Original Investigations

The Impact of the Using Culture Media Containing with Granulocyte-Macrophage Colony-Stimulating Factor on Live Birth Rates in Patients with a History of Embryonic Developmental Arrest in Previous In Vitro Fertilization (IVF) Cycles

Sipahi et al. GM-CSF for Embryonic Developmental Arrest

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Abstract

Objectives: To investigate the effect of using granulocyte-macrophage colony-stimulating factor (GM-CSF) containing culture media on embryological data and reproductive outcomes in patients with early embryonic developmental arrest.

Material and Methods: Retrospective case-control study. A total of 39 patients, whose embryos were incubated with GM-CSF containing culture media due to embryonic developmental arrest in two previous IVF cycles in-between January 2016 and November 2017 at … University IVF center, were enrolled. Control group was generated among patients with first IVF attempts due to tubal factor in the same time period. All embryos in the control group were incubated with single step culture medium (without GM-CSF). For the control group selection, matching was done 1:2 ratio considering female age, body mass index, number of M-II oocyte retrieved, and number of embryo transferred (n=80).

Results: Demographic features and embryological data were comparable between two groups. Number of fertilized oocytes (2-pronuclear) was 3.7±2.0 in GM-CSF group and 3.9±2.5 in the control (p= 0.576). Overall, number of embryos transferred (1.3±0.5 vs. 1.3±0.5, respectively) and blastocyst transfer rate (67.6% vs. 59.2%, respectively; p=0.401) were similar. For the reproductive outcomes, implantation rate (32.3% vs. 33.1%, respectively; p= 0.937), clinical pregnancy rate (33.3% vs. 32.5%, respectively; p= 0.770), and live birth rate (LBR) (25.2% vs. 26.2%, respectively; p= 0.943) were similar.

Conclusion: Using GM-CSF containing culture media in patients with previous two failed IVF attempts due to embryonic developmental arrest might rectify embryological data and

reproductive outcomes. To make solid conclusion further randomized controlled trial is warrant.
Keywords: GM-CSF, ICSI, recurrent IVF failure, embryonic developmental arrest

Introduction

Up to 20% of in vitro fertilization (IVF) cycles are cancelled at the stage of follicular growth, oocyte retrieval, fertilization and cleavage stage before embryo transfer (1). Although optimal ovarian stimulation and successful fertilization steps completed, some embryos may stop cleaving at the 2- to 4-cell stages which is called as "early embryonic developmental arrest" (2). The rate of early embryonic developmental arrest for human embryos in IVF cycles is ~10%, and 40% of patients experience at least one embryo arrest at cleavage stage (2, 3). The reasons for early embryonic developmental arrest are not exactly clear (4, 5). Chromosomal abnormalities are observed more in arrested embryos than normal developed embryos (6). Also, suboptimal culture conditions, reactive oxygen species, and inadequate oocyte maturation can be counted among the reasons of early embryonic developmental arrest. Apoptosis is a mechanism for eliminating damaged cells in the human body, however, there is no biochemical, morphological or structural evidence of apoptosis detected in embryos before the 8-cell stage (7). Immature mitochondria in embryos in the 2-to 4-cell stages causes a lack of apoptosis, which induces other mechanisms to eliminate defective cells at the duration of embryonic development (8, 9). It is also known that the early cleavage divisions are controlled maternally by transcription factors formed during oogenesis in vivo (10). Therefore, embryos, which are dependent on maternal infrastructure, become more vulnerable to environmental conditions (reactive oxygen species (ROS)) in vitro (11). During in vitro culture overrides, anti-oxidant defenses, due to lacking oviduct protection in IVF (12), lead to poor embryo quality and delayed embryonic development (5, 13). Inappropriate expression of Adenosine Deaminase (ADA) and glucose-6-phosphate dehydrogenase (G6PDH) genes (14) in embryo metabolism and disabled correction of telomeres length (15) at early stages are other options for early embryonic development arrest. Embryo implantation requires embryo-endometrium synchronization, which is a multi-step process regulated by intracellular and intermolecular relations. These interactions are modulated by various cytokines and growth factors via autocrine, paracrine, and endocrine regulations (16). Deficiencies in interleukin-1 (IL-1), IL-4, IL-6, colony-stimulating factor-1 (CSF-1), GM-CSF, tumor necrosis factor-a (TNF-a), and TNF-b may have resulted in poor embryonic growth, implantation, and pregnancy outcomes (17, 18). GM-CSF is a multi-functional cytokine synthesizes in epithelial cells of the female reproductive tract, which is essential for modulating stress response genes, heat shock proteins, and apoptosis (19-21). Culture conditions for embryonic development are generally considered suboptimal. The supplementation of these factors may accelerate embryo development, increase blastulation rates, and decrease apoptosis. This study investigated the effects of adding GM-CSF to culture medium on embryological data and reproductive outcomes in patients with previous early embryonic developmental arrest.

Materials and Methods

Patient selection

For this study, the database of the IVF Centre of … University School of Medicine was scrutinized between January 2016 and November 2017. A total of 49 couples were identified whose embryos were treated with culture media containing GM-CSF (Embryogen®, Origio, Denmark) due to previous early embryonic development arrest (n=49). Inclusion criteria were as follows: (i) female <40 years old; (ii) agonist and antagonist ovarian stimulation cycles; (iii) Day 3 or 5 fresh embryo transfer cycles; (iv) two consecutive IVF cycles with embryonic development arrest at the 2- to 4-cell cleavage stages. Embryonic development arrest was defined in couples with a history of ≥6 oocytes retrieval and ≥5 fertilized oocytes, but failure

to reach embryo transfer during two consecutive IVF cycles. Exclusion criteria included male contributions necessitating surgical sperm retrieval. The inclusion and exclusion criteria narrowed the sample size of study group to 39 patients. To generate a control group from the patients that had undergone IVF treatment in the same time period (n=80) patients with tubal factor, female age (±1 year) and antral follicle (±2) were matched in a 1:2 ratio from the database. In the control group, all embryos were incubated in a single step culture medium with human albumin solution (Sage 1-Step, Origio®, Denmark).
The primary outcome of this study was investigating the LBR in patients with a history of recurrent cycle cancelation due to embryonic development arrest after the enrichment of cultural media with GM-CSF. Secondary outcomes were embryo transfer rates on Day 5, implantation rates, and clinical pregnancy rates.

Ovarian stimulation
Recombinant follicle-stimulating hormone (r-FSH) (Gonal-F; MerckSerono GmbH, Kiel, Germany or Puregon; MSD, Haarlem, the Netherlands) or human menopausal gonadotropin (hMG) (Merional; IBSA, Lamone, Switzerland, or Menopur, Menogon; Ferring Company, Kiel, Germany) were used solely or in combination for controlled ovarian stimulation. Beginning dose was varied between 175 and 200 IU. Pituitary suppression was maintained by a GnRH antagonist (Orgalutran; MSD or Cetrotide; MerckSerono) or GnRH-agonist protocols according to physician preference. Cycles were monitored by transvaginal ultrasound (TVUSG).

Laboratory procedure
Cumulus cell-oocyte complexes (COCs) were accumulated from aspirated follicule fluids under a stereomicroscope and than COCs were washed twice Flushing Medium with heparin (Origio) that contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). At the end of the oocyte pick up, COCs were cultured in four-well dishes (Thermo Scientific™ Nunc™). Each well contained Fert solution (Origio) and covered with liquid paraffin (Origio). Received from male patient the semen sample, was delivered to laboratory on the day of OPU, and following the liquefaction sperm sample was evaluated in terms of count, motility, and morphology. Subsequently, sperm sample was prepared using SupraSperm density gradient (Origio) with the swim-up technique. Before the intracytoplasmic sperm injection (ICSI), COCs were removed from cumulus and corona cells using enzymatic (ICSI Cumulase, Origio) and mechanical digestion. After that, the oocytes were assessed in terms of nuclear maturation and to all metaphase II (MII) oocytes were performed ICSI under an inverted microscope containing with Hoffman optics at x200 magnification. These injected oocytes were cultured under two different conditions. One group (n=39) was cultured with 40 µl of EmbryoGen (Origio) media until day 3, then same embryos cultured with BlastGen (Origio) media up to day 5. The others (n=80) were cultured for 3 or 5 days with single step medium contains with human albumin solution (Sage 1-Step, Origio). Oocytes were evaluated for fertilization after 16 to 18 hours. At 42-44 and 68-72 hours after ICSI, embryos were classified according to Hardarson's morphological criteria (22). Embryo culture was extended to the Day 5, if there were at least three high quality embryos on day 3 according to the Gardner criteria (23). After cultured with BlastGen or single step media, selected embryos for transferred, were taken to the Soft-Trans Embryo Transfer Catheter with 15-30 µl of media and transferred to the patient under ultrasound.

Statistical Analysis
Mean and standard deviation, or median and 25–75th percentiles, were used for stating continuous variables. A Pearson Chi-square identified differences between cycle and pregnancy outcomes between the groups. Groups were also compared with independent samples *t*-test or Mann-Whitney U-test. A two-sided *p* value <0.05 was considered

statistically significant. All statistical analyses were performed by the SPSS version 17.0 (Chicago, IL).

Results

The demographic characteristics and ovarian reserve tests were comparable among the study and control groups (Table 1).

ART cycles in the study and control groups

GnRH-antagonist protocol was administered to 23 (59%) of the 39 cycles in women in the study group and 55 (69%) of the 80 cycles in women in the control group (Table 2). Four of the 16 agonist protocols applied in the study group cycles were micro-dose flare-up and 12 protocols were luteal long leuprolide acetate. In the control group, 5 of the 25 agonist protocols were micro-dose flare-up and 20 protocols were luteal long leuprolide acetate. On average, the duration of ovulation induction was 9.72 days and 9.70 days for the study and control groups, respectively (p: 0.961). The median (25^{h}–75^{th} percentiles) total r-FSH dose was 2834 IU (1875–3375 IU) in the study group and 2576 IU (1600–3206 IU) in the control group (p= 0.290).

Cycle and pregnancy outcomes

The mean COCs collected were 6.3±3.0 and 7.0±3.0 in the GM-CSF and control groups, respectively (p= 0.292) (Table 2). The respective figure for mean fertilized oocytes (2-pronuclear) was 3.7±2.0 and 3.9±2.5 (p= 0.576). Mean embryos transferred were 1.3 ± 0.5 in both groups. Whereas embryo transfer on Day 5 was available in 67.6% of patients in the study group, 59.2% reached the blastocyst stage in the control group (p=0.401). Implantation (32.3% vs. 33.1%, p= 0.937), clinical pregnancy rates (33.3% vs. 32.5%, p= 0.770), and LBRs (25.2% vs. 26.2%, p= 0.943) were comparable between groups.

Discussion

According to the Istanbul consensus, embryos reach to 7–9 cells by Day 3, with fragmentation under %15 and no multi-nucleation and have cleaved during the preceding 24 hours called ‘Normal’, embryos have 6 or fewer cells on Day 3 (68 ± 1 h post insemination), but have cleaved during the former 24 hours called ‘Slow’, and embryos are those that have not cleaved within 24 hours called ‘Arrest’. (24). Approximately 40% of couples that undergo treatment exhibit at least one embryonic arrest per treatment cycle and 10–15% of all human embryos arrested at the early cleavage stage (2, 3). Despite the sufficient number of oocytes and zygotes, embryonic development arrest is closely associated with assisted reproductive technology (ART) and may lead to recurrent treatment failure. The presence of arrested embryos may diminish the total number of available embryos and affect the treatment cycle outcome. This condition is destructive for the patients receiving treatment. The reasons for early embryonic developmental arrest are not exactly clear (4, 5). Chromosomal abnormalities, suboptimal culture conditions, reactive oxygen species, apoptosis and inadequate oocyte maturation can be counted among the reasons of early embryonic developmental arrest (6).

Suboptimal culture conditions are a possible cause of early embryonic development arrest (4, 5). Culture medium and ingredients are crucial for embryo development and implantation, therefore various growth factors and cytokines have been examined by supplementing the culture medium to optimize embryo development. GM-CSF may be an ideal candidate for embryo development owing to its natural existence in the surface of the fallopian tubes and endometrial cells (19-21). GM-CSF initiates biological activity by binding to its receptor which is a 18–22 kDa glycoprotein. It is a cytokine/growth factor secreted by the uterine epithelium and oviducts under the influence of estrogens. GM-CSF acts as a principal mediator of recruitment and activation of neutrophils and macrophages during the early

pregnancy (19). It likewise initiates granulocytes, mononuclear phagocytes and dendritic cells, impacts their dealing with and out of tissues, and supports cytotoxicity, phagocytosis, antigen introduction, and cytokine discharge (25). GM-CSF is involved in embryonic development, growth and viability by effecting cell proliferation, blastocyst development, hatching and implantation (26). Some genetic combinations may result in the production of polymorphic Killer immunoglobulin-like receptors (KIR) genes from maternal decidual Natural Killer (NK) cells and defective human leukocyte antigen-C (HLA-C) genes from fetal trophoblasts. Incompatibility between KIR-HLA-C can cause abnormal placentation. GM-CSF also provides higher KIR-HLA-C coupling by increasing the migration of primary trophoblasts (27).

Culture media containing GM-CSF accelerates embryo development, increases early cleavage embryo counts that develop at the blastocyst stage, and increases viable inner mass cells with less apoptosis in cultured human embryos in vitro (28). Tevkin et al. reported that using GM-CSF in the culture medium to cope with recurrent assisted reproductive technology (ART) failure increased, although not statistically significantly, the clinical pregnancy rates compared to the control group (39.1% vs. 27.8%). The implantation rate after 7 weeks of gestation (11.6% vs. 20.4%, $p<0.05$) and clinical pregnancy rate after first trimester (9.1% vs. 17.4%, $p<0.001$) were significantly higher in the GM-CSF group (29). They concluded that adding GM-CSF to the cultural medium improves implantation and enhances the success of IVF/ICSI cycles (29).

Culture medium with GM-CSF used in a randomized clinical trial to compare the effect of low (2mg/mL) and high (5mg/mL) human serum albumin (HSA) (28). Under the low HSA concentration, the ongoing implantation rate at first trimester for the GM-CSF group was significantly higher (23.5% vs. 16.7%, p=0.007) than the control group, whereas the high HSA concentrations were similar between groups (22.4% vs. 21.1%, p= 0.53), respectively. Overall, LBRs were significantly higher in the GM-CSF group compared to the control group (28.9% vs 24.1%, p=0.03). Also, failed embryonic development in previous cycles in culture medium with high HSA, implantation, and clinical pregnancy rates were similar in the subsequent cycle with GM-CSF supplemented culture medium compared to the control group (28). Also, in this study for the reproductive outcomes; implantation rates (32,3% vs. 33.1% respectively; p=0.937), clinical pregnancy rate (33.3% vs 32.5%, respectively; p=0.770) and LBR (25.2% vs. 26.2, respectively; p=0.943) were found parallel.

It has been investigated how it affects ongoing pregnancy rates by using culture media enriched with GM-CSF, heparin-binding epidermal growth factor-like factor (HB-EGF), and leukemia inhibitory factor (LIF) in ICSI cycles. Ongoing pregnancy rates were higher in the group using enriched culture media (106/224 [47%] vs. 78/219 [36%]; absolute rate difference [ARD]: 12; 95% CI, 2.5 –21). Cumulative live birth rates were also higher (132/224 [60%] vs. 97/219 [44%]; ARD: 12; 95% CI, 4-20), and pregnancy loss rates were lower (27/124 [22%] vs. 37/103 [36%]; ARD: –14; 95% CI, –26 to –2). The authors argued that less biochemical stress occurs in the enriched culture environment, so that less energy is consumed on embryo plasticity, and the reserve can be spent on post-implantation development (30).

In a retrospective study using GM-CSF in culture medium in fresh transfer cycles, the cleavage rate and the blastocyst formation rate were found to be similar comparing to control group. However, avaliable embryo rate was significantly higher in the GM-CSF group. In the subgroup analysis, cleavage rate and blastocyst formation rate were found to be significantly higher in those over 38 years old (31).

In a review article evaluating the effects of GM-CSF in subfertile patients undergoing ART, the beneficial effects of cytokine supplementation have been observed in the periods of embryonic development. However, the implantation rates and pregnancy rates were different among investigated studies. According to a Cochrane meta-analysis, there was no evidence that GM-CSF-added culture media had any superiority comparing to standard media in terms

of clinical outcomes (32). Furthermore, only one large randomized controlled trial ended with positive results in LBRs between the study and control groups (26). Similarly, in our study the number of M-II and fertilized oocytes were also comparable between the groups. Retrospective design and a small sample size were limitations in our study, GM-CSF supplemented culture medium might be recommended to patients with a history of recurrent failed IVF attempts due to arrest in embryo development.

Conclusion

Using GM-CSF containing culture media in patients with previous two failed IVF attempts due to embryonic developmental arrest might rectify embryological data and reproductive outcomes. However, further studies are needed to support the routine use and solid conclusion of GM-CSF supplemented culture medium in IVF.

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Table 1. Demographic characteristics of both groups. (Data are expressed as means±SD, p<0.05)

	GM-CSF Group (n=39)	**Control Group (n=80)**	**p**
Female age, year	32.8±4.1	31.7±5.3	0.219
AFC, n	12.2±8.3	12.9±8.0	0.412
AMH, ng/ml	2.38±1.6	2.30±1.6	0.800
BMI, kg/m^2	25.1±4.2	24.8±4.5	0.739
Duration of infertility, months	75.1±55.5	57.7±39.6	0.085
AFC: antral follicle count; AMH: anti-Müllerian hormone; BMI: Body Mass Index			

Table 2. Comparison of cycle characteristics and pregnancy outcomes of both groups

	GM-CSF Group (n=39)	**Control Group (n=80)**	**p**
Antagonist Protocol (n, %)	23/39 (59)	55/80 (69)	0.309
Total r-FSH dose (IU)	2834 (1875-3375)	2576 (1600-3206)	0.290
Cycle cancelation rate (n, %)	5/39 (12.8)	9/80 (11.2)	0.802
Number of retrieved oocytes	6.3 ±3.0	7.0±3.0	0.292
Number of M-II oocytes	4.9±2.6	5.6±3.5	0.263
Number of 2PN	3.7±2.0	3.9±2.5	0.576
Number of embryos transferred	1.3±0.5	1.3±0.5	0.707
Embryo transfer day (n, %)			0.416
Day 3	11/34 (32.4)	29/71 (40.8)	
Day 5	23/34 (67.6)	42/71 (59.2)	
Implantation rate, (%)	32.3±44.2	33.1±46.2	0.937
Clinical pregnancy rate, n (%)	13/39 (33.3)	26/80 (32.5)	0.770
Miscarriage rate, n (%)	3/13 (23.1)	5/26 (19.2)	0.997
Live birth rate, (%)	10/39 (25.6)	21/80 (26.2)	0.943
(Data are expressed as means±SD, p<0.05, median (min-max) and percentages)			