# Embryo Density and Medium Volume Effects on Early Murine Embryo Development

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***Background:*** *One-cell mouse embryos were used to determine the effects of drop size and number of embryos per drop for optimum development in vitro.*
***Methods:*** *Embryos were collected from immature C57BL6 female mice superovulated with pregnant mare serum gonadotropin and human chorionic gonadotropin and mated by CD1 males. Groups of 1, 5, 10, or 20 embryos were cultured in 5-, 10-, 20-, or 40-μl drops of CZB under silicon oil at 37.5°C in a humidified atmosphere of 5% $CO_2$ and 95% air.*
***Results:*** *Development score for embryos cultured in 10 μl was higher than that of embryos cultured in 20 or 40 μl. Embryos cultured in groups of 5, 10, or 20 had higher development scores than embryos cultured singly. The highest development score was obtained by the combination of 5 embryos per 10-μl drop. The percentage of live embryos in 20 or 40 μl was lower than that of embryos cultured in 10 μl. Additionally, the percentage of live embryos cultured singly was lower than that of embryos cultured in groups.*
***Conclusions:*** *Our results suggest that a stimulatory interaction occurs among embryos possibly exerted through the secretion of growth factors. This effect can be diluted if the embryos are cultured in large drops or singly.*

KEY WORDS: embryo culture; density; culture volume.

## INTRODUCTION

The development of murine embryos in vitro is affected by factors such as strain of mice, culture medium, and culture system. Currently, most culture systems involve the use of microdrops of medium covered with oil. Brinster (1) demonstrated that it was possible to culture mouse embryos from the two-cell to the blastocyst stage in vitro using microdrops of medium covered with oil. Embryo density can affect murine preimplantation embryo development in vitro (2). Recently Paria and Dey (3) showed that the ratio of the number of embryos per microliter of medium affected preimplantation embryo development. Therefore, our objective was to establish the optimum volume of medium and number of embryos per drop for in vitro development of one-cell mouse embryos to the hatching or hatched blastocyst stage.

## MATERIALS AND METHODS

Female C57BL6 mice (Charles River, Wilmington, MA), 3 to 4 weeks old, were superovulated with 5 IU pregnant mare serum gonadotropin (PMSG; Diosynth Inc., Chicago, IL) and, 48 hr later, 5 IU human chorionic gonadotropin (hCG; Schein Pharmaceuticals, Inc., Port Washington, NY) and mated by CD1 males. One-cell embryos ($n$ = 640) were collected 20 to 22 hr after hCG. Pooled embryos ($n$ = 40) were randomly assigned to 1 of 16 culture systems. Groups of 1 ($n$ = 40), 5 ($n$ = 8), 10 ($n$ = 4), or 20 ($n$ = 2) embryos were cultured in 5-, 10-, 20-, or 40-μl drops of CZB medium (4) under silicon oil at 37.5°C in a humidified atmosphere of 5% $CO_2$ in air for 120 hr. Embryos were evaluated every 24 hr and scored 0 to 9 (0 = degenerate; 1 = two-cell; 2 = four-cell; 3 = eight-cell; 4 = compact morula; 5 = early blastocyst; 6 = blastocyst; 7 = expanded blastocyst; 8 = hatching blastocyst; 9 = hatched blastocyst). Score rather than cell number was evaluated to reduce the skewness of the treatments. Differences in development scores and percentage of live embryos (PLE) at each time period were analyzed by analysis of variance (5). Embryos were classified live if they were at the appropriate stage of in vitro development at each time of evaluation. Embryos that had degenerated or that had arrested were not considered advancing in development.

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## RESULTS

The effect of medium volume on development score is shown in Table I. During the first 24 hr of culture, embryos cultured in the larger volumes developed more rapidly. By 72 and 96 hr embryo development was similar across culture volumes. However, the development score at 120 hr for embryos cultured in 10-μl drops (6.1 ± 0.3) was higher ($P < 0.01$) than that for embryos cultured in 20 μl (4.9 ± 0.3) or 40 μl (4.5 ± 0.3). The development score for embryos cultured in 5-μl drops (5.6 ± 0.3) was not different from that for embryos cultured in 10 or 20 μl but was higher ($P < 0.01$) than that for embryos cultured in 40-μl drops.

Embryo numbers began to have a significant impact on development by 96 hr in culture (Table II). Culturing embryos in groups of 5, 10, or 20 resulted in higher developmental scores ($P < 0.01$) than that of embryos cultured singly at 120 hr (5.5 ± 0.3, 5.6 ± 0.3, and 6.2 ± 0.3 vs 3.8 ± 0.3). The highest developmental score at 120 hr was obtained by the combination of 5 embryos per 10-μl drop (7.1 ± 0.6). The lowest development score was obtained by the combination of 1 embryo per 40-μl drop (3.0 ± 0.6; Fig. 1). Generally, multiple embryos in 10 μl had the highest development scores, while single embryos developed less except for single embryos in 5-μl drops. Variance between replicates generally decreased from $3.5^2$ to $0.8^2$ as the observation within a replicate increased from 1 to 20 embryos. However, when the data were pooled and evaluated by the ratio of embryos per unit volume, the ratio of 2 embryos/μl of medium resulted in the highest development score (Fig. 2). While not statistically different ($P > 0.10$), the ratios of 1 embryo/μl and 4 embryos/μl did not develop as well. The 2:1 ratio was greater ($P < 0.05$) than all ratios below 1 embryo/μl (1:1).

Beginning after 48 hr an 8 to 18% decline in PLE occurred at 72 hr (Table III). Percentage of live embryos after 120 hr of culture also was affected by ($P < 0.05$) both drop size and number of embryos per drop (Tables III and IV). At 120 hr the PLE cultured in 40 μl (58.7) was lower ($P < 0.05$) than that of embryos cultured in 10 μl (77.5). The PLE cultured in 5 μl (73.8) and 20 μl (64.4) was not different ($P > 0.05$) from the others. Additionally, the PLE for embryos cultured singly (51.3) was lower ($P < 0.05$) than that for embryos cultured in groups of 5 (72.5), 10 (70.6), or 20 (71.3) per microdrop.

## DISCUSSION

This study confirms and expands upon previous reports on the effect of embryonic density on murine embryo development in vitro. Quinn *et al.* (6) cultured 1, 10, or 20 mouse zygotes in 10-μl drops and found that embryos cultured in groups had higher developmental rates to blastocyst and hatching blastocyst stages than embryos cultured singly. In our study, the combination of a 10-μl drop and 10, 20, or 40 embryos per drop yielded the best development.

Our results also support the theory that mouse embryos secrete compounds that are required for optimum development in vitro. Rapolee *et al.* (7) investigated the production of growth factors by mouse preimplantation embryos. They were able to detect mRNA for platelet-derived growth factor (PDGF) and transforming growth factor alpha (TGFα) and $TGF_{\beta 1}$ in whole blastocysts, suggesting a role for these factors in early differentiation and development of mouse embryos.

More recently, Paria and Dey (3) showed that two-cell mouse embryos cultured singly in 25-μl drops had inferior development to the blastocyst stage and lower cell numbers per blastocyst than those cultured in groups of 5 or 10. Moreover, they were able to improve the inferior development of singly cultured embryos by adding epidermal

Table I. Mean (±SE) Development Score of Mouse Embryos Cultured in Various-Sized Drops of CZB Medium over Time

| Time in culture (hr) | Culture drop volume (μl) | | | |
|---|---|---|---|---|
| | 5 | 10 | 20 | 40 |
| 24 | 1.0 ± 0.03[c,*] | 1.0 ± 0.03[c] | 1.2 ± 0.03[b] | 1.6 ± 0.03[a] |
| 48 | 3.7 ± 0.07[a] | 3.7 ± 0.07[a] | 3.4 ± 0.07[b] | 3.8 ± 0.07[a] |
| 72 | 5.1 ± 0.19[a] | 5.4 ± 0.19[a] | 4.7 ± 0.19[a] | 5.0 ± 0.19[a] |
| 96 | 5.5 ± 0.23[a] | 5.9 ± 0.23[a] | 5.1 ± 0.23[a] | 5.1 ± 0.23[a] |
| 120 | 5.6 ± 0.27[a,b] | 6.1 ± 0.27[a] | 4.9 ± 0.27[b,c] | 4.5 ± 0.27[c] |

* Development scores in the same row with different superscripts differ ($P < 0.01$).

**Table II.** Mean (±SE) Development Score of Mouse Embryos Cultured in Varying Numbers in CZB Medium over Time

| Time in culture (hr) | Number of embryos per drop | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 20 |
| 24 | 1.3 ± 0.03[a,*] | 1.2 ± 0.03[a] | 1.2 ± 0.03[a] | 1.2 ± 0.03[a] |
| 48 | 3.7 ± 0.07[a] | 3.7 ± 0.07[a] | 3.4 ± 0.07[b] | 3.8 ± 0.07[a] |
| 72 | 4.8 ± 0.19[a] | 5.3 ± 0.19[a] | 5.1 ± 0.19[a] | 5.1 ± 0.19[a] |
| 96 | 4.7 ± 0.24[b] | 5.6 ± 0.24[a] | 5.5 ± 0.24[a,b] | 5.8 ± 0.24[a] |
| 120 | 3.8 ± 0.27[b] | 5.5 ± 0.27[a] | 5.6 ± 0.27[a] | 6.2 ± 0.27[a] |

* Development scores in the same row with different superscripts differ ($P < 0.01$).

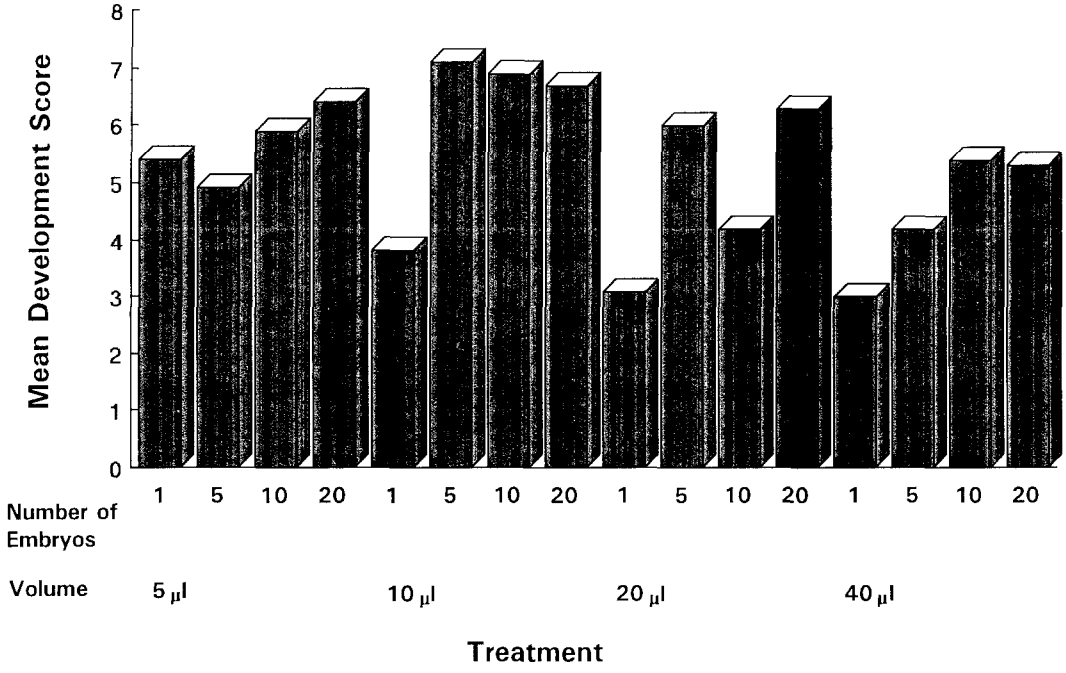


**Fig. 1.** The effect of culture drop volume and number of mouse embryos cultured per drop on embryo development score at 120 hr in culture.

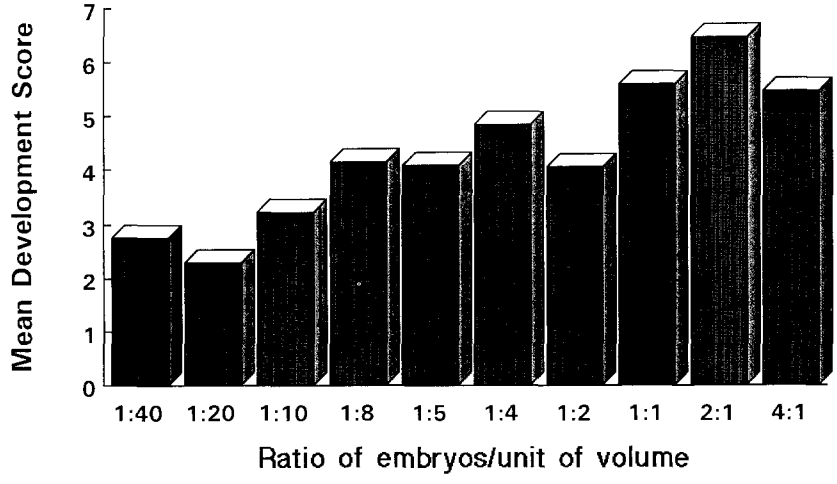


**Fig. 2.** The effect of the ratio of embryo number to unit volume on mean development score at 120 hr in culture.

Table III. Percentage of Live Embryos in Various-Sized Drops Cultured in CZB Medium over Time[a]

| Time in culture (hr) | Drop size (μl) | | | |
|---|---|---|---|---|
| | 5 | 10 | 20 | 40 |
| 24 | 95.0[a,*] | 100.0[a] | 98.8[a] | 97.5[a] |
| 48 | 93.8[a] | 98.8[a] | 95.0[a] | 96.9[a] |
| 72 | 85.6[a] | 90.0[a] | 81.9[a] | 78.8[a] |
| 96 | 78.1[a] | 83.1[a] | 72.5[a] | 71.9[a] |
| 120 | 73.8[a,b] | 77.5[a] | 64.4[a,b] | 58.7[b] |

[a] Percentage reflects the proportion of embryos that were at the appropriate stage of development at each time of evaluation. Degenerate embryos and non-stage-specific development are reflected in these values.

* Percentages in the same row with different superscripts differ ($P < 0.05$).

growth factor (EGF) or $TGF_{\alpha}$ or $TGF_{\beta 1}$. However, insulin-like growth factor (IGF) had no influence on embryo development. Additionally, when they increased the drop size to 50 μl, they observed a detrimental developmental effect on singly cultured embryos, suggesting a dilution of any beneficial compounds secreted by the embryo.

Evidence for a dilution effect also was found by Lane and Gardner (8), who reported that embryos cultured singly in 5-μl drops had higher cell numbers than those cultured in 320-μl drops. Additionally, when embryos were cultured in groups of two in 20-μl drops, there was a significant increase in cell number. However, eight embryos were required to increase the number of cells per embryo when 320-μl drops were used. Also, after transfer of cultured embryos to pseudopregnant recipients, singly cultured embryos in 20 μl yielded more implantations than those grown in 320 μl.

Table IV. Percentage of Live Embryos by Varying Numbers of Embryos Cultured in CZB Medium over Time[a]

| Time in culture (hr) | Number of embryos per drop | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 20 |
| 24 | 100.0[a,*] | 97.5[a] | 95.6[a] | 98.1[a] |
| 48 | 98.1[a] | 96.8[a] | 93.1[a] | 96.3[a] |
| 72 | 82.5[a] | 85.6[a] | 83.1[a] | 85.0[a] |
| 96 | 68.1[a] | 78.8[a] | 76.9[a] | 81.9[a] |
| 120 | 51.3[b] | 72.5[a] | 70.6[a] | 71.3[a] |

[a] Percentage reflects the proportion of embryos that were at the appropriate stage of development at each time of evaluation. Degenerate embryos and non-stage-specific development are reflected in these values.

* Percentages in the same row with different superscripts differ ($P < 0.05$).

The appearance of EGF receptors in mouse embryos occurs at the eight-cell, morula, and blastocyst stages (9). We first observed a delay in development of singly cultured embryos at 96 hr of culture (blastocyst stage; Table II), suggesting that a lack of an embryotrophic factor (possibly EGF) may play a role in this developmental delay in vitro.

In conclusion, our results and those discussed above suggest that it is important to consider the embryo numbers and medium volume as factors that influence early mammalian embryos development in vitro. Five embryos per 10 μl of medium appears optimal for the development of mouse embryos in CZB medium from the one-cell to the hatching blastocyst stage.

## REFERENCES


1. Brinster RL: A method for in vitro cultivation of mouse ova from two-cell to blastocyst. Exp Cell Res 1963;32:205–208
2. Wiley LM, Yamami Sand Munden VD: Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation embryo development in vitro. Fertil Steril 1986;45:111–119
3. Paria BC, Dey SK: Preimplantation embryo development in vitro: Cooperative interaction among embryos and role of growth factors. Proc Natl Acad Sci USA 1990;87:4756–4760
4. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I: An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fert 1989; 86:679–688
5. SAS: SAS User's Guide: Statistics. Cary, NC, SAS Institute, 1985
6. Quinn P, Hirayamada T, Marrs RP: Cooperative interaction among mouse zygotes cultured in protein-free medium: Blastocyst development and hatching. Serono Symposium on Preimplantation Embryo Development, 1991, p 68 (abstr)
7. Rapolee DA, Brenner CA, Schultz R, Mark D, Verb Z: Developmental expression of PDGF, $TGF_{\alpha}$, and $TGF_{\beta}$ genes in preimplantation mouse embryos. Science 1988;241:1823–1825
8. Lane M, Gardner DK: Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. Serono Symposium on Preimplantation Embryo Development, 1991, p 33 (abstr)
9. Dey SK, Paria BC: Chemical signals in embryo-maternal dialog: Role of growth factors. Serono Symposium on Preimplantation Embryo Development, 1991, p 21 (abstr)