Effects of granulocyte-macrophage colony-stimulating factor supplementation in culture medium on embryo quality and pregnancy outcome of women aged over 35 years

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Abstract

Purpose The purpose of this study is to explore whether a low concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF) supplementation in culture medium is beneficial to infertile women aged over 35 years.

Methods A retrospective cohort study was performed to analyze the embryo quality and pregnancy outcome of 212 controlled ovarian stimulation (COH) cycles with or without GM-CSF addition ($n=117$ [GM-CSF, 0.2 ng/mL] vs $n=95$ [control]).

Results No significant difference was observed in cleavage rate (96.2 vs 96.5 %), blastocyst formation rate (53.2 vs 54.0 %), good blastocyst rate (26.8 vs 26.8 %), or available embryo rate (54.2 vs 49.7 %) between the GM-CSF group and the control group. However, the average age of the GM-CSF group (38.41 ± 3.13 years) was significantly 1 year older than that of the corresponding control group (37.45 ± 2.74 years) ($P<0.05$). GM-CSF addition greatly decreased the occurrence of biochemical pregnancy (55.6 % [control] vs 20.8 % [GM-CSF], $P<0.05$). No case of neonatal malformation was observed in the present study.

Conclusion Although no benefit of GM-CSF on embryo quality was observed, the addition of this factor significantly

decreased the occurrence of chemical pregnancy of women aged over 35 years, indicating the role of GM-CSF in improving implantation competence of embryos derived from elderly infertile women.

Keywords Granulocyte-macrophage colony-stimulating factor (GM-CSF) · Culture medium · Embryo quality · Pregnancy outcome

Introduction

Although advancement in in vitro culture medium has greatly improved the efficacy of assisted reproductive technologies in the treatment of infertility, there is still disparity between in vitro culture and in vivo conditions [1–4]. Culture conditions are important for the outcome of assisted reproductive technologies and have an impact on pre- and postimplantation development and possibly the future health of the offspring [1, 5–8]. An inappropriate culture can lead to impaired embryo development and even recurrent implantation failure [1, 9, 10]. To provide an optimal environment for zygotes/embryos, clinical embryologists have long been involved in optimizing the culture conditions.

An important optimizing protocol is a supplementation of some embryokines to the culture medium [1–4, 11–15]. Among various embryokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) has attracted much attention. GM-CSF is expressed in female genital tract, and as a developmental programming agent, it enhances cell proliferation, embryo progression, blastocyst hatching, and embryo implantation [1, 3, 16–23]. Especially, some existing data have indicated a link between decreased GM-CSF levels and reproductive pathologies in women, including miscarriage, intrauterine fetal growth retardation, preeclampsia, and preterm delivery

Capsule Although no benefit of GM-CSF on embryo quality was observed, the addition of this factor significantly decreased the occurrence of chemical pregnancy of women aged over 35 years, indicating the role of GM-CSF in improving implantation competence of embryos derived from elderly infertile women.

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[23]. These researches suggest GM-CSF as an attractable candidate for improving the quality of the present culture medium. However, most of these results are from animal studies [1, 3, 16–18], and the effect of GM-CSF on human preimplantation embryo, especially the clinical perinatal outcome and offspring health, has yet to be elucidated [20–23]. Moreover, the present concentration of GM-CSF (2 ng/mL) applied in human researches is chosen mainly based on animal data [19, 21, 22], which cannot be exactly equal to human oocytes or embryos. Thus, we still need to know more about the precise nature of GM-CSF effect before its routine application in clinical practice for subfertility treatment.

In the present study, a retrospective cohort study was performed to explore whether a low concentration of GM-CSF (0.2 ng/mL) supplemented in the routine culture medium was beneficial to embryo developmental potential and pregnancy outcome of infertile women aged over 35. We hope this study would contribute to providing new insights for modifying the quality of the existing culture medium.

Materials and methods

Study groups

This study was approved by the Human Research Ethics Committee of Beijing Chao-Yang Hospital, and all patients had provided their informed consent. Data were collected from 189 patients over the age of 35 years with 212 controlled ovarian hyperstimulation (COH) cycles in our center from May 2013 to September 2014.

Ovarian stimulation

Before the start of COH, the patients were divided into two groups: the GM-CSF group and the control group based on their own choices. The hormonal stimulation was undertaken according to the standard long or short protocol (Decapeptyl, triptorelin acetate injection, Ferring, Switzerland, and recombinant human follitropin for injection, Merck Serono). Ovarian response was estimated according to serum estradiol level and ultrasound monitoring. Low response was defined based on the Bologna criteria [24]. Briefly, at least two of the following three features had to be present: (1) age over 40 years; (2) a previous history of ovarian poor response; (3) an abnormal ovarian reserve test (the number of antral follicle count ≤ 5 –7) [24]. High response was defined by a concentration of serum estradiol more than or equal to 5000 pg/mL on the day of human chorionic gonadotropin (HCG) injection or by the number of oocytes retrieved more than or equal to 15. HCG (250 μ g, Ovitrille, Merck Serono) was injected to induce

ovulation, when at least three follicles had reached the diameter of 18 mm or more on ultrasound examination.

Oocyte collection and culture

Oocyte pickup was performed under ultrasound guidance at 37–38 h after HCG administration. After being washed twice with G-MOPS and G-IVF (Vitrolife, Sweden), respectively, the cumulus-oocyte complexes were cultured in G-IVF in an atmosphere of 6 % CO₂ and 20 % O₂.

Sperm preparation

Semen samples were collected immediately after oocyte retrieval. Density gradient centrifugation (PureCception, 40 and 80 %, SAGE, America) was employed to isolate the motile sperms. Optimization was achieved by swimming-up technique for 15–30 min. In the present study, the optimized sperms were all from the fresh ejaculated semen samples.

Micromanipulation of IVF or ICSI

After 2–4-h incubation with G-IVF medium in an atmosphere of 6 % CO₂ and 20 % O₂, the collected cumulus-oocyte complexes were treated with standard in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

IVF was performed when the count of forward motile sperms was >1 million after optimization. Each oocyte was inseminated with 10,000 spermatozoa overnight in G-IVF medium covered with oil (Vitrolife, Sweden) at 37 °C in an atmosphere of 6 % CO₂ and 20 % O₂.

ICSI was performed when the count of forward motile sperms was ≤ 1 million after optimization. The cumulus and corona cells were removed with hyaluronidase (80 IU/mL, SAGE, America), and each mature oocyte was injected with a single mature sperm under standard magnification in a 20- μ L droplet of G-MOPS (Vitrolife, Sweden), with 7 % PVP (SAGE, America). The injected oocytes were culture in G1 medium (Vitrolife, Sweden) in microdroplets covered with oil at 37 °C in an atmosphere of 6 % CO₂ and 20 % O₂.

Embryo culture

Fertilization was checked at 16–18 h after insemination or microinjection on day 1. Normal fertilized (two pronuclei and two polar bodies) oocytes were immediately transferred into standard medium (G1, cleavage stage) at 37 °C in an atmosphere of 6 % CO₂ and 5 % O₂ or standard medium supplemented with 0.2 ng/mL GM-CSF (recombinant human GM-CSF, ORIGIO, Denmark) according to the patients' own choice before the start of COH. After being observed and selected on day 3 (68 $\pm$ 1 h after insemination), the

embryos were transferred into G2 (blastocyst stage, Vitrolife, Sweden) at 37 °C in an atmosphere of 6 % CO₂ and 5 % O₂.

Embryo evaluation

Embryo evaluation was performed at fixed time points of 17, 44, 68, 116, and 140±1 h after insemination by two professional clinical embryologists based on our previous publication and on Istanbul consensus and Gardner scoring systems [25–27].

In the cleavage stage, the grades were as follows: (1) grade 1: embryos with 8–10 blastomeres, had even division and regular morphology and fragments <5 %, and without multinucleation or vacuoles; (2) grade 2: embryos greater than six blastomeres with slightly uneven size and fragments <20 %; (3) grade 3: blastomeres with uneven size and fragments were 20–50 %; and (4) grade 4: >50 % fragments or obviously different cell diameter. In this grading system, the embryo development rate was also taken into consideration. Only embryos with 8–10 blastomeres could be categorized as grade 1. The highest score for 6–7 or >10 cells was grade 2, and embryos with blastomeres of less than six cells could only be assessed as grades 3 or 4. Embryos scored as grades 1 and 2 were recorded as high quality [25–27].

In the blastocyst stage, the scoring systems were as follows: stage 1: with a blastocoel less than half of the volume of embryo; stage 2: with a blastocoel occupying at least half of the volume of embryo; stage 3: with a full blastocoel completely filling the embryo; grade 4: an expanded blastocyst with the volume of the blastocoel larger than the early embryo; grade 5: a hatching blastocyst in which the trophoctoderm was escaping through the zona; and grade 6: a completely hatched blastocyst. Blastocysts beyond stage 2 were further classified according to their inner cell mass and trophoctoderm. For inner cell mass, grade A means a tightly packed inner cell mass with many cells; grade B, a loosely grouped inner cell mass with many cells; and grade C, an inner cell mass with very few cells. For trophoctoderm, grade A means a trophoctoderm with many cells forming a cohesive epithelium; grade B, a trophoctoderm with few cells forming a loose epithelium; and grade C, a trophoctoderm with very few cells. Stage 3–6 blastocysts with inner cell mass and trophoctoderm scored as or over grade C were defined as good quality [25–27].

Embryo transfer

Embryo assessment did not interfere with each other, and high-grade embryos were selected for transfer. Embryo transfer (ET) was normally conducted on day 3. If the number of embryos suitable for transfer was less than that of allowable

for transfer (≤ 3), transfer was conducted on day 2. There were also 10 patients who accepted ET on day 5 based on the patients' own choice.

Follow-up

Serum β -HCG was measured on the 14th day after embryo transfer. A positive result was followed by an ultrasound scan of the uterus at gestational weeks 7–8. Pregnancy outcomes were collected from each couple by a clinically professional follow-up personnel.

Outcome measurements

Embryo quality and pregnancy outcome were recorded as follows: cleavage rate=the number of cleaved embryos/that of fertilized embryos $\times 100$ %, blastocyst formation rate=the number of blastocysts/that of cultured embryos for blastocyst formation $\times 100$ %, good blastocyst rate=the number of high-quality blastocysts/that of formed blastocyst $\times 100$ %, available embryo rate=the number of transferred and frozen embryos/that of total cleaved embryos, pregnancy rate=the number of patients with clinical pregnancy/that of transferred patients, and implantation rate=the number of implanted embryos/that of transferred embryos. Positive HCG is a value of serum HCG ≥ 25 IU/mL on the 14th day after ET. Biochemical pregnancy means positive HCG but an absence of gestational sac by ultrasound detection. Clinical pregnancy means the presence of intrauterine gestational sac by ultrasound at 7–8-week gestation.

Statistical analysis

The data were statistically analyzed by SPSS 18, and a chi-square or *t* test was used to detect the differences between the two groups. Fisher's exact test was used when $n < 40$ or a value of < 5 was expected. $P < 0.05$ was considered as statistically significant.

Results

General clinical information of the patients

Overall, the average age, basal follicle stimulation hormone (FSH) level, antrum follicle count (AFC), body mass index (BMI), and number of retrieved oocytes were 38.03 ± 3.02 years, 8.53 ± 4.03 IU/mL, 10.87 ± 4.93 , 22.98 ± 3.18 , and 9.58 ± 6.22 , respectively. There were no significant differences in FSH (8.35 ± 3.83 vs 8.88 ± 4.19), AFC (11.11 ± 5.82 vs 10.72 ± 4.16), BMI (23.27 ± 3.61 vs 22.79 ± 2.81), the number of retrieved oocytes (10.53 ± 7.45 vs 8.99 ± 5.22), infertility type (primary or secondary), infertility causes (female, male,

or mixed), infertility duration (4.53 ± 3.69 years vs 4.88 ± 4.62 years), ovarian responses (low, normal, or high), injection of gonadotropin (10.59 ± 2.04 days vs 10.33 ± 1.93 days), or dosage of gonadotropin (3007.26 ± 1154.56 IU vs 2843.91 ± 1109.16 IU) between the control group and the GM-CSF group. However, the average age and treatment times of the GM-CSF group were significantly higher than that of the corresponding control group (38.41 ± 3.13 years vs 37.45 ± 2.74 years; 1.61 ± 0.94 vs 1.28 ± 0.72) (Table 1).

Effects of GM-CSF supplementation on embryo development potential derived from patients beyond the age of 35

Patients

The patients from 212 COH cycles (99 IVF cycles and 113 ICSI cycles) were divided into two groups: the routine medium and the test medium containing 0.2 ng/mL GM-CSF.

Table 1 General clinical information of patients

		Total		IVF		ICSI	
		Control	GM-CSF	Control	GM-CSF	Control	GM-CSF
Total cycles		95	117	50	49	45	68
ET cycles		67	104	40	44	27	60
Age		37.45 ± 2.74	$38.41 \pm 3.13^*$	36.64 ± 2.49	$37.96 \pm 2.19^*$	38.36 ± 2.76	38.74 ± 3.67
FSH		8.35 ± 3.83	8.88 ± 4.19	7.75 ± 1.85	7.91 ± 2.94	9.02 ± 5.18	9.09 ± 4.96
AFC		11.11 ± 5.82	10.72 ± 4.16	11.28 ± 5.09	11.56 ± 4.65	10.95 ± 6.55	10.16 ± 3.73
BMI		23.27 ± 3.61	22.79 ± 2.81	23.58 ± 4.19	22.90 ± 2.96	22.96 ± 2.92	22.69 ± 2.72
Type	Primary	41.1(39/95)	35.0(41/117)	38.0(19/50)	26.5(13/49)	40.8(20/45)	41.1(28/68)
	Secondary	58.9(56/95)	65.0(76/117)	62.0(31/50)	73.5(36/49)	51.0(25/45)	58.8(40/68)
Causes	Female	96.8(92/95)	94.0(110/117)	100.0(50/50)	98.0(48/49)	85.7(42/45)	91.2(62/68)
	PCOS	6.3(6/95)	7.7(9/117)	8.0(4/50)	6.1(3/49)	4.1(2/45)	8.8(6/68)
	Tubal	78.9(75/95)	60.7(71/117)	90.0(45/50)	69.4(34/49)	61.2(30/45)	54.4(37/68)
	Endometriosis	7.4(7/95)	6.8(8/117)	8.0(4/50)	8.2(4/49)	6.1(3/45)	5.9(4/68)
	Polyp	7.4(7/95)	5.1(6/117)	0	2.0(1/49)	14.3(7/45)	7.4(5/68)
	OFF	6.3(6/95)	9.4(11/117)	4.0(2/50)	6.1(3/49)	8.2(4/45)	11.8(8/68)
	AIH failure	9.5(9/95)	16.2(19/117)	8.0(4/50)	20.4(10/49)	10.2(5/45)	13.2(9/68)
	Hysteromyoma	3.2(3/95)	5.1(6/117)	2.0(1/50)	2.0(1/49)	4.1(2/45)	7.4(5/68)
	Adhesion	2.1(2/95)	1.7(2/117)	0	2.0(1/49)	4.1(2/45)	1.5(1/68)
	Male	47.4(45/95)	46.2(54/117)	38.0(19/50)	32.7(16/49)	53.1(26/45)	55.9(38/68)
	Mixed	44.2(42/95)	40.2(47/117)	38.0(19/50)	30.6(15/49)	46.9(23/45)	47.1(32/68)
	Unexplained	0	0	0	0	0	0
Duration of infertility		4.53 ± 3.69	4.88 ± 4.62	3.94 ± 3.42	4.70 ± 5.78	5.17 ± 3.92	5.00 ± 3.59
Times for treatments		1.28 ± 0.72	$1.61 \pm 0.94^*$	1.18 ± 0.63	1.43 ± 0.79	1.39 ± 0.81	$1.75 \pm 1.02^*$
Responses	Low	44.2(42/95)	47.0(55/117)	30.0(15/50)	32.7(16/49)	60.0(27/45)	57.4(39/68)
	Normal	35.8(34/95)	40.2(47/117)	46.0(23/50)	46.9(23/49)	24.4(11/45)	35.3(24/68)
	High	20.0(19/95)	12.8(15/117)	24.0(12/50)	20.4(10/49)	15.6(7/45)	7.4(5/68)
Gn days		10.59 ± 2.04	10.33 ± 1.93	10.60 ± 1.91	10.69 ± 1.84	10.58 ± 2.20	10.07 ± 1.97
Gn dosage (IU)		3007.26 ± 1154.56	2843.91 ± 1109.16	2810.11 ± 1037.26	2832.81 ± 843.72	3213.17 ± 1243.86	2851.87 ± 1271.95
Oocytes no.		10.53 ± 7.45	8.99 ± 5.22	11.00 ± 6.37	10.94 ± 5.10	9.35 ± 8.01	7.72 ± 5.00

The results were analyzed by *t* test

IVF in vitro fertilization, *ICSI* intracytoplasmic sperm injection, *GM-CSF* supplementation of 0.2 ng/mL granulocyte-macrophage colony-stimulating factor, *ET* embryo transfer, *Age* the average age, *FSH* the basal value of follicular stimulating hormone, *AFC* the count of antrum ovarian follicle, *BMI* body mass index, *PCOS* polycystic ovarian syndrome, *Polyp* endometrial polyp, *OFF* ovarian function failure, *AIH failure* repeated failure of artificial insemination by husband, *Adhesion* intrauterine adhesion, *Gn* gonadotropin, *Oocytes no.* the number of retrieved oocytes

* $P < 0.05$

Parameters reflecting embryo quality

Several parameters reflecting embryo quality were compared between the two groups. As shown in Table 2, there was no significant difference in cleavage rate (96.5 vs 96.2 %), blastocyst formation rate (54.0 vs 53.2 %), or good blastocyst rate (26.8 vs 26.8 %) between the control group and the GM-CSF group.

Effect of insemination manner on embryo quality

Insemination manner showed no effect on cleavage rate (96.3 vs 95.9 % [IVF]; 96.8 vs 96.5 % [ICSI]), blastocyst formation rate (56.9 vs 50.8 % [IVF]; 46.8 vs 57.8 % [ICSI]), or good blastocyst rate (28.7 vs 27.4 % [IVF]; 22.1 vs 25.6 % [ICSI]) in each subgroup. Moreover, contrary to the IVF group, the available embryo rate (50.00 vs 61.2 %, $P<0.05$) of the GM-CSF addition group was significantly higher than that of the corresponding control group in the ICSI patients.

Effects of GM-CSF supplementation on pregnancy outcomes of patients beyond the age of 35

Overall results

Overall, as shown in Table 3, fresh ET was performed on 117 patients, including 67 control cycles and 104 GM-CSF addition cycles, respectively. There was no significant difference in the number of transferred embryos (2.30 ± 0.76 vs 2.42 ± 0.75), pregnancy rate (37.3 vs 38.5 %), or implantation rate (26.6 vs 23.4 %) between the control group and the GM-CSF group. However, in HCG-positive women, GM-CSF significantly decreased the occurrence of biochemical

pregnancy (37.5 [control] vs 18.4 % [GM-CSF]). Although no statistical difference was detected in the percentage of ongoing/delivery, the GM-CSF group had a higher ongoing/delivery rate (61.2 %) compared with the control group (52.5 %). Moreover, similar observation was made in both the IVF subgroup (57.7 % [control] vs 68.2 % [GM-CSF]) and the ICSI subgroup (42.9 % [control] vs 55.6 % [GM-CSF]). Unfortunately, after being diagnosed as clinical pregnancy at 7–8 weeks, 11 women experienced pregnancy loss (two from the control group and nine from the GM-CSF group). Among these, chromosome examination of the aborted tissue was carried out in four GM-CSF patients. Chromosome abnormalities were detected in three cases.

Day 2 results

Day 2 transfer was performed in 96 patients (36 control cycles vs 60 GM-CSF cycles). In these patients, there was no significant difference in transferred numbers (2.06 ± 0.83 vs 2.23 ± 0.85), pregnancy rate (22.2 vs 31.7 %), or implantation rate (14.9 vs 19.3 %) between the control group and the GM-CSF group. Although compared to the control group, the biochemical pregnancy rate significantly decreased after the addition of GM-CSF to the culture medium (55.6 [control] vs 20.8 % [GM-CSF]), six cases of pregnancy loss were recorded in the GM-CSF group.

Day 3 results

Day 3 transfer was performed in 65 cycles (27 control cycles vs 38 GM-CSF cycles). Because of the limitation of the present sample size, no significant difference was detected in transferred numbers (2.81 ± 0.39 [control] vs 2.79 ± 0.47

Table 2 Effects of GM-CSF on the developmental potential of human zygotes

	Total		IVF		ICSI	
	Control	GM-CSF	Control	GM-CSF	Control	GM-CSF
COH cycles	95	117	50	49	45	68
ET cycles	67	104	40	44	27	60
Zygote no.	567	629	351	340	216	289
CR (%)	96.5 (547/567)	96.2 (605/629)	96.3 (338/351)	95.9 (326/340)	96.8 (209/216)	96.5 (279/289)
BFR (%)	54.0 (143/265)	53.2 (143/269)	56.9 (107/188)	50.8 (91/179)	46.8 (36/77)	57.8 (52/90)
GBR (%)	26.8 (71/265)	26.8 (72/269)	28.7 (54/188)	27.4 (49/179)	22.1 (17/77)	25.6 (23/90)
AER (%)	49.7 (282/567)	54.2 (341/629)	49.6 (174/351)	48.2 (164/340)	50.0 (108/216)	61.2* (177/289)

The results were analyzed by chi-squared test. Fisher's exact test was used when $n<40$ or a value of <5 was expected

IVF in vitro fertilization, ICSI intracytoplasmic sperm injection, COH controlled ovarian hyperstimulation, ET embryo transfer, GM-CSF supplementation with 0.2 ng/mL granulocyte-macrophage colony-stimulating factor, No. number, CR cleavage rate, BFR blastocyst formation rate, GBR good blastocyst rate, AER available embryo rate

* $P<0.05$

Table 3 Effects of GM-CSF addition on pregnancy outcome of patients over the age of 35

		All		IVF		ICSI	
		Control	GM-CSF	Control	GM-CSF	Control	GM-CSF
All	COH cycles	95	117	50	49	45	68
	ET cycles	67	104	40	44	27	60
	No. of ET embryos	2.30±0.76	2.42±0.75	2.38±0.74	2.45±0.66	2.19±0.79	2.40±0.81
	Clinical pregnancy (%)	37.3 (25/67)	38.5 (40/104)	42.5 (17/40)	40.9 (18/44)	29.6 (8/27)	36.7 (22/60)
	Implantation rate (%)	26.6 (41/154)	23.4 (59/252)	31.6 (30/95)	25.9 (28/108)	18.6 (11/59)	22.2 (31/144)
	Positive HCG (%)	59.7 (40/67)	47.1 (49/104)	65.0 (26/40)	50.0 (22/44)	51.9 (14/27)	45.0 (27/60)
	Biochemical pregnancy	37.5 (15/40)	18.4* (9/49)	34.6 (9/26)	18.2 (4/22)	42.9 (6/14)	18.5 (5/27)
	Ongoing/delivery (%)	52.5 (21/40)	61.2 (30/49)	57.7 (15/26)	68.2 (15/22)	42.9 (6/14)	55.6 (15/27)
	Pregnancy loss (%)	5.0 (2/40)	18.4 (9/49)	0	13.6 (3/22)	14.3 (2/14)	22.2 (6/27)
	Ectopic pregnancy (%)	5.0 (2/40)	2.0 (1/49)	7.7 (2/26)	0	0	3.7 (1/27)
D2 ET	ET cycles	36	60	16	17	20	43
	No. of ET embryos	2.06±0.83	2.23±0.85	2.13±0.89	2.35±0.70	2.00±0.80	2.21±0.86
	Clinical pregnancy (%)	22.2 (8/36)	31.7 (19/60)	31.3 (5/16)	29.4 (5/17)	15.0 (3/20)	32.6 (14/43)
	Implantation rate (%)	14.9 (11/74)	19.3 (26/135)	20.6 (7/34)	22.5 (9/40)	10 (4/40)	17.9 (17/95)
	Positive HCG	50.0 (18/36)	40.0 (24/60)	56.3 (9/16)	47.1 (8/17)	45.0 (9/20)	37.2 (16/43)
	Biochemical pregnancy	55.6 (10/18)	20.8* (5/24)	44.4 (4/9)	37.5 (3/8)	66.7 (6/9)	12.5* (2/16)
	Ongoing/delivery (%)	38.9 (7/18)	50.0 (12/24)	44.4 (4/9)	50 (4/8)	33.3 (3/9)	50 (8/16)
	Pregnancy loss (%)	0	25.0* (6/24)	0	12.5 (1/8)	0	31.3 (5/16)
	Ectopic pregnancy (%)	5.6 (1/18)	4.2 (1/24)	11.1 (1/9)	0	0	6.3 (1/16)
D3 ET	ET cycles	27	38	20	21	7	17
	No. of ET embryos	2.81±0.39	2.79±0.47	2.70±0.47	2.71±0.56	2.71±0.49	2.88±0.33
	Clinical pregnancy (%)	59.3 (16/27)	52.6 (20/38)	55.0 (11/20)	57.1 (12/21)	71.4 (5/7)	47.1 (8/17)
	Implantation rate (%)	38.4 (28/73)	30.2 (32/106)	38.9 (21/54)	31.6 (18/57)	36.8 (7/19)	28.6 (14/49)
	Positive HCG	70.4 (19/27)	63.2 (24/38)	70.0 (14/20)	61.9 (13/21)	71.4 (5/7)	64.7 (11/17)
	Biochemical pregnancy	15.8 (3/19)	16.7 (4/24)	21.4 (3/14)	7.7 (1/13)	0	27.3 (3/11)
	Ongoing/delivery (%)	68.4 (13/19)	70.8 (17/24)	71.4 (10/14)	76.9 (10/13)	60.0 (3/5)	63.6 (7/11)
	Pregnancy loss (%)	10.5 (2/19)	12.5 (3/24)	0	15.4 (2/13)	40.0 (2/5)	9.1 (1/11)
	Ectopic pregnancy (%)	5.3 (1/19)	0	7.1 (1/14)	0	0	0
D5 ET	ET cycles	4	6	4	6	0	0
	No. of ET embryos	1.86±0.38	1.83±0.41	1.75±0.50	1.80±0.45	0	0
	Clinical pregnancy (%)	25.0 (1/4)	16.7 (1/6)	25.0 (1/4)	16.7 (1/6)	0	0
	Implantation rate (%)	28.6 (2/7)	9.1 (1/11)	28.6 (2/7)	11.1 (1/9)	0	0
	Positive HCG (%)	75.0 (3/4)	16.7 (1/6)	75.0 (3/4)	16.7 (1/6)	0	0
	Biochemical pregnancy	66.7 (2/3)	0	66.7 (2/3)	0	0	0
	Ongoing/delivery (%)	33.3 (1/3)	100 (1/1)	33.3 (1/3)	100 (1/1)	0	0
	Pregnancy loss (%)	0	0	0	0	0	0
	Ectopic pregnancy (%)	0	0	0	0	0	0

The results were analyzed by chi-squared test. Fisher's exact test was used when $n < 40$ or a value of < 5 was expected

IVF in vitro fertilization, ICSI intracytoplasmic sperm injection, COH controlled ovarian hyperstimulation, ET embryo transfer, GM-CSF supplementation with 0.2 ng/mL granulocyte-macrophage colony-stimulating factor, ET embryo transfer, HCG human chorionic gonadotropin

* $P < 0.05$

[GM-CSF]), pregnancy rate (59.3 % [control] vs 52.6 % [GM-CSF]), implantation rate (38.4 % [control] vs 30.2 % [GM-CSF]), biochemical pregnancy rate (15.8 % [control] vs 16.7 % [GM-CSF]), or ongoing/delivery rate (68.4 % [control] vs 70.8 % [GM-CSF]).

Day 5 results

There were also 10 patients accepting embryo transfer on day 5 (four control cycles and six GM-CSF cycles). The number was too small (10) to merit statistical analysis. Only two

progressed to full pregnancies (one from the control group and one from the GM-CSF group).

Comparison of birth and infant characteristics according to GM-CSF supplementation

Birth and infant characteristics were also investigated in the present study (Table 4). No significant difference was observed in taking baby home rate (17.9 vs 21.1 %), gestational age at birth (37.17 ± 1.90 week vs 37.74 ± 1.74 weeks), or birth weight (2819.17 ± 626.28 g vs 2925.14 ± 547.25 g) between the control group and the GM-CSF group. The same results were observed in the singleton or twin subgroup. In the singletons, the gestational age at birth and birth weight were 37.83 ± 2.32 weeks (control) vs 38.18 ± 1.67 weeks (GM-CSF) and 3299.17 ± 442.05 g (control) vs 3217.00 ± 448.10 g (GM-CSF), respectively. In the twins, the gestational age at birth and birth weight were 36.50 ± 1.22 weeks (control) vs 36.80 ± 1.30 week (GM-CSF) and 2579.17 ± 573.45 g (control) vs 2496.00 ± 355.03 g (GM-CSF), respectively. No case of malformation was observed in the present study.

Discussion

The recognition of the Developmental Origins of Health and Disease induced by in vitro manipulation of gametes or embryos has stimulated the continuous improvement of embryo culture systems. Based on ‘the back to nature’ strategy, growth factors have attracted much attention because of their intrinsic existence in female genital tracts [1, 28]. Now, whether it is

necessary to supplement a specific growth to culture medium in human IVF procedure is a hot spot issue.

The present study aimed to evaluate whether supplementation of GM-CSF, a potential growth factor, was beneficial to infertile patients aged over than 35 years. Unfortunately, compared to the control group, no significant benefit to embryo quality was observed. The present result differed from several previous preclinical studies in which the development-promoting and apoptosis-decreasing effect of GM-CSF on human embryos was observed [20, 29]. The main causes of this difference might be the following. Firstly, in Sjöblom’s studies [29], the donated embryos had experienced freezing-thawing procedure before treatment of GM-CSF. It was shown that the observed benefit of GM-CSF supplementation was only apparent in culture medium containing a low concentration of human serum albumin (2 mg/mL HSA), not when a higher concentration of HSA (5 mg/mL) was used, indicating the possibility that the effect of GM-CSF was to alleviate culture stress induced by suboptimal conditions [19, 27]. Thus, cryopreservation exerting extra stress to the embryos might be an affecting factor. Secondly, the clinical characteristics of patients in this study could not be compared to previous preclinical researches lacking the donor’s clinical information. Thirdly, the competence of embryos derived from senior aged women (>35 years) was generally weaker than young women, and a low concentration of GM-CSF supplementation (0.2 ng/mL) might be insufficient to improve the embryo quality. Different researches following different methods might lead to different results. More rigorous studies with the usage of standard evaluating indicators and end points need to be undertaken in future studies.

Table 4 Birth and infant characteristics of patients over the age of 35 according to GM-CSF supplementation

		Control	GM-CSF
Taking baby home rate (%)		17.9 (12/67)	21.1 (22/104)
No. of deliveries		12	23
No. of live deliveries		12	22
Gestational ages (weeks)		37.17 ± 1.90	37.74 ± 1.74
Mean Birth weight (g)		2819.17 ± 626.28	2925.14 ± 547.25
Malformation		0	0
Singletons	Gestational age (weeks)	37.83 ± 2.32	38.18 ± 1.67
	Mean birth weight (g)	3299.17 ± 442.05	3217.00 ± 448.10
	Male	4	11
	Female	2	6
Twins	Gestational age (weeks)	36.50 ± 1.22	36.80 ± 1.30
	Mean birth weight (g)	2579.17 ± 573.45	2496.00 ± 355.03
	Male	6	5
	Female	6	5

The results were analyzed by chi-squared test. Fisher’s exact test was used when $n < 40$ or a value of < 5 was expected

GM-CSF supplementation with 0.2 ng/mL granulocyte-macrophage colony-stimulating factor, No. number

Apart from its effect on embryo development potential, GM-CSF also affected pregnancy outcome. It significantly decreased the incidence of chemical pregnancy (a decrease of early abortion rate). However, there was no difference in pregnancy rate (37.3 % [control] vs 38.5 % [GM-CSF]) or taking baby home rate (17.9 % [control] vs 21.1 % [GM-CSF]) between the two groups. Although not statistically significant, HCG was positive in 47.1 % in the GM-CSF group and in 59.7 % in the control group. This discordant result may be explained by the fact that the average age in the corresponding control group (37.45 ± 2.74 years) was significantly 1 year younger than that of the GM-CSF group (38.41 ± 3.13 years) ($P < 0.05$). Thus, it might be speculated that GM-CSF exerts beneficial effects on implantation competence of the embryos derived from elderly subfertile women. Several previous clinical researches also confirmed the beneficial role of GM-CSF addition in pregnancy rate and/or live birth rate in patients who experienced at least one previous miscarriage or unsuccessful IVF cycles [1, 20]. In the present study, all patients recruited were aged over 35 and the probability to achieve successful pregnancy was greatly reduced compared to the normal population. Comparing our results with previous researches, we could conclude the possibility that GM-CSF possessed embryotrophic properties, especially to congenitally deficient embryos derived from elderly subfertile women. Contrary to previous studies [20] and owing to extraneous expenses for GM-CSF addition, only a relatively lower concentration of GM-CSF (0.2 ng/mL) was employed in this study. It was confirmed that a culture with 2 ng/mL GM-CSF did not disturb the chromosomal constitution of embryos when compared with the control [22]. Whether a relatively lower concentration of GM-CSF affected the embryonic chromosome could not be elucidated in the present investigation. The present study demonstrated no difference in gestational age, birth weight, sex ratio, or the occurrence of malformation between the GM-CSF group and the control group. Nevertheless, GM-CSF addition was an artificial intervention, especially during the specific period susceptible to epigenetic changes whose consequences might be obvious later in life. Therefore, the effectiveness and safety of its addition needs both large-scale optimization bench experiments and well-designed clinical studies.

One limitation of this study was the following. GM-CSF supplementation meant adding expenses to the patient. Therefore, its use was based on the patient's willingness to pay. Because of this, the clinical conditions between the two groups could not be matched and we only retrospectively analyzed the effects of GM-CSF addition on embryo quality and pregnancy outcome. Another limitation was related to the optimal concentration of GM-CSF. The optimal concentration of GM-CSF in an in vivo environment containing a complexity of various factors and always in a dynamic metabolic

balance could not be simply equal to the effective concentration of GM-CSF in in vitro culture. Moreover, because of the specificity of human samples and ethical issues, it was difficult to perform direct in vitro experiments to explore the natural response of preimplantation embryos to GM-CSF stimulation. To obtain more precise information, a randomized clinical trial (RCT) with a greater population from multicenters was proposed, concentrating especially the effects of GM-CSF on embryos of different stages (day 2, day 3, day 5); a long-term follow-up with the offsprings also should be considered. Last but not the least, in association with the reduction of chemical pregnancy (earlier abortion rate, 37.5 vs 18.4 %), we also observed an increase of later pregnancy loss in day 2 transfer patients (5 vs 18 % and 0 vs 25 %). When correlating the risk of chromosome aneuploidies with women age, only 4 out of 11 patients who experienced pregnancy loss accepted chromosomal analysis of the aborted tissue. Chromosome abnormalities were detected in three cases. Thus, in our opinion, these results further suggested the beneficial effects of GM-CSF on embryo implantation competence rather than decreasing the occurrence of chromosome abnormalities. In the present study, we could not exclude the possibility that the increase of later abortion might be related to the decrease of chemical pregnancies. Thus, in future, we recommend fully informed consent and preimplantation genetic screening/preimplantation genetic diagnosis if the patients choose GM-CSF supplementation. Therefore, there is still much to be achieved before the routine application of GM-CSF in ART.

Conclusions

Although no benefit of GM-CSF on embryo quality was observed, an addition of GM-CSF significantly decreased the occurrence of chemical pregnancy of women aged over 35 years, indicating the role of GM-CSF in improving implantation competence of congenital deficiency embryos. To achieve safe application of GM-CSF in clinical practice, we recommend further strict investigations, especially long-term multicenter follow-up studies with unified evaluation standards and focus on offspring health.

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Compliance with ethical standards

Competing interest The authors declare that they have no competing interests.

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