

Effect of supplementation of leukemia inhibitory factor and epidermal growth factor on murine embryonic development in vitro, implantation, and outcome of offspring

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Objective: To evaluate the supplementation of leukemia inhibitory factor (LIF) or epidermal growth factor (EGF) in a single culture system (IVF50) and a sequential culture system (IVF50-S2) to determine whether they had any additional benefit on embryo development and implantation.

Design: Prospective controlled animal study.

Setting: University research laboratory.

Subject(s): Two-cell embryos from F1(CBA × C57BL) mice.

Intervention(s): Embryos were randomly cultured in different growth factor-treated or untreated in vitro systems.

Main Outcome Measure(s): Blastocyst formation and morphology, total cell numbers of day 5 blastocysts, birth rates, and characteristics of the offspring.

Result(s): The beneficial effects of LIF or EGF on blastocyst development and morphology were observed only in IVF50 medium but not in sequential IVF50-S2 media. In addition, blastocysts generated from the LIF-supplemented cultures had higher total cell numbers and higher total birth rates after transfer compared to those from IVF50 medium alone. No significant effect on fetal development was observed but pups born after treatment in LIF-supplemented sequential cultures had prolonged gestation and increased birth weights.

Conclusion(s): The beneficial effects of LIF and EGF on in vitro blastocyst development are mainly seen under suboptimal culture conditions (simple medium only) and their effects are masked in an improved in vitro system (sequential culture media). Supplementation of culture media with such factors, however, is still an attractive approach in enhancing embryo viability through increased total cell numbers. The beneficial effect of LIF in improving implantation and subsequent increased birth rates should be further explored. (Fertil Steril® 2003;80(Suppl 2):727–35. ©2003 by American Society for Reproductive Medicine.)

Key Words: Growth factors, cytokines, in vitro culture

Despite years of experience with the assisted reproductive techniques (ART) and many technical innovations, a low implantation rate is still one of the major obstacles resulting in a large proportion of embryonic loss in human ART programs. Recent evidence suggests that transfer of one to two embryos at the blastocyst stage gives high implantation rates of up to 40%–50% and at the same time significantly reduces multiple pregnancy rates (1, 2). However, the problem remains that only 25%–30% of embryos develop to the blastocyst stage in conventional simple culture media (3). Embryo fragmentation, blastomere irregularity, retarded rates of embryo

cleavage and blastulation, along with reductions in total cell numbers observed in blastocysts grown in vitro (4), have been attributed to suboptimal in vitro conditions, which in turn contribute significantly to reduced embryo viability. Co-culture systems were shown to increase blastulation rates of as high as 55%–70% (5, 6), but such systems have been fraught with the fear of disease transmission and the labor involved in establishing and maintaining cell lines.

Better understanding of the modes of action of co-culture systems and the basic metabolic requirements of the preimplantation embryo at

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different embryonic stages have led to the formulation of new cell-free, serum-free sequential culture media that can generate high blastulation rates of 50%–70% and improved implantation rates of up to 50% (1, 2). However, some of the embryotrophic factors that have been shown to be released by the co-cultured cells, such as growth factors, cytokines, and low molecular weight glycoproteins, are still absent in these new generation sequential culture media. Barmat et al. (7) showed the secretion of colony-stimulating factor-1, interleukin-6 (IL-6), leukemia inhibitory factor (LIF), and epidermal growth factor (EGF) from human tubal epithelial cells and hypothesized that these were responsible for the positive effects of co-culture. In addition, some of these factors such as LIF and EGF have increasingly been shown to be involved in preparing a receptive endometrium and in the blastocyst–endometrium dialogue, which are required for successful implantation (8, 9). Addition of these factors into the current sequential media may see a further improvement in cell-free, in vitro systems that could contribute significantly to the success of assisted reproductive programs.

However, previous studies in sheep (10, 11) and cattle (12, 13) suggested that in vitro embryo culture may induce adverse outcomes on the offspring including prolonged gestation, increased birth weights, and increased perinatal death rates after embryo transfer. Factors responsible for the production of abnormal offspring after in vitro handling have not been identified, although serum has been suggested to be a potential contributing factor (11). It is still not clear whether serum, or some unknown factors in it, are the source of the problem. It is therefore important to carefully study the development of offspring before any supplementation is introduced into the culture media. The supplementation of growth factors and cytokines to culture media require more caution because of their effect on cell proliferation and differentiation. Preliminary animal studies should be undertaken to carefully evaluate the risks and benefits of these factors before they are applied to human embryo culture.

Therefore, this study was designed to evaluate the supplementation of two of these putative factors, LIF and EGF, in a simple single culture medium (IVF50: Scandinavian IVF Science AB, Göteborg, Sweden) and a newly developed sequential culture media formulation (IVF50-S2: Scandinavian IVF Science AB) to study their effects both individually and in combination on [1] preimplantation mouse embryo development in terms of blastulation and hatching rates, embryo morphology, and total cell numbers (TCN) of day 5 blastocysts; [2] embryo viability and implantation potential after transfer to pseudopregnant recipients; and [3] the immediate and long-term outcome of the offspring. For the first time, their effects in two different in vitro culture systems, the conventional single culture medium and the new sequential culture media, are being compared.

Embryo Collection

Four to 8-week-old F1 (CBA × C57BL) hybrid female mice were superovulated by intraperitoneal injections of 10 IU of pregnant mare serum gonadotropin (PMSG) (Folligon; Intervet, Boxmeer, Holland) followed 48 hours later with 10 IU of hCG (Chorulon; Intervet). On the same day of hCG injection, which was designated as day 0, the female mice were paired with male mice of the same strain to allow mating. The mated females were then killed 44 hours after hCG administration and two-cell stage embryos harvested from the oviducts in prewarmed HEPES-buffered ASP250 medium (Scandinavian IVF Science AB), followed by washing in IVF50 medium before incubation at 37°C in a 5% CO₂ in air atmosphere.

In Vitro Culture

Two thousand seven hundred two-cell mouse embryos with regular blastomeres and no fragments were pooled together and evenly distributed to the different in vitro systems. A total of 16 replicates were carried out in two stages. The growth factors were compared with single IVF50 medium in stage A and sequential IVF50-S2 media in stage B. In each replicate, approximately 30–60 embryos were randomly allocated to each culture group. Embryos were cultured in 20 μL microdroplets of medium covered with pre-equilibrated oil (Ovoil; Scandinavian IVF Science AB). The number of embryos cultured in each microdroplet varied from 10 to 15 in different replicates, but the same number was used between the treatment groups. The cultures were then incubated at 37°C in 5% CO₂ in air. The culture media were changed daily in both the study and the control groups.

Stage A: Single Culture Medium System

The IVF50 medium was used as the simple single medium throughout the period of in vitro culture of embryos to the blastocyst stage. Embryos were allocated to four in vitro systems in stage A. Group 1 embryos were cultured in IVF50 medium supplemented with 10 ng/mL of recombinant murine LIF (Sigma L5158, St. Louis, MO) from day 2 onward. Group 2 embryos were cultured in IVF50 medium supplemented with 10 ng/mL of murine natural EGF (GIBCO E4127, Tissue Culture Grade, Grand Island, NY) from day 2 onward. Group 3 embryos were cultured in IVF50 medium supplemented with 10 ng/mL of recombinant murine LIF and 10 ng/mL of murine natural EGF from day 2 onward. Group 4 was the control group and the embryos were cultured in IVF50 medium alone without supplementation of growth factors.

Stage B: Sequential Culture Media System

The embryos were grown sequentially in IVF50 medium on day 2, a 1:1 mixture of IVF50 and S2 medium on day 3, and then S2 medium from day 4 onward. Embryos were allocated to five in vitro systems in stage B. Group 1 em-

bryos were cultured in sequential IVF50-S2 media supplemented with 10 ng/mL of recombinant murine LIF from day 2 onward. Group 2 embryos were cultured in sequential IVF50-S2 media supplemented with 10 ng/mL of murine natural EGF from day 2 onward. Group 3 embryos were cultured in sequential IVF50-S2 media supplemented with 10 ng/mL of recombinant murine LIF and 10 ng/mL of murine natural EGF from day 2 onward. Group 4 embryos were cultured in sequential IVF50-S2 media alone without supplementation of growth factor. Group 5 embryos were cultured in IVF50 medium alone without supplementation of growth factor.

Embryo Monitoring

The embryos were monitored twice daily from day 3 to day 6 at the same time in the morning and afternoon for embryo morphology, cleavage rate, and the percentage progression to different embryonic stages. The monitoring was performed by the same investigator using Normarski's inverted optics. The embryo morphology was graded at the blastocyst stage according to good, fair, and poor. Good blastocysts were classified as those with a distinct inner cell mass (ICM), a well-differentiated trophoectoderm, and a single large blastocoelic cavity without degenerative foci on day 5 of development. Fair blastocysts were classified as transitional blastocysts that developed more slowly but eventually became the good type on day 6 of development. Poor blastocysts did not have a distinct ICM and clear trophoectoderm but had a number of degenerative foci and a poorly developed blastocoelic cavity.

Evaluation of TCN

In two of the random replicates, all the morphologically normal blastocysts on day 5 were evaluated for total cell number using a slight modification of the method of Tarkowski (14). Briefly, the blastocysts were first exposed to a hypotonic aqueous solution of 0.5% sodium citrate and incubated at 37°C for 30 minutes. Each embryo was then placed on a precleaned grease-free slide and excess sodium citrate was removed. A few drops of freshly prepared fixative (3:1, methanol:glacial acetic acid) previously cooled at -70°C was placed directly over the blastocyst, allowing it to swell and flatten. Excess fixative was avoided to prevent overscattering and loss of nuclei. The slides were then air dried and stained with 10% Giemsa in phosphate buffer for 5 minutes. The total number of cell nuclei were then counted at ×400 magnification.

Uterine Transfer to Pseudopregnant Recipients

In another nine replicates selected randomly, all the morphologically normal blastocysts on day 5 were transferred to the uterine horns of pseudopregnant recipient female mice to evaluate their implantation potential. Natural cycling Swiss Albino female mice weighing >20 g were mated with the vasectomized Swiss Albino males to produce the pseudo-

pregnant recipients. The females were examined the next morning for the presence of a vaginal plug. If present, this was designated to be day 1 of pseudopregnancy. Female mice at day 3 and day 4 of pseudopregnancy were evenly distributed among the different treatment groups for transfer of blastocysts. The method of transfer was as described by Hogan et al. (15). Briefly, the recipients were anesthetized by intraperitoneal (IP) injection of 0.05 mL of CRC (Clinical Research Centre) cocktail (one part of hypnorm, one part of midazolam, and two parts of distilled water) per 10-g mouse body weight. A dorsal incision was made to pull out one side of the uterine horn. A 25-gauge needle attached to a 1-mL syringe was then used to puncture a hole through the wall of the uterus into its lumen at the mesenteric side of the uterus near the uterotubal junction. About three to five blastocysts were then loaded and transferred to each uterine horn. The same procedure was then repeated on the other side.

Pregnancy was then monitored and the pseudopregnant recipients were allowed to give birth. The parameters measured at the time of birth were the litter size, the gestation length, the birth weight of the offspring, and the incidence of stillbirths and abnormal fetuses. The subsequent growth of the offspring was also monitored. A few female and male offspring in each of the treatment groups were then randomly selected and mated when they grew up to examine their fertility potential.

Statistical Evaluation

The proportion of embryos reaching each developmental stage, the embryo morphology, and some of the characteristics of the offspring such as total births, live births, and stillbirths were analyzed using χ^2 or Fisher's exact test. The TCN of day 5 blastocysts, the gestation length and the birth weights of the offspring were compared by one-way analysis of variance (ANOVA). All post-hoc analyses were corrected by the Bonferroni method for multiple group comparisons.

RESULTS

Blastocyst Development and Morphology

Tables 1 and 2 illustrate the effect of LIF and EGF on blastocyst development and morphology in different in vitro culture conditions.

In the single culture medium system (Table 1), the IVF50 medium after supplementation with either LIF or EGF resulted in higher blastulation rates (LIF + IVF50 vs. IVF50: 64.9% vs. 36.6%, $P < .0001$; EGF + IVF50 vs. IVF50: 59.5% vs. 36.6%, $P < .0001$) and higher percentage of good blastocysts (LIF + IVF50 vs. IVF50: 65.4% vs. 34.9%, $P < .0001$; EGF + IVF50 vs. IVF50: 59.6% vs. 34.9%, $P < .0001$). The stimulatory effect of LIF or EGF on embryo development was first observed in the eight-cell/compacted morula transition on day 3 of development (Cheung et al., unpublished data), and after this stage, embryos treated in the LIF or EGF-supplemented IVF50 medium had faster

TABLE 1

The effect of LIF and EGF on in vitro blastocyst development and quality when supplemented into single culture medium.

In vitro systems	Blastulation rate		Hatching rate		Blastocyst morphology on day 6		
	Day 5*	Day 6**	Day 5*	Day 6***	Good**	Fair**	Poor**
LIF + IVF50	144/222 ^a (64.9%)	118/162 (72.8%)	41/222 ^{de} (18.5%)	35/66 (53.0%)	106/162 ^{gh} (65.4%)	8/162 ^k (4.9%)	4/162 (2.5%)
EGF + IVF50	119/200 ^b (59.5%)	100/142 ^c (70.4%)	47/200 ^f (23.5%)	31/55 (56.4%)	85/142 ^{ij} (59.6%)	14/142 (9.9%)	1/142 (0.7%)
LIF + EGF + IVF50	93/208 (44.7%)	87/150 (58%)	29/208 ^d (13.9%)	30/63 (47.6%)	59/150 ^{ei} (39.3%)	27/150 ^k (18.0%)	1/150 (0.7%)
IVF50	78/213 ^{ab} (36.6%)	64/146 ^c (43.8%)	22/213 ^{ef} (10.3%)	21/52 (40.4%)	51/146 ^{hj} (34.9%)	11/146 (7.5%)	2/146 (1.4%)
<i>P</i> value (χ^2 test)	<.0001	.0022	<.0001	.3643	<.0001	.001	.471

Note: * Data from 6 replicates, **4 replicates, and ***2 replicates. Post-hoc multiple group comparisons made by χ^2 test with Bonferroni correction. a,b,d,e,f,g,h,j *P*<.0001; c *P*=.0006; i *P*=.0007; k *P*=.0005.

Cheung. Embryotrophic effects of LIF and EGF in vitro. Fertil Steril 2003.

cleavage rates than those in the single IVF50 medium alone. However, the combined supplementation of both LIF and EGF in the IVF50 medium did not result in any additional benefit on blastocyst development or morphology.

In the sequential culture media system (Table 2), the IVF50-S2 media combination generated higher blastulation and hatching rates (72.6% vs. 51.0%, *P*<.0001 and 48.8% vs. 8.9%, *P*<.0001, respectively) and increased number of good blastocysts (68.5% vs. 40.1%, *P*<.0001) when compared to the single IVF50 medium alone. Similar beneficial effects were also observed in LIF- or EGF-supplemented sequential cultures when compared with the single IVF50 medium. However, the supplementation of LIF or EGF ei-

ther individually or in combination in the sequential IVF50-S2 media did not result in any additional improvement in blastocyst development and morphology when compared to the sequential IVF50-S2 media alone.

TCN of Day 5 Blastocysts

The results of TCN for day 5 blastocysts in different in vitro systems are shown in Table 3. The TCN of day 5 blastocysts cultured in sequential IVF50-S2 media were not statistically different from those cultured in the single IVF50 medium alone. However, the TCN were generally higher in embryos treated with growth factor-supplemented sequential cultures (LIF + IVF50-S2 vs. IVF50: 96.0 ± 23.8 vs. 63.6

TABLE 2

The effect of LIF and EGF on in vitro blastocyst development and quality when supplemented into sequential culture media.

In vitro systems	Blastulation rate		Hatching rate		Blastocyst morphology on day 6		
	Day 5*	Day 6*	Day 5*	Day 6**	Good*	Fair*	Poor*
LIF + IVF50-S2	254/379 ^a (67.0%)	285/379 (75.2%)	173/379 ^e (45.6%)	47/85 (55.3%)	239/379 ⁿ (63.1%)	41/379 ^r (10.8%)	5/379 (1.3%)
EGF + IVF50-S2	267/380 ^b (70.3%)	297/380 ⁱ (78.2%)	195/380 ^f (51.3%)	50/86 ^l (58.1%)	254/380 ^o (66.8%)	39/380 ^s (10.3%)	4/380 ^u (1.1%)
LIF + EGF + IVF50-S2	256/384 ^c (66.7%)	296/384 ^j (77.1%)	158/384 ^g (41.1%)	51/86 ^m (59.3%)	233/384 ^p (60.7%)	52/384 (13.5%)	11/384 (2.9%)
IVF50-S2	281/387 ^d (72.6%)	300/387 ^k (77.5%)	189/387 ^h (48.8%)	49/94 (52.1%)	265/387 ^q (68.5%)	29/387 ^t (7.5%)	6/387 (1.6%)
IVF50	196/384 ^{abcd} (51.0%)	253/384 ^{ijk} (65.9%)	34/384 ^{efgh} (8.9%)	31/93 ^{lm} (33.3%)	154/384 ^{nopq} (40.1%)	80/384 ^{rst} (20.8%)	19/384 ^u (4.9%)
<i>P</i> value (χ^2 test)	<.0001	.0003	<.0001	.0025	<.0001	<.0001	.0017

Note: * Data from 10 replicates and **2 replicates. Post-hoc multiple group comparisons made by χ^2 test or Fisher's exact test with Bonferroni correction. a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u *P*<.0001; i,r *P*=.0002; j *P*=.0008; k *P*=.0005; l *P*=.0015; m *P*=.0009; s *P*=.0001; u *P*=.001.

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TABLE 3

Total cell numbers of day 5 blastocysts in different in vitro systems.

In vitro systems	Number of embryos	TCN (mean ± SD)
LIF + IVF50-S2	32	96.0 ± 23.8 ^a
EGF + IVF50-S2	24	90.3 ± 22.2
EGF + LIF + IVF50-S2	31	92.2 ± 28.2 ^b
IVF50-S2	27	78.9 ± 27.7
IVF50	9	63.6 ± 17.3 ^{ab}

Note: Data from 2 replicates. Comparison made by one way ANOVA; $P = .004$. Post-hoc multiple group comparison with Bonferroni correction. ^a $P = .009$; ^b $P = .033$.

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± 17.3, $P = .009$; EGF + IVF50-S2 vs. IVF50: 90.3 ± 22.2 vs. 63.6 ± 17.3, $P = .078$; LIF + EGF + IVF50-S2 vs. IVF50: 92.2 ± 28.2 vs. 63.6 ± 17.3, $P = .033$), indicating that embryos cultured with the supplementation of growth factors may have an additional benefit on embryo viability.

There was also a tendency toward higher TCN in the day 5 blastocysts cultured in sequential IVF50-S2 media supplemented with LIF or EGF compared to those in sequential IVF-S2 media alone. However, this had not reached statistical significance after Bonferroni correction for post-hoc multiple group comparisons.

Birth Rates and Perinatal Outcome

The effect of LIF and EGF on the birth rates and perinatal outcome after transfer of blastocysts to pseudopregnant recipients are shown in Tables 4 and 5. The total birth rate was defined as the total number of pups born over the total number of embryos transferred per group. The live birth rate was defined as the number of normal live births excluding stillbirths and abnormal fetuses over the total number of embryos transferred per group.

The supplementation of LIF in the single IVF50 medium resulted in embryos with a higher implantation potential as reflected by an increase in the total birth rates after transfer (LIF + IVF50 vs. IVF50: 41.1% vs. 13.9%, $P = .0065$). However, this beneficial effect disappeared in the sequential culture media system. Conversely, the supplementation of EGF in either single IVF50 medium or sequential IVF50-S2 media did not result in any significant effect on the birth rates.

With respect to perinatal outcome, although the supplementation of LIF or EGF in either single IVF50 medium or sequential IVF50-S2 media did not affect the incidence of perinatal death and congenital abnormalities, pups born after treatment in LIF-supplemented sequential cultures had prolonged gestation (LIF + IVF50-S2 vs. IVF50-S2: 20.82 ± 0.63 days vs. 20.24 ± 0.43 days, $P < .001$) and increased birth weights (LIF + IVF50-S2 vs. IVF50-S2: 1.64 ± 0.15 g vs. 1.50 ± 0.13 g, $P = .02$; LIF + IVF50-S2 vs. IVF50: 1.64 ± 0.15 g vs. 1.50 ± 0.16 g, $P = .03$). Similarly, pups born after treatment in EGF-supplemented sequential cultures also had prolonged gestation (EGF + IVF50-S2 vs. IVF50-S2: 20.75% vs. 20.24 days, $P < .001$) when compared to those treated in sequential IVF50-S2 media alone.

The offspring were followed up further for their growth and all of them developed normally. When mature female and male offspring in each of the treatment groups were randomly mated, all revealed normal fertility and produced normal pups.

DISCUSSION

Effect of Sequential Culture on Preimplantation Embryo Development

In this study, we have used commercially available simple and sequential culture media as a model to study the influence of growth factors and cytokines on mouse embryo development. Although this is not an ideal model as these commercial culture media are mainly defined for human

TABLE 4

The effect of LIF and EGF on birth rates and perinatal outcome when supplemented into single culture medium.

In vitro systems	Total birth rate ¹	Live birth rate ²	Stillbirth rate ³	Gestation length (days) ⁴ (mean ± SD)	Live birth weight (g) ⁵ (mean ± SD)
LIF + IVF50	37/90 ^a (41.1%)	34/90 (37.8%)	3/90 (3.3%)	20.26 ± 0.45	1.66 ± 0.18
EGF + IVF50	23/68 (33.8%)	23/68 (33.8%)	0	20.39 ± 0.78	1.68 ± 0.18
LIF + EGF + IVF50	15/47 (31.9%)	13/47 (27.6%)	2/47 (4.3%)	20.38 ± 0.51	1.70 ± 0.15
IVF50	5/36 ^a (13.9%)	5/36 (13.9%)	0	20.80 ± 0.45	1.73 ± 0.29
<i>P</i> value	.0345	.0618	.2609	.807	.284

Note: Data from 4 replicates. Comparison made by ^{1,2,3} χ^2 test/Fisher's exact test and ^{4,5} one-way ANOVA. Post-hoc multiple group comparison with Bonferroni correction.

^a $P = .0065$.

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TABLE 5

Effect of LIF and EGF on birth rates and perinatal outcome when supplemented into sequential culture media.

In vitro systems	Total birth rate ¹	Live birth rate ²	Stillbirth rate ³	Gestation length (days) ⁴ (mean ± SD)	Live birth weight (g) ⁵ (mean ± SD)
LIF + IVF50-S2	35/91 (38.5%)	34/91 (37.4%)	1/91 (1.1%)	20.82 ± 0.63 ^a	1.64 ± 0.15 ^{de}
EGF + IVF50-S2	50/83 (60.2%)	48/83 (57.8%)	2/83 (2.4%)	20.75 ± 0.44 ^b	1.55 ± 0.18
LIF + EGF + IVF50-S2	34/80 (42.5%)	33/80 (41.3%)	1/80 (1.3%)	20.85 ± 0.75 ^c	1.55 ± 0.15
IVF50-S2	40/86 (46.5%)	38/86 (44.2%)	2/86 (2.3%)	20.24 ± 0.43 ^{abc}	1.50 ± 0.13 ^d
IVF50	32/83 (38.6%)	32/83 (38.6%)	0	20.50 ± 0.51	1.50 ± 0.16 ^e
<i>P</i> value	.0263	.0536	.6678	.001	<.001

Note: Data from 5 replicates. Comparison made by ^{1,2,3} χ^2 test/Fisher's exact test and ^{4,5} one-way ANOVA. Post-hoc multiple group comparison with Bonferroni correction.

^{a,b,c} $P < .001$; ^d $P = .02$; ^e $P = .03$.

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embryos, they were chosen because of their advantages of stringent quality control and smaller risk of batch-to-batch variation compared to in-house culture media. In addition, their compositions are well defined and serum-free.

Both IVF50 and S2 are modified human tubal fluid (HTF) media supplemented with human serum albumin as the protein source. IVF50 is a simple medium containing only salts, energy substrates, and EDTA. It contains high pyruvate and low glucose levels to suit the metabolic requirements of embryos at early cleavage stages. S2 is a complex medium with lower pyruvate but higher glucose levels and has additional supplementation of amino acids, vitamins, hormones, insulin, and taurine. It does not contain LIF or EGF. It is used for the culture of embryos after embryonic genome activation to the blastocyst stage. The basic compositions of these two culture media are quite different because of the changing metabolic requirements for embryos at different embryonic stages. Therefore, it seems that a combined sequential medium approach will be more physiological than the use of a single medium throughout. Our results also support this finding. The sequential culture media were found to be more successful than the simple single medium alone in improving the blastocyst development and quality.

Effect of LIF and EGF on Preimplantation Embryo Development

The results of our study suggest that growth factors and cytokines may have different effects on embryo development in different in vitro systems. The effect of growth factors and cytokines on preimplantation embryo development has been extensively studied by different workers using in vitro and gene knockout studies. Interestingly, the results obtained so far from in vitro studies are conflicting and both LIF and EGF have been demonstrated to be either stimulatory (16–21) or have no effect (22–25) on blastocyst development in vitro. Although their effects on embryo development may be cell stage and concentration dependent, species specific, and masked in medium supplemented with serum

(26, 27), these could not fully explain the contradictory literature.

Two observations in the present data may explain the contradictory literature on the effect of growth factors and cytokines on preimplantation embryo development. First, we have shown that the beneficial effects of LIF or EGF on blastocyst development and morphology can only be observed in suboptimal single culture media but these beneficial effects disappeared in the sequential culture media system, demonstrating that the effect of growth factors and cytokines can vary between different culture media. We speculate that the beneficial effects of growth factors and cytokines on embryo development can only be observed under stressful conditions, either due to suboptimal in vitro culture conditions or poor embryo quality, consistent with the findings of Paria and Dey (20) who demonstrated that in mouse embryos cultured under stressful conditions, that is, singly cultured embryos, EGF can reverse the inferior development and stimulate blastocyst formation. Similarly, it has been shown that the beneficial effects of co-culture systems can also disappear when different culture media are used, or when the preparation of media is done more rigorously (28).

Second, it appears that morphological criteria and cleavage rates are not very reliable markers to assess blastocyst quality and predict subsequent developmental competence. Previous studies using these as the only outcome measures may be potentially misleading. It was shown in this study that although blastocyst development and embryo morphology were not different between the growth factor-treated and untreated groups in sequential culture media, examination of TCN values on day 5 blastocysts showed that the embryos grown in growth factor-supplemented sequential cultures were generally more mitogenic despite being morphologically indifferent from those cultured in sequential media alone. These effects are subtle and may become evident only after more thorough assessment. Further studies using dif-

ferential counting of ICM and trophoectoderm cells would be helpful to validate this finding.

Effect of LIF and EGF on Implantation

Although the embryos cultured in the LIF-supplemented media resulted in significantly higher total birth rates when compared with the control group in the single culture medium system, its effects were not obvious in the sequential culture media system. It thus appears that the supplementation of LIF in culture media does not necessarily increase the implantation potential of the embryos as one would have expected from previous studies.

There is compelling evidence from a number of observations that LIF has a direct role on blastocyst implantation. First, in most species examined to date, there was expression of LIF in the endometrial epithelium with maximal expression at the time of implantation (8, 9). Second, in vitro studies demonstrated that LIF treatment of mouse embryos cultured to blastocysts increased trophoblastic outgrowth in vitro and improved implantation rate after ET (18). Third, using LIF knockout mice, Stewart et al. (29) showed that homozygous females lacking a functional LIF gene were unable to support implantation, although the blastocysts were viable and could implant normally and develop to term when transferred to wild-type pseudopregnant recipients. Implantation was, however, rescued by infusing exogenous LIF into the uterine lumen of the homozygous LIF-deficient females, resulting in successful pregnancy. These results strongly supported a crucial role of LIF in the process of implantation.

However, it should not be overlooked that uterine LIF expression is under maternal control. The same pattern of LIF expression has been reported in pseudopregnant animals even in the absence of implanting embryos (30). Exogenous supplementation of LIF to normal pseudopregnant recipients may not necessarily have a direct impact on implantation if it can be compensated by endogenous LIF production. Supplementation of media with LIF, however, is still an attractive approach in augmenting embryo viability through increased TCN and improved embryo quality in suboptimal in vitro culture conditions. Moreover, in human studies, increasing evidence suggests that abnormalities in growth factor expression may underlie endometrial dysfunction, which may in turn cause subfertility. It has been shown that secretion of LIF by endometrial explants of women with repeated implantation failure or unexplained infertility was reduced when compared with that of endometrium from normal fertile women (31, 32). Supplementation of LIF in media may help to reverse the problem of implantation failure in these types of patients.

Although there is some evidence in both in vitro and genetic studies to support the role of EGF in the process of trophoblastic proliferation and implantation, exogenous addition of EGF in either single medium (IVF50) or sequential

media (IVF50-S2) did not result in any beneficial effect on birth rates in this study.

Epidermal growth factor was shown to be an important mitogenic factor for cytotrophoblast cells as demonstrated from its ability to stimulate proliferation of a normal placental cytotrophoblast cell line (33). In vitro studies also demonstrated that EGF treatment of mouse embryos cultured to blastocysts could enhance zona hatching and trophoblast outgrowth (34, 35). Moreover, it was shown that mouse blastocysts of the CF-1 strain, which lacked the receptor for EGF, failed to attach and implant, indicating that EGF receptor may play an important role in embryo–endometrial signaling during the process of implantation (9). Threadgill et al. (36) also demonstrated that the knockout of the EGF receptor in CF-1 strain of mice resulted in peri-implantation death due to degeneration of the ICM.

It was also shown that blastocysts from transgenic mice lacking functional EGF receptors were incapable of implantation; gene deletions that involved the EGF ligands had no effect on implantation (9). This is probably related to the fact that even in the absence of any one ligand, other members of the EGF family such as transforming growth factor- α (TGF- α), heparin-binding EGF, and amphiregulin are still available to activate the EGF receptors. This complex growth factor network may explain the contradictory results of this study and demonstrate how nature has evolved an enormous number of possible mechanisms to enhance reproductive efficiency, which may appear as redundant in normal circumstances but may have species-preserving effect under a drastically changed environment.

Effect of LIF and EGF on Outcome of the Offspring

The minimal concentrations of growth factors and cytokines that do not cause undue harm to the offspring need to be carefully studied. In animal studies, overexpression of LIF has been associated with some detrimental effects both in vitro and in vivo. In vitro, overexpression of LIF inhibits the formation of primitive ectoderm, while permitting the differentiation of primitive endoderm (18, 37). In vivo, excessive LIF expression has been shown to cause a fatal syndrome characterized by cachexia, excessive new bone formation, calcification in heart and skeletal muscle, pancreatitis, thymic atrophy, abnormalities in the adrenal cortex and ovarian corpora lutea, resulting in suppression of ovulation (38).

In the present study, although no significant effect on fetal development was observed in pups born after treatment in growth factor-supplemented cultures, the clinical significance of prolonged gestation and increased birth weights is still unknown. The preliminary results suggest that the long-term outcome of the offspring is not compromised. Despite the fact that disturbances of ovulation have been described

with overexpression of LIF (38), the female offspring in this study were proven to have normal fertility.

In conclusion, the findings in this study suggest a beneficial effect of LIF and EGF on mouse blastocyst development in vitro. Their effect was more obvious in suboptimal culture conditions but could be masked in the improved in vitro systems. Additional synergistic effects could not be observed by combining LIF and EGF. Although gene knockout studies suggest that preimplantation embryo development to the blastocyst stage was independent of exogenous growth factors and cytokines (8, 39), in vitro studies including the present study showed that growth factors and cytokines may facilitate embryo development in artificial in vitro conditions.

The importance of growth factors and cytokines in the process of implantation is beyond any doubt; however, it is still a matter of controversy whether exogenous supplementation of these factors in culture media would have any additional benefit on uterine receptivity as it may be compensated by maternal endogenous production. The beneficial effect of LIF in improving implantation by increased birth rates in suboptimal in vitro conditions or underlying endometrial dysfunction should be further explored in humans.

Because of its beneficial effects on embryo viability and implantation, supplementation of LIF into current culture media may see a further improvement in cell-free, in vitro systems. Nevertheless, as the clinical significance of prolonged gestation and increased birth weights of the offspring is still unclear, it is strongly recommended that more animal studies should be undertaken to evaluate its safety, with particular attention to its potential teratogenic effects and the long-term outcome of the offspring, before it is applied to human-assisted reproductive programs.

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