

## Effects of gas atmosphere, platelet-derived growth factor and leukemia inhibitory factor on cell numbers of rabbit embryos cultured in a protein-free medium

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(Received 21 July 1996; accepted 23 October 1996)

**Summary** — While culture conditions for embryos have improved, development *in vitro* is not equivalent to *in vivo* growth. Platelet-derived growth factor (PDGF), mouse (mLIF) or human (hLIF) leukemia inhibitory factor and 10% fetal bovine serum (FBS) added to protein-free culture medium, as well as two gas atmospheres were evaluated for their effect on rabbit embryo development. Adding PDGF, mLIF or hLIF to the culture medium did not result in detectable differences in total cell number for blastocysts. The culture of rabbit embryos under a gas atmosphere of 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> resulted in improved total cell numbers ( $P < 0.01$ ) for blastocysts compared to those developing under 5% CO<sub>2</sub>: 95% air (230 vs 159, respectively). Supplementing RD (RPMI-1640 and Dulbecco's MEM, 1:1) medium with 10% FBS improved the number of total cells (264 vs 155) and inner cell mass cells (71 vs 47). These results indicate that when defined culture conditions promote a high proportion of two-cell embryos developing into blastocysts, the addition of certain growth factors did not have a detectable beneficial effect, while 10% FBS improved culture conditions.

**rabbit blastocyst / inner cell mass cell / oxygen / PDGF / LIF**

**Résumé** — Effet des conditions de culture (environnement gazeux, présence de PDGF et LIF) sur le développement d'embryons de lapin *in vitro*. L'objectif de ce travail est d'optimiser les conditions de culture *in vitro* pour les embryons de lapins. Aussi, les effets du facteur de croissance dérivé des plaquettes (PDGF), du facteur inhibiteur de la leucémie d'origine murine (mLIF) ou humaine (hLIF) et de 10 % de sérum de veau foetal ont été étudiés. Deux environnements gazeux pendant la culture ont été également comparés. Aucun des traitements avec le PDGF et les LIF n'a affecté les nombres de cellules des blastocystes. En revanche, l'environnement 10 % CO<sub>2</sub> : 5 % O<sub>2</sub> : 85 % N<sub>2</sub> avait un effet favorable sur les effec-

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tifs cellulaires des blastocytes (230 cellules) par rapport à un gazage avec 5 % CO<sub>2</sub> : 95 % air (159 cellules). L'addition de sérum de veau fœtal avait également un effet bénéfique sur les nombres de cellules (264 versus 155) et sur le nombre de cellules du bouton embryonnaire (71 versus 47). Les résultats démontrent que dans un système de culture optimisé pour embryons de lapins, seul le sérum de veau fœtal (mais pas le PDGF et le LIF) améliore encore les résultats.

## **blastocyste / lapin / oxygène / LIF / PDGF**

### **INTRODUCTION**

The development of rabbit embryo culture has progressed from media containing plasma or serum to semi-defined media containing bovine serum albumin (BSA) and finally to defined media containing no macromolecules (reviewed by Kane, 1987; Carney and Foote, 1991). Although the majority of rabbit embryos cultured in these defined media will develop into blastocysts, the number of cells is reduced compared to embryos developing in vivo (Carney and Foote, 1990, 1991; Collas et al, 1991; Giles and Foote, 1995), indicating that the culture systems are suboptimal.

Improvements in total cell numbers have been accomplished by decreasing the oxygen concentration and/or increasing the carbon dioxide concentration of the gas atmosphere under which rabbit embryos are cultured (Hallden et al, 1992; Li and Foote, 1993; Farrell and Foote, 1995). Further improvements in in vitro development may be dependent on the identification and addition of biological factors produced in the oviducts during embryo development. In fact, the expression of ligand and receptor genes for many growth factors in preimplantation stage embryos or in the oviduct supports the claim for their involvement during early embryonic development (Rappolee et al, 1988; Watson et al, 1992; Schulz and Heyner, 1993). Two such factors are platelet-derived growth factor (PDGF) and leukemia inhibitory factor (LIF).

The addition of PDGF to the culture medium has been reported to enhance development of bovine embryos past the eight-to-16-cell stage (Larson et al, 1992; Thibodeaux et al, 1993) or to increase the proportion of embryos developing into blastocysts (Yang et al, 1993). Other investigators, however, have not observed a beneficial effect of PDGF on development of mouse embryos (Colver et al, 1991) or cow embryos (Flood et al, 1993). The LIF, a glycoprotein expressed on cultured mouse blastocysts (Murray et al, 1990) and in the uterine endometrial glands of mice by the fourth day of pregnancy, is maternally controlled and is essential for implantation (reviewed by Stewart, 1994). The addition of human LIF (hLIF) to the culture medium increased the number of eight-cell stage mouse embryos which hatched and increased the size of blastocyst outgrowths in vitro (Robertson et al, 1991; Lavranos et al, 1995). Similarly, the culture of embryos in the presence of hLIF resulted in fewer degenerate embryos and a greater proportion of hatched blastocysts in the sheep (Fry et al, 1992) and cow (Fukui and Matsuyama, 1994; Han et al, 1995). Furthermore, using an antibody to hLIF, Yang et al (1994) detected LIF in luminal epithelium of rabbit uteri.

The present study was designed to test the hypothesis that PDGF and human or mouse LIF (hLIF or mLIF) added to a defined protein-free medium could improve rabbit embryo development into blastocysts when cultured under different gas environments, in comparison with fetal bovine serum (FBS) in the medium.

## MATERIALS AND METHODS

### Animals, superovulation and embryo recovery

Sexually mature Dutch-belted and an albino strain of Dutch-belted rabbits were superovulated as previously described (Kennelly and Foote, 1965). Semen was collected from fertile males and used for artificial insemination at the time of injection of luteinizing hormone (LH; day 0, d0) to induce ovulation. Embryos were collected, following midventral laparotomy, by flushing the oviducts with Dulbecco's phosphate-buffered saline (PBS; Gibco BRL, Grand Island, NY) + 0.1% polyvinyl alcohol (PVA; Sigma, St Louis, MO, USA) 24–26 h (predominantly two-cell but some four-cell embryos) or 48–50 h (16-cell embryos) after injection of LH.

### Embryo culture

Embryos were cultured in 50  $\mu$ L droplets (five to seven embryos/droplet) of modified RD medium (Carney and Foote, 1991) overlaid with silicone medical fluid (Dow Corning, Midland, MI, USA) at 39 °C under a humid gas atmosphere of 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> or 5% CO<sub>2</sub>: 95% air. Modifications of RD medium were the use of low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) and the addition of 0.1% PVA (Li and Foote, 1993). The two-cell with a few four-cell embryos were cultured for 3 days (d4 of development) with the exception of those in experiment 3, which were cultured for 5 days (d6 of development). Sixteen-cell embryos in experiment 3 were cultured for 4 days (d6 of development). At the end of culture, the embryos were allocated for study with immunosurgery and differential staining procedures.

### Immunosurgery and differential staining

Immunosurgery of blastocysts and differential labeling of trophectoderm (TE) cells and inner

cell mass (ICM) cells was accomplished as previously described (Handyside and Hunter, 1984; Giles and Foote, 1995). Briefly, the mucin coat and zona pellucida were removed and the embryos were incubated with mouse-antirabbit serum in the absence of a complement, washed and treated with rabbit serum as a source of complement. Embryos were fixed, stained with bisbenzimidazole (Hoechst 33342, Sigma Chemical Co, St Louis, MO, USA) and propidium iodide (PI; Sigma), mounted on a glass slide and examined using a fluorescence microscope. The lysed TE cells should be permeable to bisbenzimidazole and PI and fluoresce pink, whereas the ICM cells, unaffected by the immunosurgery procedure, should be permeable only to bisbenzimidazole, resulting in blue nuclei.

### Experimental design

Experiment 1 was a 3 x 2 factorial study designed to determine if embryo development could be enhanced by adding 0, 1 or 5 ng/mL of recombinant human PDGF-BB (R & D Systems, Minneapolis, MN, USA) to the protein-free culture medium. In addition, the effects on embryo development of two gas atmospheres, 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> or 5% CO<sub>2</sub>: 95% air, were examined.

Experiment 2 was a 2 x 2 x 4 factorial study designed to determine if embryo development could be enhanced by adding 0 or 1 000 U of mLIF or 0 or 1 000 U of recombinant hLIF (Gibco BRL), all with 0 or 10% FBS in the RD culture medium. This was the only experiment in which FBS was used. Again, the effects on embryo development of two gas atmospheres, 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> or 5% CO<sub>2</sub>: 95% air, were examined.

Experiment 3 was a 2 x 2 factorial study designed to determine the effects on embryonic development by adding 0 or 1 000 U of recombinant hLIF to the RD culture medium. Both two- and 16-cell stage embryos were utilized since the embryonic stage at the time of exposure to hLIF was previously reported to affect devel-

opment (Fukui and Matsuyama, 1994; Han et al, 1995; Lavranos et al, 1995). Furthermore, the culture interval was increased such that all embryos were cultured until d6 of development, in an effort to discern any effects of hLIF on blastocyst hatching, as had been reported for embryos of the mouse (Robertson et al, 1991; Lavranos et al, 1995), sheep (Fry et al, 1992) and cow (Han et al, 1995). However, the embryos were also examined earlier (d4 of development). The gas atmosphere was 10% CO<sub>2</sub>:5% O<sub>2</sub>: 85% N<sub>2</sub>.

Experiment 4 was a 2 x 2 factorial study where embryos were cultured in medium containing 0 or 5 ng/mL of PDGF and 0 or 1 000 U/mL recombinant hLIF. Again, embryos were cultured under a gas atmosphere of 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub>.

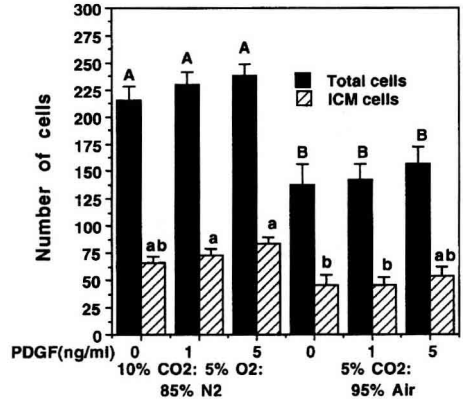
### Statistical analysis

Percentages of embryos developing into blastocysts were recorded daily. Following the described culture interval, blastocysts were stained and the number of ICM and TE cells were counted and total cells calculated. Data were analyzed using the general linear models procedure of SAS (1985). Differences among means were detected using the Tukey hsd option, with  $P < 0.05$  being considered as statistically significant.

## RESULTS

### Experiment 1

Two to four-cell embryos recovered from four donors, cultured in a defined medium supplemented with 0, 1 or 5 ng/mL PDGF and under a gas atmosphere of 10% CO<sub>2</sub>:5% O<sub>2</sub>: 85% N<sub>2</sub> or 5% CO<sub>2</sub>: 95% air, developed into morulae or blastocysts with 72/82 (88%) blastocysts on d4. Three blastocysts were lost while staining. Cell numbers were greater ( $P < 0.01$ ) for embryos developing under 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> gas atmosphere (fig 1)

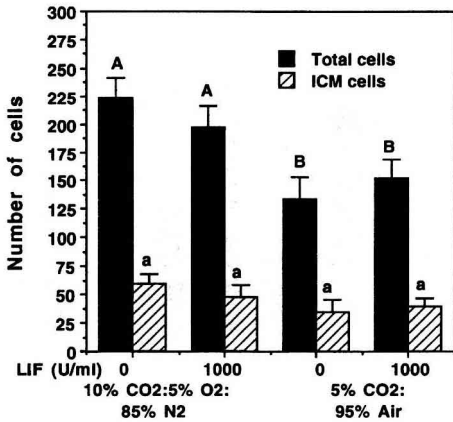


**Fig 1.** Experiment 1 ( $n = 69$ ). Effect of 0, 1 or 5 ng/mL platelet-derived growth factors (PDGF) on the number of total cells and inner cell mass (ICM) cells for blastocysts developing in protein-free RD medium under 10% CO<sub>2</sub>:5% O<sub>2</sub>:85% N<sub>2</sub> or 5% CO<sub>2</sub>:95% air gas atmospheres. Values are expressed as means  $\pm$  SEM. Bars with different superscripts (A vs B and a vs b) differ significantly ( $P < 0.05$ ).

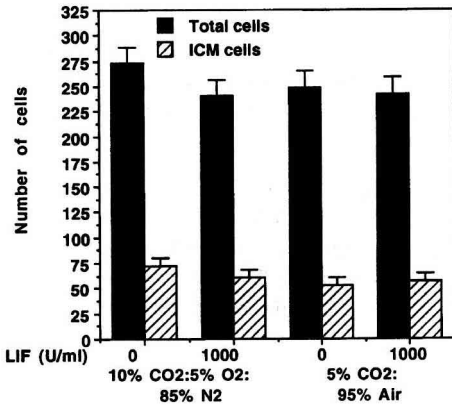
compared to 5% CO<sub>2</sub>: 95% air (230 total and 75 ICM cells compared to 159 total and 48 ICM cells, respectively). The addition of PDGF had no significant ( $P > 0.10$ ) effect on the number of cells in the blastocysts.

### Experiment 2

Of the 149 two- to four-cell embryos recovered from six donors, 144 (97%) developed into morulae or blastocysts with 76% blastocysts on d4. Four blastocysts were lost while staining. The percentage of embryos developing to the blastocyst stage was not significantly ( $P > 0.10$ ) affected by gas atmosphere, or mLIF, hLIF or 10% FBS in the culture medium. Cell numbers for blastocysts developing in medium supplemented with mLIF ( $n = 236$ ) were not significantly ( $P > 0.05$ ) different from hLIF ( $n = 194$ ), nor different from the total cell numbers for control blastocysts ( $n = 224$ ). Therefore, data for blastocysts developing in the presence of mLIF or



**Fig 2.** Experiment 2 ( $n = 50$ ). Effect of 0 or 1 000 U/mL mouse (mLIF) or human leukemia inhibitory factor (hLIF) and 10% CO<sub>2</sub>:5% O<sub>2</sub>:85% N<sub>2</sub> or 5% CO<sub>2</sub>:95% air gas atmospheres on the number of total cells and inner cell mass (ICM) cells for blastocysts developing in RD medium without fetal bovine serum. Values are expressed as means ± SEM. Bars with different superscripts (A vs B and a vs b) differ significantly ( $P < 0.05$ ).



**Fig 3.** Experiment 2 ( $n = 59$ ). Effect of 0 to 1 000 U/mL mouse (mLIF) or human (hLIF) leukemia inhibitory factor (hLIF) and 10% CO<sub>2</sub>:5% O<sub>2</sub>:85% N<sub>2</sub> or 5% CO<sub>2</sub>:95% air gas atmospheres on the number of total cells and inner cell mass (ICM) cells for blastocysts developing in RD medium with 10% fetal bovine serum. Values are expressed as means ± SEM. Bars within total cells and ICM cells were not different ( $P > 0.10$ ).

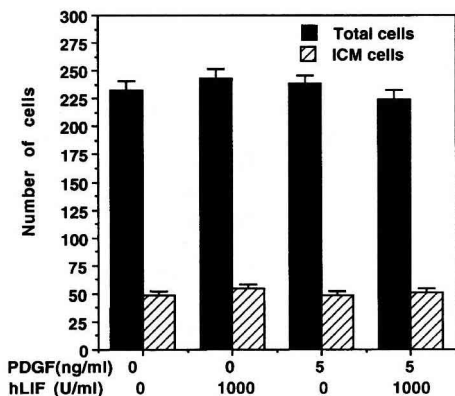
hLIF are combined in figure 2 without FBS and in figure 3 with FBS. In the absence of FBS (fig 2), the number of total cells in the blastocysts developing under 10% CO<sub>2</sub>:5% O<sub>2</sub>:85% N<sub>2</sub> was greater than for blastocysts under 5% CO<sub>2</sub>:95% air (206 vs 155;  $P < 0.05$ ). In the presence of FBS (fig 3), there was no effect of gas atmosphere on the number of cells. Figures 2 and 3 also show that the addition of 10% FBS vs no FBS increased total cell numbers (251 vs 176;  $P < 0.01$ ) as well as the number of ICM cells (62 vs 46;  $P < 0.05$ ).

**Experiment 3**

In experiment 3, embryos were recovered from 13 donors at the two- to four or 16-cell stage ( $n = 160$ ) and cultured in defined medium without FBS, to compare the treatment of embryos at different stages with hLIF. Development of 16-cell embryos exposed to hLIF was reduced ( $P < 0.05$ ) at d4 compared to controls (75 vs 98% blastocysts). Two-cell embryos cultured with hLIF resulted in 100% blastocysts by d4 and were greater ( $P < 0.05$ ) than 88% for controls. Although embryos were cultured to d6 and then stained and examined for numbers of ICM and TE cells, these counts are not reported because the blastocysts were observed to be undergoing degeneration.

**Experiment 4**

In experiment 4, 170 two- to four-cell embryos recovered from eight donors and cultured in defined medium supplemented with 0 or 5 ng/mL PDGF and 0 or 1 000 U/mL hLIF, resulted in 98% blastocysts. The addition of either PDGF or hLIF or both to the culture medium had no beneficial effect on cell numbers (fig 4). In addition, the percentages of hatching blastocysts on d4 listed in the same order of treatments shown in fig 4 were 26, 39, 36 and 26, respectively ( $P > 0.05$ ).



**Fig 4.** Experiment 4 ( $n = 167$ ). Effect of 0 or 5 ng/mL platelet-derived growth factors (PDGF) and 0 or 1 000 U/mL human leukemia inhibitory factor (hLIF) on the number of total cells and inner cell mass (ICM) cells for blastocysts developing in RD medium under a gas atmosphere of 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub>. Values are expressed as means  $\pm$  SEM. Bars within total cells and ICM cells were not different ( $P > 0.10$ ).

## DISCUSSION AND CONCLUSION

Most rabbit embryos cultured in RD medium develop into blastocysts (Carney and Foote, 1991; Hallden et al, 1992; Li and Foote, 1993). However, cell numbers are often compromised compared to embryos developing *in vivo* (Fischer, 1987; Fischer et al, 1990; Carney and Foote, 1991). Carney and Foote (1990) observed embryos to have a mean of 242 cells at 3.5 days; at d4, Collas et al (1991) found 310 cells and Giles and Foote found 414 cells. In the present study, the number of ICM and TE cells comprising blastocysts developing in RD medium supplemented with growth factors and/or FBS under two gas atmospheres was determined.

In agreement with other studies (Hallden et al, 1992; Li and Foote, 1993; Lindenau and Fischer, 1994; Farrell and Foote, 1995), we observed that the gas atmosphere utilized for rabbit embryo culture affects embryo development. Blastocysts produced under a gas

atmosphere of 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub>, and without serum, had a greater number of total cells than those cultured under 5% CO<sub>2</sub>: 95% air (figs 1 and 2). The improved development with 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> could be due to the decreased oxygen concentration (Li and Foote, 1993; Lindenau and Fischer, 1994; Farrell and Foote, 1995), or to the increase in carbon dioxide (Hallden et al, 1992). In fact, with rabbit embryos, the improved development is probably the effect of both gases.

In the present study, differences in blastocyst cell numbers due to gas atmosphere were affected by the presence of 10% FBS. The effect seen in figure 2 without FBS was eliminated when FBS was included (fig 3). One explanation may be that more free radicals are formed in the 5% CO<sub>2</sub>: 95% air atmosphere (approximately 20% O<sub>2</sub>) than when 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> is used and these radicals may be toxic to the embryo in a protein-free medium (Li and Foote, 1993). The radicals, however, may be bound or modified by high molecular weight proteins present in FBS and, therefore, differences in embryo development due to gas atmosphere may be reduced or eliminated.

The FBS may provide a variety of growth and other factors. Improved cell numbers for blastocysts were observed if RD medium (fig 2) was supplemented with 10% FBS (fig 3). In this experiment, the addition of 10% FBS improved the number of total cells and ICM cells for embryos cultured under 5% CO<sub>2</sub>: 95% air, but not the ratio between the two. These results are consistent with other reports in which the addition of biological fluids improved embryo cell numbers (Collas et al, 1991).

In the present study, culture medium supplemented with PDGF and/or LIF had no effect on the proportion of embryos developing to the blastocyst stage or on cell numbers (figs 1–4). The differences in response reported here and in the literature could be due to several factors. Species differences

and differences in culture conditions, including medium, protein source, gas atmosphere and embryo density, may affect embryo response to PDGF and/or LIF supplementation.

In addition, the growth factors utilized were not proteins isolated from rabbits since these were not available. However, we used recombinant human PDGF-BB which has been shown to have biological activity in cultured rabbit gastric epithelial cells (Watanabe et al, 1996) and smooth muscle cells (Fabunmi et al, 1996). Similarly, LIF was detected in the luminal epithelium of the rabbit uterus on d5 of pregnancy using an antibody to recombinant hLIF (Yang et al, 1994).

In conclusion, these studies indicate that the protein-free medium provides a nutrient environment suitable for two-cell embryos to be cultured into a high proportion of blastocysts. The gas atmosphere of 10% CO<sub>2</sub>: 5% O<sub>2</sub>: 85% N<sub>2</sub> enhanced blastocyst development in both total cell numbers and ICM cells, compared with 5% CO<sub>2</sub>: 95% air. However, development was less than optimal compared to media containing 10% FBS. Under these conditions, the inclusion of PDGF or LIF did not improve culture conditions of rabbit embryos.

## ACKNOWLEDGMENTS

The authors thank Dr GA Presicce for helpful suggestions and D Bevins for assistance with manuscript preparation. This research was supported in part by the NICHD National Cooperative Program on Non-human In Vitro Fertilization and Preimplantation Development, funded by NICHD through Cooperative Agreement HD21939. The follicle-stimulating hormone and luteinizing hormone were supplied by the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development and the US Department of Agriculture.

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