

Dose-dependent embryotrophic effect of recombinant granulocyte-macrophage colony-stimulating factor and brain-derived neurotrophic factor in culture medium for mouse preimplantation embryo

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Objective

To evaluate the dose effect of recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) or brain-derived neurotrophic factor (BDNF) in culture medium on the development of *in vitro* fertilized mouse embryos.

Methods

Mature oocytes were retrieved from superovulated female BDF1 mice and inseminated by sperm from male BDF1 mice. On day 1, two-cell stage embryos were divided and cultured until day 5 in the embryo maintenance medium supplemented with 0, 1, 2, 5, or 10 ng/mL of rmGM-CSF or supplemented with 0, 5, 10, or 20 ng/mL of BDNF. Blastocyst formation rate and their cell numbers were assessed.

Results

The blastocyst formation rate and the total cell count in blastocyst was similar in all the rmGM-CSF treatment groups when compared with the control. However, the blastocyst formation rate and the total cell count was significantly higher in the group supplemented with 10 ng/mL of BDNF compared with the control (63.9%, 45.8±11.5 vs. 52.3%, 38.0±6.8; $P<0.05$, respectively).

Conclusion

Supplementation of 10 ng/mL of BDNF enhanced the developmental potential of mouse preimplantation embryos, but supplementation of rmGM-CSF did not.

Keywords: Brain-derived neurotrophic factor; Culture medium; Embryotrophic effects; Granulocyte-macrophage colony-stimulating factor

Introduction

Recent progress in *in vitro* fertilization (IVF) techniques involves the culture of human embryos to the blastocyst stage. Blastocyst transfer has been known to improve uterine and embryonic synchronicity and enable self-selection of viable embryos, resulting in higher implantation rates [1,2]. To obtain more blastocysts with a high implantation potential, optimal *in vitro* culture condition might be essential.

The growth and development of the preimplantation embryo is regulated by various cytokines and endogenous growth-promoting substances which are secreted from lu-

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minial epithelial cells of oviducts in human and other species [3]. Corresponding receptors for these growth factors have been known to be expressed by preimplantation embryos, supporting the close interaction between growth factors and embryos. Many studies have demonstrated the addition of growth factors to embryo culture medium can improve the development of the preimplantation embryo and the efficacy of IVF [4-6].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine originally identified as a regulator of the proliferation, differentiation, and activation of myeloid hemopoietic cells [7]. In hemopoietic precursors, GM-CSF acts as a survival factor through suppressing apoptosis [8-10]. In reproduction, GM-CSF, synthesized by estrogen-primed epithelial cells in human uterus and oviducts [11,12] has been known to improve the embryo development. Previous experiments showed that *in vitro* culture under GM-CSF increases the blastulation rate and blastomeres in mouse and human embryos [13,14].

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of proteins, which activate the high-affinity tyrosine kinase receptor (TrkB) and the low-affinity pan-neurotrophin receptor p75 [15]. It is widely distributed throughout the brain in diverse cell types and mediates the neuronal survival and differentiation [16]. Recently, it has been shown that BDNF also plays a role in the development of various non-neuronal tissues including reproductive systems [17]. Previous studies suggested that BDNF regulates folliculogenesis [18], promotes oocyte maturation and *in vitro* development of zygotes to preimplantation embryos [19,20] and the development of preimplantation embryos to the blastocysts with the increased proliferation and decreased apoptosis [21].

Several reports denoted a different effect according to the concentration of recombinant GM-CSF (rGM-CSF) for the embryo development in the different species [22,23]. In the study of porcine embryos, highest proportion of blastulation was observed in the presence of 10 ng/mL of rGM-CSF among different concentrations of 2, 10, and 100 ng/mL [23]. In naturally fertilized murine embryos, blastulation was impeded at the concentration of GM-CSF in the culture media as high as 5 ng/mL [22]. Regarding BDNF, there has been no study to evaluate the dose effect of BDNF as far as we know.

Herein, we attempted to evaluate the effect of adding different concentrations of recombinant mouse (rm) GM-CSF and BDNF to blastocyst culture in the *in vitro* fertilized murine embryos. To determine the developmental competence, the

blastulation rate and the total number of blastomeres were assessed.

Materials and methods

1. Animal and *in vitro* fertilization protocol

Five- to six-week-old female BDF₁ mice (Orient Co., Seoul, Korea) were used in this study. Animal care and use were in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Seoul National University of Bundang Hospital. Superovulation and oocyte retrieval from mice was performed as previously described [24]. Briefly, BDF₁ female mice were injected with 5 IU of Pregnant Mare's Serum Gonadotropin (Sigma-Aldrich, St Louis, MO, USA), followed by intraperitoneal 5 IU of human Chorionic Gonadotropin (Sigma-Aldrich) 48 hours later. After 13 to 14 hours, the mice were sacrificed by cervical dislocation. Oocytes were collected from the ampullae and incubated in 1 mL of modified mouse tubal fluid (mMTF) containing 0.4% bovine serum albumin (BSA, Sigma-Aldrich).

The epididymal sperm were retrieved from the cauda epididymis of 8- to 10-week-old BDF₁ mice, and the sperm suspensions were preincubated for 1.5 hours in capacitation medium (mMTF supplemented with 0.8% BSA). The oocytes were then inseminated by sperm at a final dilution of 2×10^6 /mL and incubated at 37°C in humidified 5% CO₂ in air. Inseminated oocytes were washed by pipetting 6 hours later and cultured in mMTF medium supplemented with 0.8% BSA overnight. On day 1, fertilization was assessed by the formation of 2-cell stage.

2. Culture medium

Embryos reached 2-cell stage were transferred to embryo maintenance medium (Global medium supplemented with 10% human serum albumin; Life Global, Guilford, CT, USA) containing 0, 1, 2, 5, or 10 ng/mL of rmGM-CSF (Sigma-Aldrich) or containing 0, 5, 10, or 20 ng/mL of BDNF (Sigma-Aldrich). On day 5 after insemination, development to blastocyst was assessed. In all cultures, groups of up to 10 embryos were placed in 50- μ L microdrops of medium under mineral oil (Sigma-Aldrich) in 35 \times 10-mm Petri dishes (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) and were placed at 37°C in humidified 5% CO₂ in air. All produced blastocysts were mounted on the slides and stained by 4,6-diamidino-2-phenylindole (DAPI; Vector-mounting medium for fluorescence with

DAPI/H-1200) for 15 minutes for nuclear counting.

3. Statistical analysis

It was calculated that at least 150 *in vitro* fertilized 2-cell embryos in each group achieves 80% power to reject the null hypothesis using an error of 0.05 and the chi-square test, assuming a baseline blastocyst rate of 50% and a difference of 5% between the two groups. Data were analyzed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The percentage of blastocyst in each treatment group was compared with that in the untreated (control) group by using the chi-square test. Numeric data were compared by the Student's *t*-test after performing the normality test. The result was considered significant when the *P*-value was less than 0.05.

Results

Sixty mice were used for rmGM-CSF experiment; 1,120 mature oocytes were obtained and overall IVF rate was 74.5%. The blastocyst formation rate and the total cell count in blastocyst was similar in all rmGM-CSF treatment groups when compared with control (Table 1). The blastocyst formation rate was highest in the group supplemented by 2 ng/mL of rmGM-CSF (57.2%), however, the differences did not reach a statistical significance when compared with control. The blastocyst formation rate was lowest in the 5-ng/mL-rmGM-CSF treatment group (43.4%) and this was significantly different when compared with 2-ng/mL-rmGM-CSF treatment group (*P*=0.02).

For BDNF experiment, fifty mice were used; 1,330 mature oocytes were obtained and overall IVF rate was 54.8%. In the

Table 1. Developmental outcomes after culture of mouse preimplantation embryos supplemented with rmGM-CSF

	rmGM-CSF (ng/mL)				
	0	1	2	5	10
No. 2-cell	162	162	159	159	160
No. blastocyst	80	86	91	69	79
Early (%)	60.3	48.0	54.3	44.4	61.5
Expanded (%)	36.5	49.3	44.3	51.9	33.8
Hatching (%)	3.2	2.7	1.4	3.7	4.6
% per 2-cell (pooled)	49.4	53.1	57.2	43.4 ^{a)}	49.4
% per 2-cell ^{b)}	46.6±17.8	53.9±22.2	55.6±23.2	45.3±18.7	49.9±25.9
No. blastomeres ^{b)}	35.0±15.6	27.0±7.4	30.8±12.6	32.8±11.0	31.2±14.5

Fourteen replicates were performed; Pooled percentages were not significant when compared with the control. rmGM-CSF, recombinant mouse granulocyte-macrophage colony-stimulating factor.

^{a)}*P*=0.02 when compared with 2-ng/mL-rmGM-CSF treatment group; ^{b)}Presented as mean±standard deviation and no differences were found when compared with each other.

Table 2. Developmental outcomes after culture of mouse preimplantation embryos supplemented with BDNF

	BDNF (ng/mL)			
	0	5	10	20
No. 2-cell	155	155	155	155
No. blastocyst	81	77	99	83
Early (%)	43.2	46.0	49.5	47.6
Expanded (%)	46.9	47.4	47.5	46.4
Hatching (%)	9.9	6.6	3.0	6.0
% per 2-cell (pooled)	52.3	49.7	63.9 ^{a,b)}	53.5
% per 2-cell ^{c)}	49.0±22.5	47.3±22.3	60.8±21.1	48.5±24.4
No. blastomeres ^{c)}	38.0±6.8	40.3±11.6	45.8±11.5 ^{d)}	39.8±7.7

Twelve replicates were performed.

BDNF, brain-derived neurotrophic factor.

^{a)}*P*=0.049 when compared with the control; ^{b)}*P*=0.017 when compared with 5-ng/mL-BDNF treatment group; ^{c)}Presented as mean±standard deviation and no differences were found when compared with each other except; ^{d)}*P*=0.015 when compared with the control.

group supplemented by 10 ng/mL of BDNF, the blastocyst formation rate (63.9%) was significantly higher when compared with control (52.3%) and 5-ng/mL-BDNF treatment group (49.7%) (Table 2). Total cell count in 10-ng/mL-BDNF treatment group (45.8 ± 11.5) was significantly higher when compared with the control.

Discussion

Our results suggest that BDNF may play embryotrophic role in a specific concentration, 10 ng/mL, but rmGM-CSF may not. Our study showed that adding GM-CSF to the culture media did not have any significant positive impact on the blastulation rate or cell number, which is in accordance with previous report [22,25,26]. Behr et al. [25] tested the effect of a range of concentrations of GM-CSF (0.0625–2 ng/mL) on mouse embryo development and found that concentrations from 0.25 to 2 ng/mL did not promote blastocyst development and the best effect was reported in the 0.125 ng/mL group. Elaimi et al. [22] reported no difference in the blastulation potential was noted with the addition of 1 and 2 ng/mL of GM-CSF compared with the controls but, GM-CSF exerted a negative impact on the blastulation rate at 5 and 10 ng/mL concentrations. On the other hand, some studies reported significant effects of adding 2 ng/mL of GM-CSF to embryo culture [14,27]. These different effects might come from the use of a different type of culture medium. Karagenc et al. [28] examined the effect of adding GM-CSF (0, 2, 4, 8, and 16 ng/mL) on the development of mouse embryos from different strains and under different culture conditions. They found no marked effect of supplementing the media with GM-CSF using different concentrations. However, in the absence of protein source, the stimulatory effect of GM-CSF was observed on the total number of blastomeres.

Regarding BDNF in our study, supplementation of 10 ng/mL of BDNF showed the highest blastocyst formation rate and the total cell number when compared with the control. However, when cultured with 20 ng/mL of BDNF, the embryotrophic effect was eliminated. This peculiar tendency was also observed by other researcher [21]; hatched blastocyst formation rate was the highest under 10 ng/mL rather than 30 ng/mL. This observation could be partly explained by the negative regulation of the receptors when the growth factors are present at high concentrations.

The mechanisms underlying the embryotrophic actions of

GM-CSF was suggested by Sjoblom et al. [29]. They suggested that GM-CSF acts mediated via an interaction with the GM-CSF-receptor alpha subunit (GM-R α) that may occur independently of GM-CSF-receptor beta common subunit (β c). In that study, by using reverse transcription-polymerase chain reaction and immunocytochemistry, expression of mRNA and protein for GM-R α was identified in embryos from the first-cleavage through blastocyst stages of development, but the GM-CSF-receptor β c could not be detected at any stage.

When neutralizing antibodies reactive with GM-R α were added to embryo culture medium, the development-promoting effect of GM-CSF was ablated [29]. In contrast, GM-CSF activity in embryos was not inhibited either by antibodies to β c or by E21R, a synthetic GM-CSF analogue that acts to antagonize β c-mediated GM-CSF signaling. Unexpectedly, E21R was found to mimic native GM-CSF in promoting blastulation. These indicate that GM-CSF regulates cell viability through the GM-R α that is independent of β c. GM-CSF receptor has been known to act through β c, which forms a high-affinity complex when associated with ligand-coupled GM-R α . Thus, in embryos, GM-R α may act with ligation stimulating glucose uptake through a kinase-independent pathway.

Kawamura et al. [21] demonstrated the embryotrophic actions of BDNF. According to their study, BDNF reached at its highest levels in the blastocyst stages and the corresponding receptor, TrkB were detectable throughout the early embryonic stages with an increase after the early blastocyst stage. Both BDNF and TrkB are expressed in trophoctoderm cells and ligand-binding studies indicated the specific binding of BDNF to trophoctoderm cells. Treatment with BDNF promoted the development of 2-cell stage embryos into blastocysts showing increased proliferation and decreased apoptosis. The effects of BDNF were blocked by the TrkB ectodomain or a Trk receptor inhibitor.

Among the several growth factors, recombinant human GM-CSF has been applying to the human IVF clinics. A recent research using commercially available culture medium containing recombinant human GM-CSF (2 ng/mL) was accomplished and showed the beneficial effect on reducing miscarriage rate in patients with a previous pregnancy loss [30]. For the wider application of GM-CSF-containing culture medium to human IVF, safety concerns should be resolved. A previous murine study denoted that GM-CSF does not affect mosaicism/aneuploidy, but increases the percentage of aneuploid cells within the mosaic embryos [22]. The latter finding might be attributed to anti-apoptotic effect of GM-CSF, by which more

cells with aneuploid cells in the mosaic embryos would survive. Although a recent human study indicates no increment of cytogenetically abnormal embryos [31], adding GM-CSF to the culture media for clinical purpose requires further studies either on human or animal models to evaluate its long-term effects.

The present study demonstrated that GM-CSF exerted no impact on the blastulation rate at any concentration but, BDNF showed the embryotrophic effects of at a specific concentration. For the clinical application of BDNF, safety profiles such as chromosomal constitution and long-term fetal effects should be studied in advance. Further studies are needed regarding the effective concentration in human embryo culture and whether combination of these growth factors could improve the embryo development more than one.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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References

1. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996;65:349-53.
2. Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB. Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil Steril* 1998;69:84-8.
3. Richter KS. The importance of growth factors for preimplantation embryo development and in-vitro culture. *Curr Opin Obstet Gynecol* 2008;20:292-304.
4. Aflalo ED, Sod-Moriah UA, Potashnik G, Har-Vardi I. EGF increases expression and activity of PAs in preimplantation rat embryos and their implantation rate. *Reprod Biol Endocrinol* 2007;5:4.
5. Papayannis M, Eyheremendy V, Sanjurjo C, Blaquier J, Raffo FG. Effect of granulocyte-macrophage colony stimulating factor on growth, resistance to freezing and thawing and re-expansion of murine blastocysts. *Reprod Biomed Online* 2007;14:96-101.
6. Zhou P, Liu DJ, Cang M, Ma YZ, Yang DS, Li HJ, et al. TG-Falpa and EGFR in ovine preimplantation embryos and effects on development. *Anim Reprod Sci* 2008;104:370-81.
7. Ruef C, Coleman DL. Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness. *Rev Infect Dis* 1990;12:41-62.
8. Caceres-Cortes J, Rajotte D, Dumouchel J, Haddad P, Hoang T. Product of the steel locus suppresses apoptosis in hemopoietic cells: comparison with pathways activated by granulocyte macrophage colony-stimulating factor. *J Biol Chem* 1994;269:12084-91.
9. Rajotte D, Haddad P, Haman A, Cragoe EJ Jr, Hoang T. Role of protein kinase C and the Na⁺/H⁺ antiporter in suppression of apoptosis by granulocyte macrophage colony-stimulating factor and interleukin-3. *J Biol Chem* 1992;267:9980-7.
10. Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* 1990;343:76-9.
11. Giacomini G, Tabibzadeh SS, Satyaswaroop PG, Bonsi L, Vitale L, Bagnara GP, et al. Epithelial cells are the major source of biologically active granulocyte macrophage colony-stimulating factor in human endometrium. *Hum Reprod* 1995;10:3259-63.
12. Zhao Y, Chegini N. Human fallopian tube expresses granulocyte-macrophage colony stimulating factor (GM-CSF) and GM-CSF alpha and beta receptors and contain immunoreactive GM-CSF protein. *J Clin Endocrinol Metab* 1994;79:662-5.
13. Robertson SA, Sjoblom C, Jasper MJ, Norman RJ, Seemark RF. Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. *Biol Reprod* 2001;64:1206-15.
14. Sjoblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development in vitro. *Hum Reprod* 1999;14:3069-76.

15. Barbacid M. The Trk family of neurotrophin receptors. *J Neurobiol* 1994;25:1386-403.
16. Kafitz KW, Rose CR, Thoenen H, Konnerth A. Neurotrophin-evoked rapid excitation through TrkB receptors. *Nature* 1999;401:918-21.
17. Seifer DB, Feng B, Shelden RM, Chen S, Dreyfus CF. Brain-derived neurotrophic factor: a novel human ovarian follicular protein. *J Clin Endocrinol Metab* 2002;87:655-9.
18. Dissen GA, Hirshfield AN, Malamed S, Ojeda SR. Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. *Endocrinology* 1995;136:4681-92.
19. Anderson RA, Bayne RA, Gardner J, De Sousa PA. Brain-derived neurotrophic factor is a regulator of human oocyte maturation and early embryo development. *Fertil Steril* 2010;93:1394-406.
20. Kawamura K, Kawamura N, Mulders SM, Sollewijn Gelpke MD, Hsueh AJ. Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. *Proc Natl Acad Sci U S A* 2005;102:9206-11.
21. Kawamura K, Kawamura N, Fukuda J, Kumagai J, Hsueh AJ, Tanaka T. Regulation of preimplantation embryo development by brain-derived neurotrophic factor. *Dev Biol* 2007;311:147-58.
22. Elaimi A, Gardner K, Kistnareddy K, Harper J. The effect of GM-CSF on development and aneuploidy in murine blastocysts. *Hum Reprod* 2012;27:1590-5.
23. Kwak SS, Jeung SH, Biswas D, Jeon YB, Hyun SH. Effects of porcine granulocyte-macrophage colony-stimulating factor on porcine in vitro-fertilized embryos. *Theriogenology* 2012;77:1186-97.
24. Jee BC, Jo JW, Lee JR, Suh CS, Kim SH, Moon SY. Effect of trichostatin A on fertilization and embryo development during extended culture of mouse oocyte. *Zygote* 2012;20:27-32.
25. Behr B, Mooney S, Wen Y, Polan ML, Wang H. Preliminary experience with low concentration of granulocyte-macrophage colony-stimulating factor: a potential regulator in preimplantation mouse embryo development and apoptosis. *J Assist Reprod Genet* 2005;22:25-32.
26. Desai N, Kattal N, AbdelHafez FF, Szeptycki-Lawson J, Goldfarb J. Granulocyte-macrophage colony stimulating factor (GM-CSF) and co-culture can affect post-thaw development and apoptosis in cryopreserved embryos. *J Assist Reprod Genet* 2007;24:215-22.
27. Sjoblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology* 2005;146:2142-53.
28. Karagenc L, Lane M, Gardner DK. Granulocyte-macrophage colony-stimulating factor stimulates mouse blastocyst inner cell mass development only when media lack human serum albumin. *Reprod Biomed Online* 2005;10:511-8.
29. Sjoblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. *Biol Reprod* 2002;67:1817-23.
30. Ziebe S, Loft A, Povlsen BB, Erb K, Agerholm I, Aasted M, et al. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. *Fertil Steril* 2013;99:1600-9.
31. Agerholm I, Loft A, Hald F, Lemmen JG, Munding B, Sorensen PD, et al. Culture of human oocytes with granulocyte-macrophage colony-stimulating factor has no effect on embryonic chromosomal constitution. *Reprod Biomed Online* 2010;20:477-84.