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Animal Experimentation

Preliminary Experience with Low Concentration of Granulocyte-Macrophage Colony-Stimulating Factor: A Potential Regulator in Preimplantation Mouse Embryo Development and Apoptosis

Barry Behr,[1,2] Stephen Mooney,[1] Yan Wen,[1] Mary Lake Polan,[1] and Hongbo Wang[1]

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***Purpose*:** To investigate the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) on the development of preimplantation mouse embryos.
***Methods*:** Mouse 2-cell embryos were collected and cultured in P-1 medium supplemented with GM-CSF at different concentrations. Using reverse transcription-polymerase chain reaction, expression Bcl-2 and Bax mRNA in blastocyst were evaluated in the GM-CSF group and control group. Apoptosis detection was performed using the in situ apoptosis detection kit in mouse blastocysts. The statistical significance of the data was analyzed using *t*-test and chi-square test.
***Results*:** The development of blastocyst increased to 89% in the addition of GM-CSF (0.125 ng/mL), compared to controlled group (80%). The number of cells staining for apoptosis was lower in GM-CSF group than that in the control group. Bcl-2 expression was found to be upregulated in blastocysts in the GM-CSF supplemented group compared to the control group.
***Conclusion*:** These results suggest that GM-CSF might be an important regulator in embryo development.

KEY WORDS: Apoptosis; blastocyst development; GM-CSF; in vivo blastocyst.

INTRODUCTION

Formation of a mammalian blastocyst involves cell division, differentiation and death. The phenomenon of cell death by apoptosis has been observed during preimplantation embryogenesis both in vivo and in vitro in a range of species (1). Studies have shown that approximately 50% of human embryos arrest during the first six days following in vitro fertilization (2). Jurisicova *et al.* (3) suggested that apoptosis might be involved in this early embryonic arrest. Recently, a number of studies have demonstrated that suboptimal culture conditions may be implicated in preimplantation arrest and cell apoptosis. In particular, there is increasing evidence that growth factors play an important role in preimplantation embryo development (4). Granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion from epithelial cells lining the female reproductive tract is induced during early pregnancy by ovarian steroid hormones and constituents of seminal plasma (5). The receptor of GM-CSF is comprised of two subunits that belong to the superfamily of cytokine receptors typified by the growth hormone receptor. The a-chain (GM-Ra) confers low affinity binding whereas the B-common chain (Bc) does not bind

[1] Stanford University School of Medicine, Stanford, California.
[2] To whom correspondence should be addressed at Department of Obstetrics and Gynecology, Stanford University School of Medicine, 900 Welch Road, Ste 14, Palo Alto, California 94304; e-mail: behr@stanford.edu.

to GM-CSF itself but forms a high affinity complex when associated with the ligand-coupled a-chain (6). Studies in mice have demonstrated that the GM-Ra expression in preimplantation embryos and exogenous GM-CSF increased mouse blastocyst formation, hatching, and subsequent attachment to the culture dish and were associated with enhanced proliferation and viability of the inner cell mass cells (7).

GM-CSF is associated with suppression of apoptosis has been studied in much greater detail in other cell types, such as lymphocytes and neutrophils (8,9). Epling-Burnette *et al.* (10) found that GM-CSF induced a time-dependent increase in the mRNA and protein expression of the anti-apoptotic Bcl-2 family in human neutrophiles. Although potential apoptotic signals are numerous vast and vary with cell type, in most case the ratio of proapoptotic to antiapoptotic Bcl-2 homologues such as Bax and Bcl-2 within a cell determines whether the cell will live or die (11). In mouse, expression of Bcl-2 apoptotic family has been detected in every stage preimplantation embryos and the ratio of Bcl-2 to Bax was altered in apoptotic embryos (12). In the present study, we attempted to investigate the effect of GM-CSF on blastocyst development and apoptosis and to describe the analysis of gene transcripts such as Bcl-2 and Bax that regulate apoptosis in preimplantation embryos. On the other hand, our preliminary experiment showed that low concentration of GM-CSF (<2 ng/mL) in culture media might be suitable for mouse embryo development. We designed the experiment at low concentrations of GM-CSF, which was different from the previous works by Sjoblom *et al.* (13), and also compared our results of blastocyst cell number and number of apoptotic cells in our experiment to in vivo derived blastocysts.

MATERIALS AND METHODS

Animals

Female mice (strain CB6F1) at 6–8 weeks of age and proven male breeders were obtained from the Charles River Animal Central (Wilmington, MA). Female mice were housed 5 per cage at a constant temperature ($22 \pm 1^{\circ}C$), humidity ($55 \pm 5\%$), and light schedule (12L:12D). Commercial food (Rat Chow 5012; Purina Mills, St. Louis, MO) and water were provided ad libitum. Institutional review board approval was obtained for this study.

Collection of Embryos

Female mice were given single injections of superovulatory doses of pregnant mouse serum gonadotropin (10 IU of PMSG, Sigma, St. Louis, MO) followed 48 h later by a dose of hCG (10 IU; Sigma) to synchronize the timing of ovulation. Two female mice were housed immediately with a male for mating. The mice were anesthetized with CO_2 and killed by cervical dislocation on the next morning. The uteri and fallopian tubes were removed and transferred to 5 mL of PBS + 10% SSS (Irvine Science, Santa Ana, CA). Embryos were collected and pooled, then allocated into the control and experiment groups. Embryos cultured in Blastocyst medium with 10% SSS was used as the control group (group 1). Embryos cultured in Blastocyst medium with different concentrations of GM-CSF were designated experimental groups (group 2, 0.0625 ng/mL; group 3, 0.125 ng/mL; group 4, 0.25 ng/mL; group 5, 0.5 ng/mL; group 6, 1 ng/mL; and group 7, 2 ng/mL). There was no SSS or albumin supplement in these experiment groups. All embryos were placed in 150 μL drop of medium under oil and incubated in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37°C and 98% humidity.

In vivo derived blastocysts were obtained identically to the 1-cell embryos except that they were flushed from the uterus at 96 h post-hCG. The developmental stage of cultured embryos was assessed visually with the aid of an inverted microscope at 8 h intervals. Preimplantation embryos were scored as 2-cell, 4-cell, morula, or expanded blastocyst according to conventional criteria.

Determination of Cell Numbers

The numbers of cells in blastocysts were determined by staining with bisbenzimide. After brief incubation in acid Tyrode's solution containing 8 mg/mL to remove zonae pellucidae, blastocysts were washed in PBS supplemented with 10 mg/mL BSA (PBS-BSA) and incubated in bisbenzimide (Sigma; 1 μg/mL, 5 min at room temperature). Following a final wash in PBS-BSA, embryos were fixed briefly in 1% paraformaldehyde in PBS, then mounted on a microscope slide under a coverslip. Nuclei were viewed and counted with a confocal laser-scanning microscope (Zeiss LSM-400).

Apoptosis Detection

The zonae were removed from the embryos with acidified Tyrode's after they had been cultured to the blastocyst stage. Embryos were fixed in 4% paraformaldehyde for 15 min and apoptosis detection was performed using the fluorescein-isothiocyanate (FITC) APOPTAG in situ apoptosis detection kit (Oncor Inc., Gaithersburg, MD) according to previously described procedures. Staining and washing of embryos was performed under a Nikon SMZ-2B dissecting microscope in Falcon organ tissue culture dishes. Negative controls for nonspecific binding of the antibody were performed on samples by omission of the terminal deoxynucleotidyltransferase. This enzyme catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the free 3'-OH ends of double-stranded or single-stranded DNA that result from DNA degradation, typical of early apoptotic events. Fluorescent antibody staining was observed at 520 nm as distinct bright green fluorescence of the FITC label.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Single blastocysts ($n = 20$) were examined for Bcl-2 and Bax mRNAs by one round of RT followed by two rounds of PCR using a modification of methods described previously (14). In addition, the β-actin transcript was also amplified and identified to ensure the presence of intact mRNA and acted as inner control. The primer cDNA sequences and the size of the amplified fragments are listed in Table I. Paired outer primers were used for the first round PCR while the inner primers were used for the second round PCR. Primer sets used were designed to span at least two exons to exclude the possibility of amplifying genomic DNA from contamination during RNA extraction. The identification of all PCR products was confirmed by sequence analysis. RT was modified for single embryo analysis according to previous protocols. In all cases, the negative control of culture medium without an embryo was used in RT. For the first PCR, 5 μL of RT product from an individual embryo was added to the first PCR mastermix to a total volume of 80 μL containing 0.24 μM 3′ and 5′ primers mixture of each specific outer pair and 2.5 U AmpliTaq DNA polymerase (Applied Biosystem, Foster City, CA). The mixture then was subjected to 40 cycles of amplification for Bcl-2, Bax, and β-actin. First-round PCR products were stored at -20°C until the second round of PCR.

For the second round PCR, 20 μL of the initial PCR products were added to the second PCR mastermix to a total volume of 80 μL containing 0.24 μM 3′ and 5′ primers mixture of each corresponding inner pair and 2.5 U AmpliTaq DNA polymerase. After completing the second round PCR using the same program, samples were stored at -20°C until electrophoresis.

Agarose Gel Electrophoresis

An aliquot 27 μL of The PCR product was size-fractionated on a 2% agarose gel and visualized using ethidium bromide. Photocopies of the agarose gel were printed on the GelDoc 1000 system (Bio-Rad Laboratories, Hercules, CA). Single blastocysts from groups 1 to 3 were examined for Bcl-2 and Bax mRNAs by one round of RT followed by two rounds of PCR. The PCR products were subjected to electrophoresis. The intensity of each band was

Table I. Bcl-2, Bax, and β-actin Primer Sequences

Gene		Primer sequence	Product size
Bcl-2	External	5′ TACCGTCGTGACTTCGCAGAG 3′ GGCAGGCTGAGCAGGGTCTT	180bp
	Internal	5′ CGAGGGGACGCTTTG 3′ GCAGATGCCGGTTCA	
Bax	External	5′ CGGCGAATTGGAGATGAACTG 3′ GCAAAGTAGAAGAGGGCAACC	216bp
	Internal	5′ ATGGCTGGGGAGACACCT 3′ ACTGGGGCCGCGTGGTTG	
β-actin	External	5′ GTGGGCCGCTCTAGGCACCAA 3′ CTCTTTGATGTCACGCACGATTTC	408bp
	Internal	5′ CAAGGTGTGATGGTGGGAATGG 3′ CAGGATGGCGTGAGGGAGAGCA	

assessed by densitometry using an image analysis program. The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each group by the intensity of the β-actin band for the corresponding group. A total of eight replicates were performed for each specific gene transcript from an individual embryo. This allowed calculation of statistically significant differences between the control and the treatment groups for each transcript (15).

Statistical Analysis

Differences between the percentages of embryos reaching the blastocyst stage were compared by χ^2-analysis. Differences in the distribution of cell number per blastocyst, total number of apoptotic nuclei per blastocyst were compared using *t*-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The Effect of GM-CSF on In Vitro Preimplantation Embryo Development

The percentage of embryos reaching the blastocyst stage in the control group and the experiment groups are shown in Table II. In group 3, the blastocyst rate (89%, 93/104) was significantly increased compared to the control group (80%, 86/108) ($p < 0.05$). However, the increased concentration of GM-CSF from 0.25 to 2 ng/mL in the medium did not further promote preimplantation embryo development to the blastocyst stage ($p > 0.05$).

Total Number of Nuclei in Day 5 Blastocyst

Embryos from groups 1 to 3 were determined for the number of cells in each blastocyst. On day 5, the number of nuclei was counted for each embryo. The total number of nuclei ranged from 28 to 33 (30.4 ± 3.0, $n = 17$) and 29 to 35 (33 ± 2.5, $n = 18$) in groups 1 and 3, respectively, which was significantly different ($p < 0.05$).

The Effect of GM-CSF on Blastocyst Apoptosis

To determine the effect of GM-CSF on the incidence of cell death, blastocysts from the group 1 and group 3 were TUNEL labeled and examined by laser-scanning confocal microscopy. Nuclei labeled by TUNEL showed the fragmented nuclear morphology characteristic of apoptosis, indicating that the observed cell death was likely to be apoptotic rather than necrotic (Fig. 1). The incidence of cell apoptosis was significantly higher in blastocysts of the control group (7.9 ± 2.3, $n = 17$) compared to blastocysts in group 1 (4.6 ± 1.5, $n = 18$) ($p < 0.05$).

GM-CSF Affect Bcl-2 and Bax mRNA Expression Pattern in Blastocyst

Both Bcl-2 and Bax gene transcripts were detected in all blastocysts from group 1 and group 3. The relative abundance of Bcl-2 mRNA was significantly increased in group 3 compared to group 1 ($p < 0.05$). However, the relative amount of Bax transcript showed no significant difference between the both groups. ($p > 0.05$) (Fig. 2).

In vivo Derived Blastocysts

In order to determine how the control and GM-CSF derived blastocysts compared to the in vivo developed blastocysts, a total of 22 in vivo blastocysts were analyzed for apoptosis and cell number. The number of apoptotic cells was significantly higher in the control IVF developed blastocysts ($p < 0.05$) versus the GM-CSF and in vivo group, 5.9, 3.9, and 1.3 cells per blastocyst, respectively. In addition, the cell numbers in the control IVF derived blastocysts were significantly lower ($p < 0.05$) than the GM-CSF and

Table II. Effect of GM-CSF on the Development of Embryo to Blastocyst

	Group 1	Group 2	Group 3*	Group 4	Group 5	Group 6	Group 7	Total
2-cell	108	59	104	72	59	68	74	543
Blastocyst	86	50	93	56	50	57	63	455
Blastocyst rate (%)	80	84	89	77	84	84	85	

* Group 3 compared to group 1, $p < 0.05$.

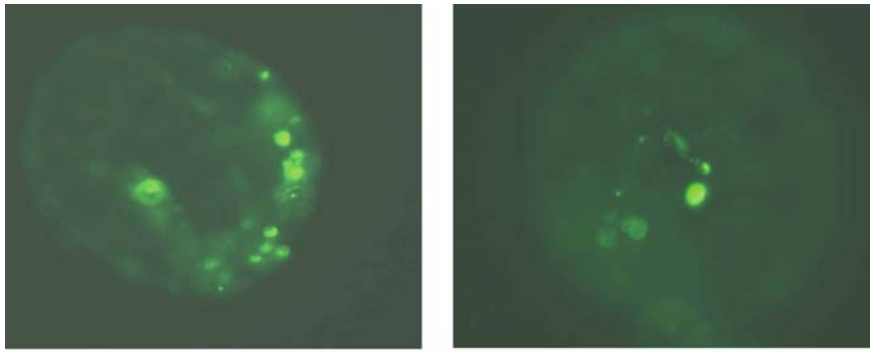

Fig. 1. The effect of GM-CSF on cell apoptosis in mouse embryos. Photomicrographs showing TUNEL assay of (A) embryos of group 1 (control) and (B) group 3 (culture medium supplement with 0.125 ng/mL GM-CSF) at the blastocyst stage. Nuclei of apoptotic cells fluoresce brightly. Seventeen embryos from group 1 and 18 embryos from group 3 were subjected to the assay. Typical results were shown.

in vivo group, 33, 37, and 38 cells per blastocysts, respectively (Table III).

DISCUSSION

In the present study, low dose GM-CSF acted to promote the development of preimplantation murine embryo, likely resulting from decreased apoptosis. We also described the analysis of the genes Bcl-2 and Bax, which regulate apoptosis in preimplantation embryo showing that GM-CSF may act as a regulator in preimplantation embryo development and apoptosis.

The oviduct provides the preimplantation mammalian embryo with a unique environment, which nurtures the early embryo and thereby influences its further development. In vitro experiments suggest the growth and development of the preimplantation embryo is regulated by an array of cytokines and growth factors secreted from epithelial cells lining the oviduct and uterus (16,17). GM-CSF is a cytokine originally identified as a product of activated T-lymphocytes involved in the proliferation and differentiation of myeloid hematopoietic cells (18). In reproductive system, GM-CSF is produced by estrogen-primed epithelial cells in the oviduct and uterus in mice, sheep, and women (5,19,20). Recent findings implicate a physiologic role for GM-CSF in regulating preimplantation embryo development. The effects of GM-CSF on ovine embryos include increasing their potential for implantation through enhancing expression of interferon τ in trophectoderm cells (21). Previous studies also suggested that GM-CSF increased blastocyst formation and maybe associated with increased glucose metabolism and enhanced proliferation of inner cell mass cells (13).

The present study demonstrated that low dose GM-CSF (0.125 ng/mL) promotes the development of preimplantation embryo in mouse, which support the conclusion that GM-CSF acts to regulate blastocyst development. Several repeated experiments by our group showed that increased concentrations of GM-CSF from 0.25 to 2 ng/mL in the medium did not further promote the preimplantation embryo development to the blastocyst stage. When the concentrations of GM-CSF in culture medium reached to 20 ng/mL, the embryonic development appeared to be inhibited (data not shown). Analysis of the GM-CSF receptor in mice indicated that the low-affinity α subunit of GM-CSF receptor was present in blastocyst stage embryos (7). Therefore, the capability of binding ligand of GM-CSF receptor in embryos is limited. Interestingly, study showed GM-CSF downmodulates its receptor in cultured human mast cells. The negative regulation of GM-CSF versus GM-CSF receptor may be another explanation why increased concentrations of GM-CSF do not promote embryonic development in vitro. Previous works by Sjoblom and colleagues suggested that incubated with 2 ng/mL GM-CSF promoted human and mouse embryos development. The different results in GM-CSF concentration may due to the use of different mouse strain, base culture media, or different experimental method.

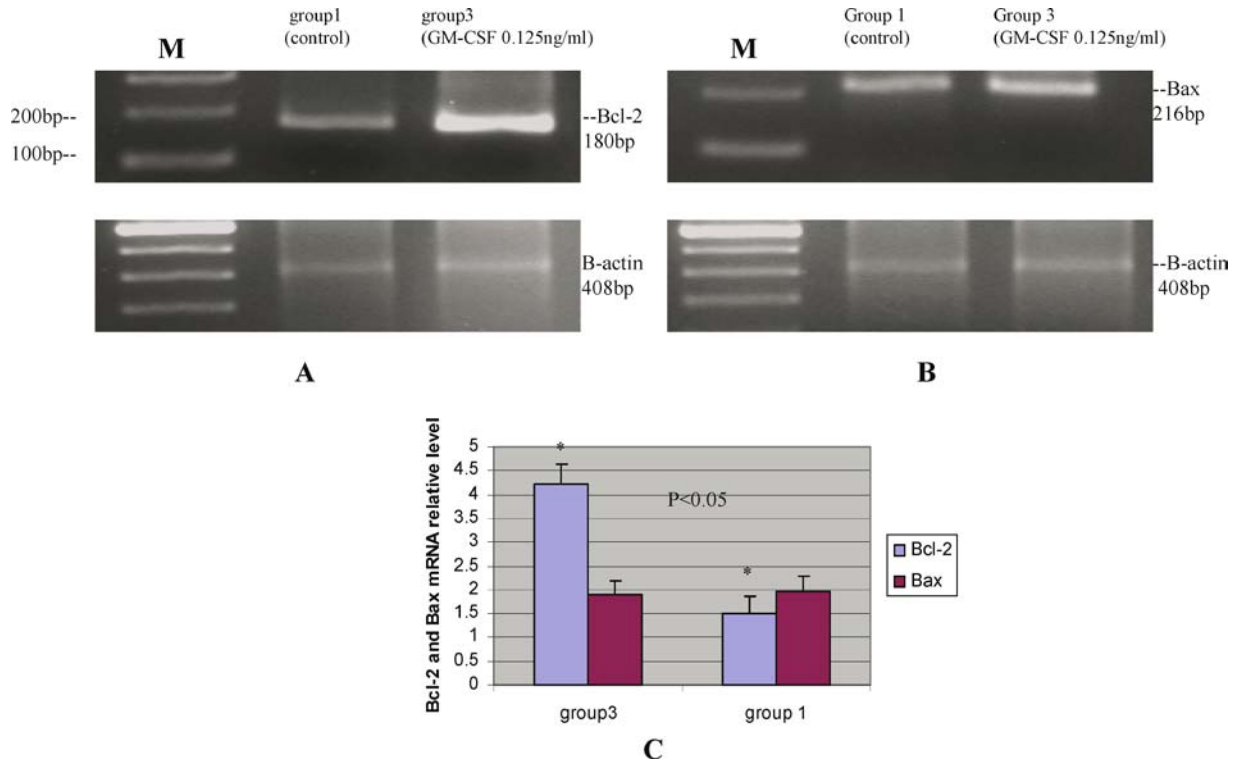

Fig. 2. RT-PCR analysis of Bcl-2 and Bax in mouse preimplantation embryos. (A) Bcl-2 transcripts as well as β-actin standards in blastocyst of group 3 with 0.125 ng/mL GM-CSF and group 1, control; (B) Bax transcripts as well as β-actin standards in blastocyst of group 3 with 0.125 ng/mL GM-CSF and group 1, control; (C) Alterations of Bcl-2 and Bax mRNA abundance attributed to the effect of GM-CSF in the control group (group 1) and the experimental group (group 3). ($p < 0.05$ denotes significant difference).

Studies in mice and humans have shown that the blastomeres of preimplantation embryos are susceptible to apoptosis (1). Several experiments in mice demonstrated that cytokines and growth factors may play a key role in regulating levels of cell death in preimplantation embryo (3). In vivo and in vitro experiments indicated that IGF-1 and TNFa and influence the incidence of apoptosis (22,23). One of the major functions of GM-CSF in various cell lines is to promote survival by suppressing apoptosis (24). The present studies show a significantly lower cellular apoptosis in embryos incultured with 0.125 ng/mL of GM-CSF compared to the control group. Together, these experiments bring us closer to understanding how GM-CSF may act as a regulator in preimplantation embryo development and apoptosis.

Although the array of potential apoptotic signals is vast and varies with cell type, all apoptotic pathways appear to terminate in the activation of the capspase family (25), whose activity is regulated by the Bcl-2 family. At least 15 mammalian Bcl-2 family members have been identified and categorized into two subgroups: those that exert anti-apoptotic effect such as Bcl-2 and those that are proapoptotic such as Bax. Studies suggest that in most cases the ratio of proapoptotic to antiapoptotic Bcl-2 homologues within a cell determines whether the cell will live or die (26).

Table III. Total Cell Number and Apoptosis in Blastocyst

	In vivo group	Control group	GM-CSF group
Number of embryos examined	22	17	18
Average number of apoptosis per embryo	$1.3 \pm 1.3^{*}$	5.9 ± 2.3	$3.9 \pm 2.0^{*}$
Average cell number per embryo	$38.1 \pm 5.9^{**}$	33 ± 4.6	$37.8 \pm 6.0^{**}$

*,** Group 1 compared to Group 3, $p < 0.05$.

In the present studies, Bcl-2 transcripts in embryos incultured with 0.125 ng/mL GM-CSF of were significantly increased compared to the control group while Bax mRNA expression was similar in both groups. Thus, altered expression of Bcl-2 by GM-CSF might affect the process of cell apoptosis in preimplantation embryos. In many tissues, apoptosis performs the function of regulating cell number or eliminating inappropriate or damaged cells. Apoptosis is thought to be the default pathway of cell fate in embryos as well as other cell types. In vitro culture system itself may induce apoptosis in good quality embryos. Supplement with low concentration of GM-CSF may protect embryos from the stress condition. On the other hand, the elimination of unwanted cells is essential in development, but conversely, apoptosis has the potential to eliminate viable cells, leading to the death of the organism (27). Thus, the incidence of apoptotic cell death is likely to be a key indicator of the viability and developmental competence of embryos (28). The precise cause of apoptosis in preimplantation development remains unknown, although the evidence presented here suggests that cell death can be modulated by the addition of low dose GM-CSF, recognizing that there are other modulators of apoptosis.

The discovery that GM-CSF that improves cell number and inhibits apoptosis in the IVF preimplantation embryo may have clinical significance. Our data demonstrating that the number of apoptotic cells and the total cell number in the blastocysts of the GM-CSF supplemented group more closely mimic the in vivo embryos than our controlled embryos did, thereby reinforcing the potential clinical application of growth factor supplemented media in IVF. Of course, before routine clinical application of any growth factor supplemented media, appropriate clinical trials need to be conducted to demonstrate its safety in human IVF.

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