubal fluid medium (MTF; Gardner and Leese, 1990), (Table I). Embryos were washed three times in this medium and once in HEPES-free MTF (Table I) before being placed in culture. In all experiments each female contributed equal numbers of embryos to each treatment group. Embryos were cultured in MTF medium either singly or in groups (2, 4, 8 and 16) in microdrops under paraffin oil and incubated at 37°C in 5% CO_2 in air. In some experiments, embryos were cultured singly in calibrated

Table I. Composition of embryo handling and culture media

Component (mM)	HEPES–MTF	MTF
NaCl	114.19	114.19
KCl	4.78	4.78
KH_2PO_4	1.19	1.19
$CaCl_2.2H_2O$	1.71	1.71
$MgSO_4.7H_2O$	1.19	1.19
$NaHCO_3$	5.00	25.00
Sodium lactate	4.79	4.79
Sodium pyruvate	0.37	0.37
Glucose	3.40	3.40
HEPES	20.00	0.00
BSA (mg/ml)	3.00	4.00

MTF = mouse tubal fluid medium. BSA = bovine serum albumin.

glass capillary tubes (Micropet; Becton-Dickinson and Company, Parsippany, NJ, USA). Calibrated glass capillary tubes were soaked in Milli-Q water for 24 h and autoclaved before use. All media were equilibrated in 5% CO_2 in air overnight before culture. Microdrops of medium (5, 10, 20, 40 and 80 μl) were set up under paraffin oil (BDH, Poole, Dorset, UK) in 35 mm Petri dishes (Nunclon, Roskilde, Denmark), whilst microdrops of 160 and 320 μl were placed in 4-well dishes (Nunclon) under paraffin oil. Medium for culture in capillary tubes was equilibrated under paraffin oil in drops of 160 μl for 12 h prior to being taken up into the capillary. The morphology of embryos cultured under oil was assessed daily.

Cell number determination

Embryo cell number was determined at either 90 h (day 4) or 114 h post-HCG (day 5). Embryos were placed in 0.4% sodium citrate for ≤2 min depending upon the stage of development, transferred to a pre-cleaned glass slide and fixed using glacial acetic acid: ethanol (1:3). Blastomere nuclei were subsequently stained with 10% Giemsa, in Gurr's buffer at pH 6.8, and counted.

Assessment of embryo viability

Embryos cultured singly in volumes of 5, 20 or 320 μl, and in groups in 20 μl, were transferred 90 h post-HCG (day 4), after 44 h of culture, to the uteri of day 3 pseudopregnant female recipients. In-vivo developed embryos, collected at 90 h post-HCG, were transferred as controls. On day 15 of pregnancy, implantation sites were assessed and fetuses weighed.

Statistical analysis

The results were analysed by analysis of variance, Student's *t*-test and chi-square test.

Results

Incubation volume

Incubation volume had no effect on the percentage of individually cultured two-cell embryos reaching the morula stage by day 4 of development. However, morulae grown in 5 or 20 μl had a significantly higher cell number than embryos developed in 320 μl ($P < 0.001$) (Figure 1).

On day 5 of development, embryos cultured singly in 5−320 μl showed no significant difference in the percentage which developed into blastocysts. However, embryos cultured singly in 10 μl or 20 μl had higher cell numbers than embryos cultured in larger volumes ($P < 0.05$ and $P < 0.01$ respectively) (Figure 2). Culture in 5 μl produced a significantly lower blastocyst cell number compared to embryos grown in 10 or 20 μl ($P < 0.01$). Given the large surface area: volume ratio of the 5 μl drop and the reduced cleavage rate of embryos observed in this volume, we wanted to eliminate any possible detrimental effects of the overlaying oil. Embryos were therefore cultured singly in 5, 10, 20, 40 or 80 μl in calibrated glass capillary tubes. Embryos cultured in 5 μl in capillary tubes had a significantly higher blastocyst cell number ($P < 0.01$) than embryos cultured in 5 μl under paraffin oil and had a cell number comparable to embryos grown in 10 and 20 μl in capillary tubes (Figure 2).

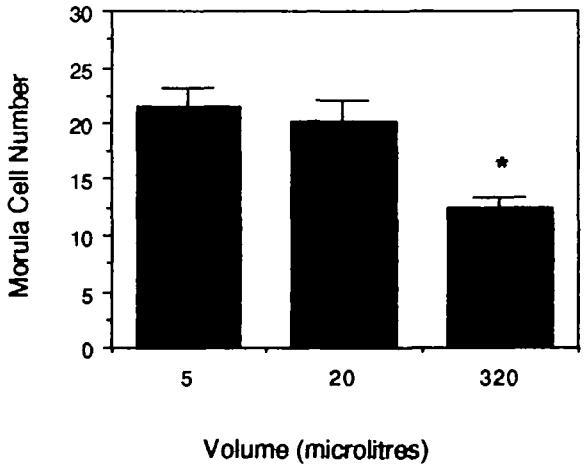

Fig. 1. Cell numbers of morulae cultured individually from the two-cell stage in 5, 20 or 320 μl, at 90 h post-HCG. Number of embryos/treatment = 40. *Significantly different to 5 and 20 μl ($P < 0.01$). Error bars represent standard errors.

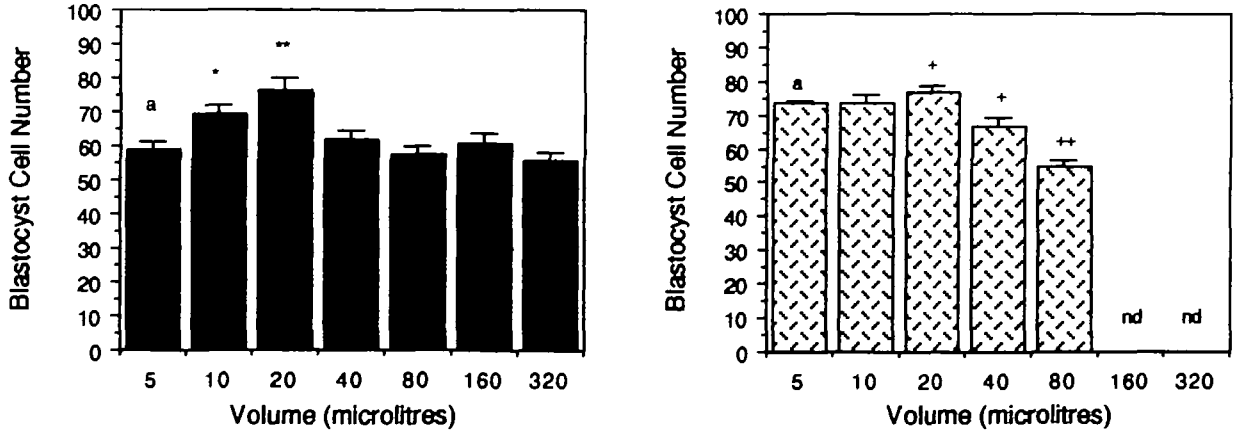

Fig. 2. Cell numbers of blastocysts cultured individually from the two-cell stage in drops of medium under paraffin oil (solid bars), or in capillary tubes (hatched bars), at 114 h post-HCG. Number of embryos/treatment = 50. *Blastocyst cell number in 5 μl significantly different between groups ($P < 0.01$). *·+Significantly different to all treatments within the same group ($P < 0.05$); **·++Significantly different to treatments within the same group ($P < 0.01$); nd, Not determined. Error bars represent standard errors.

Embryos cultured individually in 20 μl gave rise to significantly more implantation sites and fetuses ($P < 0.01$) than embryos which developed in 5 or 320 μl under oil (Table II).

Embryo density

Increasing the number of embryos per drop had no effect on development to the morula. Two-cell embryos cultured in 20 μl at a density of two and 16 embryos had higher cell numbers (30.1 ± 1.3 and 31.8 ± 1.3 respectively; $P < 0.01$) than embryos cultured individually (21.4 ± 2.8) after 44 h of culture. Culturing embryos in groups in 5 or 320 μl resulted in a significant increase ($P < 0.05$) in the percentage of two-cell embryos which developed into blastocysts. In contrast, grouping embryos had no effect on blastocyst formation in a volume of 20 μl (Figure 3).

In experiments to determine the effect of embryo density on blastocyst development, three volumes were employed: 5, 20 and 320 μl. In these volumes, blastocyst cell number increased with embryo density (Figure 4). The beneficial effects of increasing embryo numbers diminished at high embryo densities, the same plateau level of ~110 blastomeres being reached for each volume. In-vivo developed blastocysts contained significantly more blastomeres, ~160 ($P < 0.01$). The optimum blastomere number *in vitro* was attained with an embryo density of 16 in 5 μl, two in 20 μl and eight in 320 μl. An elevation in implantation and fetal development was observed when embryos were cultured in increasing numbers in 20 μl drops of medium ($P < 0.06$). The viability and fetal weight in all groups was significantly lower than that of in-vivo developed embryos ($P < 0.001$) (Table II).

Table II. Effect of incubation volume and embryo density on the viability of cultured mouse embryos

Treatment	Embryo density	Implantations % (*n*)	Fetuses %	Fetal weights (g)
5 μl	individual	15.2 (105)^{a,e,f}	10 5^{i,g,h}	0.2396
20 μl	individual	27.6 (105)^{a,b}	21.0^{i,j}	0.2630
320 μl	individual	7.8 (90)^{b,c,d}	6.7^{j,k,l}	0.2416
20 μl	groups of 2	32.7 (60)^{c,e}	23.3^{g,k}	0.2603
20 μl	groups of 16	38.0 (50)^{d,f}	28.0^{h,l}	0 2597
In vivo		54.2 (50)*	48.6*	0.3109*

Numbers of embryos transferred given in parentheses.
Like pairs significantly different from each other: a, c, e, g, i ($P < 0.05$); b, d, f, h, j, k, l ($P < 0.01$).
*Significantly different to all values ($P < 0.001$).

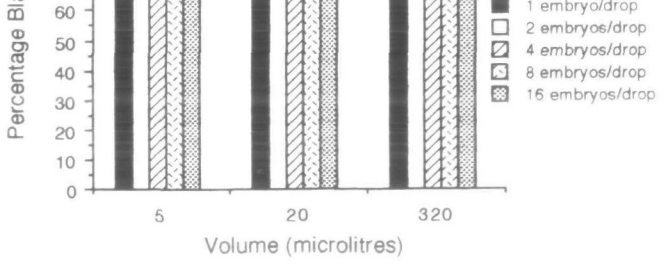

Fig. 3. Effect of incubation volume and embryo density on the percentage of two-cell embryos which develop into blastocysts. Number of embryos/treatment = 100. *Significant differences within 5 and 320 μl ($P < 0.05$).

Discussion

This study provides indirect evidence that the preimplantation mouse embryo produces a factor(s) which stimulates development in culture and enhances subsequent viability after transfer. This conclusion is based on the observations that embryo cleavage and viability are significantly increased by decreasing the incubation volume, and that blastocyst cell number is increased with increasing embryo density in culture.

Decreasing the incubation volume from 320 to 20 μl had a significant effect on the cell number of embryos by day 4 of culture (Figure 1). This observation indicates that the preimplantation mouse embryo produces a mitogenic factor, the effect of which is diluted in the larger volume. The benefits of a reduced incubation volume on embryo cleavage rates was extended to day 5 of culture (Figure 2). Poor blastocyst development and the reduction of cell numbers for embryos cultured in 5 μl was overcome by culturing embryos in glass capillary tubes instead of under paraffin oil, in order to remove any effect of the larger surface area:volume ratio of the smaller drop. It would appear, therefore, that the embryo-derived factor(s) is either lipophilic or is readily denatured at the oil/aqueous interface.

The effect of the embryonic factor(s) on subsequent viability after transfer was significant (Table II). At the time of transfer (day 4 of culture), there was no difference in embryo cell number between 5 and 20 μl (Figure 1). In contrast, embryos developed in 20 μl gave rise to significantly increased implantation and fetuses than those in 5 μl. It would appear, therefore, that the embryo-derived factor(s) has at least two but clearly separate

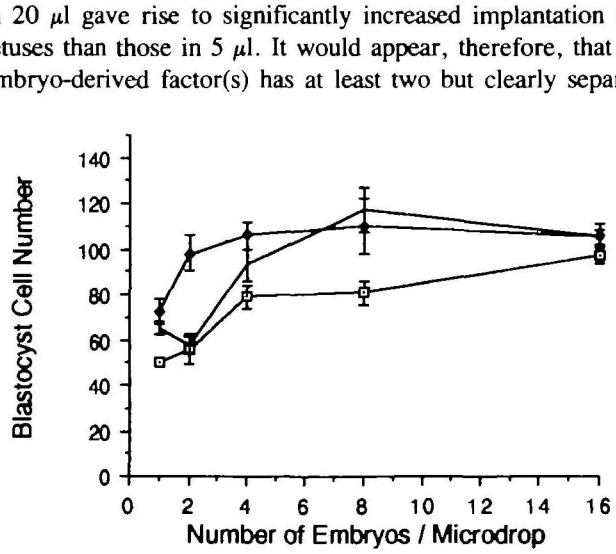

Fig. 4. Cell numbers of blastocysts cultured from the two-cell stage in either 5 (—□—), 20 (—♦—) or 320 μl with increasing embryo density. Number of embryos/treatment = 50. Cell numbers between volumes are significantly different for all embryo densities except for 16 embryos/drop. Within each volume there were significant increases in cell number prior to embryo densities of 16 in 5 μl ($P < 0.01$), 2 in 20 μl ($P < 0.05$), 8 in 320 μl ($P < 0.01$). Error bars represent standard errors.

effects on the developing embryo; firstly as a mitogen, increasing cell number; secondly, it enhances embryo viability as indicated by an increase in the number of embryos which implant. Alternatively, the data are not inconsistent with the production of two distinct factors by the embryo. The largest volume employed, 320 μl, gave the lowest embryo viability, consistent with the hypothesis of the dilution of an embryo-derived factor(s).

Increasing the number of embryos cultured in the same drop significantly increased the number of embryos reaching the blastocyst stage (Figure 3) and their cell number (Figure 4). These observations are also consistent with the production of an embryo-derived factor(s). In the three volumes used for examining the effects of embryo density on development, 5, 20 and 320 μl, the same maximum response was observed, embryos having ~ 110 blastomeres. In-vivo developed blastocysts of the same age have ~ 160 blastomeres. Therefore, the in-vitro cultured embryos were less than one cell cycle behind the controls. As the same cell number was attained in the three volumes, it appears that the response to the mitogen has been maximized. Any further increase in cleavage rate will possibly require modifications of another component of the culture system.

With respect to embryo viability, increasing the number of embryos per drop elevated the number of implantations and fetuses, but this was not significant ($P < 0.06$).

Whilst residing in the lumen of the female reproductive tract, the preimplantation mouse embryo is exposed to maternal paracrine factors, such as insulin (Heyner *et al.*, 1989). It has also been demonstrated that the mouse embryo possesses highly specific receptors for insulin-like growth factor-2 (Harvey and Kaye, 1991a) and insulin (Rosenblum *et al.*, 1986; Harvey and Kaye, 1991b) at the two-cell and compacted eight-cell stage respectively.

Addition of exogenous growth factors to embryo culture medium has been shown to stimulate several cell functions. In the mouse embryo, insulin increases cleavage of the inner cell mass (Harvey and Kaye, 1990) and stimulates protein and nucleic acid synthesis from compaction onward (Harvey and Kaye, 1988; Heyner *et al.*, 1989; Rao *et al.*, 1990). Epidermal growth factor (EGF) and transforming growth factor (TGF) one α and β1 all improve blastocyst development in the mouse, whilst EGF and TGFα are mitogenic (Paria and Dey, 1990). Furthermore, EGF has been shown to increase protein synthesis by the trophectoderm (Wood and Kaye, 1989). In the bovine embryo, TGFα increases blastocyst formation, whilst platelet-derived growth factor (PDGF) shortens the fourth cell cycle, during which the embryonic genome is activated (Larson *et al.*, 1991). It is not known which of the above growth factors are present in the female reproductive tract. However, Rappolee *et al.* (1988) demonstrated that in the mouse the transcripts for PDGF A-chain and TGFα are present in the oocyte, and PDGF A-chain, TGFα and TGFβ1 are found in the blastocyst.

A possible candidate for the putative embryo-derived factor responsible for the observed data in this study is the ether phospholipid, platelet-activating factor, which has been reported to be produced by the preimplantation embryo of several species (O'Neill, 1985; O'Neill *et al.*, 1985). Supplementation of embryo culture medium with exogenous platelet-activating factor increased blastocyst cell number and subsequent embryo viability after transfer (Ryan *et al.*, 1990), whilst the addition of platelet-activating factor antagonists to the culture medium significantly reduces blastocyst development *in vitro* and *in vivo* (Spinks *et al.*, 1990). Furthermore, platelet-activating factor, being a phospholipid, is lipophilic and will therefore be readily lost to an oil overlay. However, data regarding the ability of the mouse embryo to synthesize this factor are conflicting (Smal *et al.*, 1990). We are currently investigating the nature of the embryo-derived factor(s) whose effects are described in this study.

Although development *in vitro* and *in vivo* were significantly improved by decreasing the incubation volume and increasing embryo density, the cleavage rate and post-implantation development of cultured embryos were retarded compared to in-vivo developed controls. Subsequent improvements in embryo culture are therefore likely to come from optimization of medium composition.

This study has important implications for clinical work, where in-vitro fertilization and subsequent embryo culture occur in relatively large volumes of ≤ 1 ml, and where embryos are grown individually. Although there is usually only one embryo residing in the human reproductive tract at a time, there may still be beneficial effects conferred by an individual human embryo to its siblings *in vitro*, the healthier embryos being able to stimulate the less viable.

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