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The Impact of Cytokine Additives in Embryo Culture Media on Intracytoplasmic Sperm Injection Outcomes and Reproductive Success

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Running title: Outcome of ICSI after enrichment of culture media by granulocyte-monocyte CSF.

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Abstract

Background: Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is a cytokine that plays a crucial role in immune regulation and influencing embryo development by enhancing cellular communication and growth. In the context of assisted reproductive technologies like Intracytoplasmic Sperm Injection (ICSI), optimizing culture conditions is essential to improve embryo quality and increase pregnancy success rates. Recent studies have suggested that GM-CSF supplementation in culture media may offer benefits by promoting embryo viability and developmental potential. **Objective:** To explore the impact of GM-CSF-enriched culture media on ICSI outcomes, including embryo cleavage, blastocyst formation, and pregnancy rates. **Methods:** A randomized, double-blind clinical trial was conducted involving 100 women undergoing assisted reproductive technology over two years (January 2022 - January 2024). Participants were divided into two groups: a control group receiving standard culture media and a treatment group receiving GM-CSF-supplemented culture media. ICSI outcomes were compared between the two groups. **Results:** Significant differences were observed in the number of cleaved oocytes, top-cleaved oocytes, and blastocysts between the control and GM-CSF-supplemented groups ($p=0.001$, $p=0.03$, and $p=0.003$, respectively). The GM-CSF group demonstrated higher cleavage rates ($p<0.04$), top cleavage rates ($p=0.02$), and blastocyst formation rates ($p=0.007$) compared to the control group. Furthermore, the GM-CSF group had a significantly higher number of transferred embryos ($p=0.037$) and pregnancy rates ($p=0.04$).

Conclusions: GM-CSF supplementation in culture media significantly improves key developmental and clinical outcomes in ICSI, including cleavage rates, top cleavage rates, blastocyst formation, and pregnancy rates, compared to standard culture media.

Keywords: GM-CSF, ICSI, Embryo Development, Pregnancy Outcomes.

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Introduction:

Assisted reproductive technologies (ART) are fertility therapies that include the alteration of eggs or embryos, according to the Centers for Disease Control and Prevention (CDC). Interventions like ovarian stimulation without egg extraction and sperm-only techniques like intrauterine insemination are not included in this definition. A single sperm cell is directly injected into the cytoplasm of a mature egg as part of the sophisticated IVF procedure known as intracytoplasmic sperm injection (ICSI), which helps in fertilization [1]. It is frequently used to treat non-male factor infertility, unexplained infertility, and severe or borderline male infertility [2].

As they grow, embryos must get their energy from outside sources. This function is carried out by oviductal secretions in the natural world, however, in vitro, embryo culture mediums are used. These media must fulfill crucial physicochemical conditions in addition to serving as energy substrates [3]. A more physiologic culture medium loaded with cytokines—biological chemicals made by leukocytes and epithelial cells—may be developed to improve ICSI results. To control cellular stress, apoptosis, late embryonic development, and implantation, cytokines are essential. The effect of cytokine-enriched culture medium on enhancing IVF results is still being investigated [4].

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), leukemia inhibitory factor (LIF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are important cytokines that affect embryonic development in vivo. Research indicates that granulocyte monocyte-CSF enhances the progression of the placental health of mice embryos, and it has been demonstrated to have long-lasting positive effects on human embryo implantation [5]. Cytokines' regulatory mechanisms are yet unknown, though, and their dysregulation can have detrimental effects on the development of the embryo and its progeny. Although apoptosis occurs naturally during embryogenesis, it may also result from less-than-ideal culture conditions, and cytokines can help lessen its consequences. Further research is necessary to determine if the lack of cytokines contributes to the differences between offspring generated in vitro and in vivo, as the relevance of cytokines in decreasing apoptosis under culture-induced stress remains contentious [6].

This study intends to evaluate the association between ICSI success rates and GM-CSF enrichment, taking into account the possible advantages of adding GM-CSF to the culture medium to improve embryonic development and pregnancy outcomes. The results may offer important new information for enhancing the quality of culture medium and increasing the success of reproduction.

Patients and Methods:

Sittings and duration: This investigation encompassed female patients who were pursuing assisted reproductive technology at the Royal Fertility Center in Mansoura, Egypt. Over the course of two years, from January 2022 to January 2024, the investigation was implemented. All patients provided written informed assent. The investigation was conducted with the approval of the Ethical Committee and institutional reviewing board (IRB) at the Faculty of Medicine, Benha University, under the code number Sci-Z-M-102-2022.

Inclusion criteria: The investigation encompassed women who met the subsequent criteria: Age ranging from 20 to 42 years; Consistent menstrual cycles; Body mass index (BMI) between 20 and 33; Prolactin, testosterone, estradiol, and luteinizing hormone are all within normal limits. No fibroids, polyps, or polycystic ovarian syndrome (PCOS); First ICSI attempt or previous failed cycles, abortion; Reasons for fertility were unexplained factors, tub-al factors, and male factors (excluding surgically extracted sperm, globozoospermia, or frozen- thawed/pinhead sperm); the presence of at

least two good quality embryos for transfer was also necessary.

Exclusion criteria: The study excluded women who met any of the following criteria: Anti-Müllerian hormone (AMH) concentrations below 1 ng/ml; Thin endometrium (<7 mm) with fewer than 2 aspirated oocytes.

Sample Size: Cochran's sample size formula was utilized to ascertain the appropriate size of the sample. Initially, we determined the optimal sample size by calculating the total number of patients who met our inclusion criteria, which typically consists of approximately 1,000 patients per year. The optimal sample size was determined to be 140 patients, with a 3% margin of error and a 95% confidence interval.

Randomization Method: The allocation sequences were concealed from both the researchers and the staff undertaking the statistical analysis, and randomized frequencies were generated using STATA 9.0 statistical software. A gynecologist who was not involved in the treatments conducted participant recruitment following the randomization criteria.

Type of Randomization and Allocation:

Double-blind randomization was implemented and conducted on a one-to-one basis.

- **Patients' Recruitment and Grouping (Figure 1):** Initially, 140 patients were eligible for ICSI. 100 patients were chosen for the research after the inclusion and exclusion criteria were applied. The oocytes from each patient were divided, with half being cultured in standard single-step global media and the other half in single-step media supplemented with granulocyte-monocyte colony-stimulating factor (GM-CSF). Randomization was implemented on the day of oocyte retrieval. Study Group: This group consisted of 52 patients whose embryos were transferred from GM-CSF-enriched culture media.
- Control Group: This group consisted of 48 participants where embryos transferred from conventional CM without granulocyte monocyte-CSF enrichment.

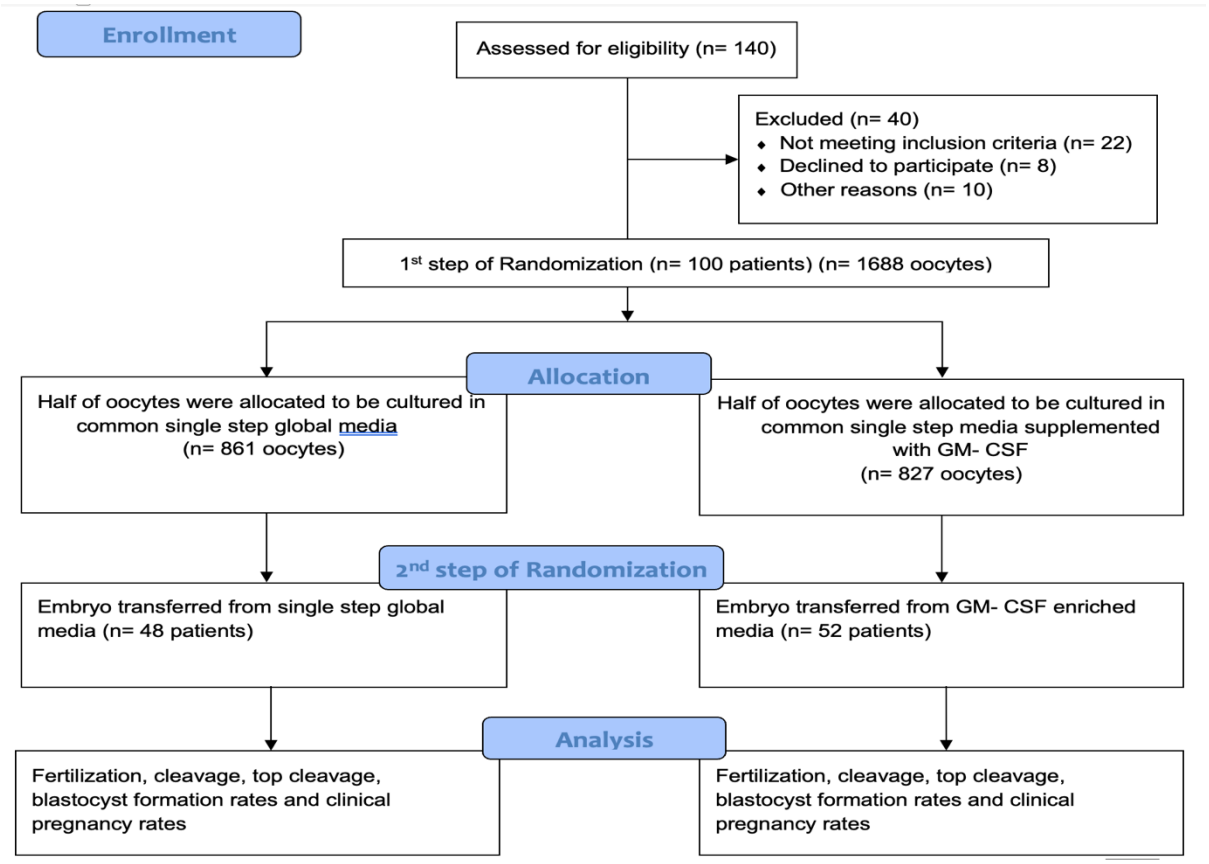

Figure (1): Consort Flow chart of the included patients

ICSI procedure:

Ovarian stimulation protocol and oocyte collection:

GnRH A protocol (Cetrotide, Merck Serono) was employed in conjunction with recombinant follicle-stimulating hormone (FSH) (GONAL-f, Merck Serono) to induce ovarian stimulation. Oocytes were collected via vaginal ovarian pickup (OPU) under ultrasound guidance (GE Healthcare, LOGIQ V5) 36 to 37 hours post-administration of human chorionic gonadotropin (hCG). The cumulus-oocyte complexes were swiftly denuded by exposure to a 25 IU/ml hyaluronidase solution (Ferticult, Fertipro Group) post-retrieval, and the corona radiata was eliminated through repeated pipetting. The oocytes were subsequently analyzed using a stereomicroscope (OLYMPUS SZ 61), and only those in the metaphase II (MII) stage, indicative of mature eggs, were selected for injection 40 hours post hCG delivery.

Semen preparation procedure: 2010 guidelines WHO, following a three to five days period of sexual abstinence, semen samples were collected from both groups. To ensure complete liquefaction, the samples were maintained at 37°C for 20–30 minutes following collection. The samples were subsequently assessed using a Nikon Eclipse E200

phase-contrast microscope. To evaluate the motility and sperm count, a 10 µl aliquot of sperm suspension was obtained. Subsequently, the conventional density gradient technique was implemented to prepare the semen samples.

Intracytoplasmic sperm injection (ICSI) and embryo transfer (ET):

At 37°C, the intracytoplasmic sperm injection (ICSI) procedure was performed under a 400x magnification inverted microscope (Olympus TH4-200). An oil-hydraulic-assisted microinjection system (Narashige, IM-9C) was employed to conduct the microinjection. The oocytes from each patient were divided into two groups following ICSI.

Group I (Control Group): The injected oocytes were cultured in a standard single-step culture medium (SSCM; Global, LifeGlobal).

Group II (Study Group): The other half of the injected oocytes were cultured in the same single-step culture medium (SSCM; Global, LifeGlobal) supplemented with 2 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; G5035 Sigma) [4].

Patient Randomization and Embryo Transfer Procedure:

Patients were randomized to one of two treatment groups on the day of oocyte retrieval:

1. **Intra-Uterine Injection:** Intrauterine administration of a 1 ml medium containing 2 ng/ml GM-CSF was performed fifth day.
2. **Embryo Selection:** All embryos chosen for transfer were exclusively selected from the study cohort.

The Labotect Embryo Transfer Catheter was employed to perform the embryo transfer (ET) procedure under transabdominal ultrasound guidance (GE Healthcare, LOGIQ V5), 3–5 days post-ovum pick-up (OPU). For the purpose of implantation, one to two embryos were transferred.

Reproductive outcomes:

Through the use of an inverted microscope (Olympus TH4-200), the rates of fertilization and cleavage were assessed. Testing of two pronuclei (2PN) was done 16–18 hours after in vitro fertilization (ICSI), and testing of embryo growth was done 44–46 hours after insemination. On the third day, the cleavage was assessed. Pregnancy was ascertained by quantifying blood hCG levels 12–14 days post-embryo transfer (ET). Women who tested positive for hCG were followed two weeks later via transvaginal ultrasound screening. At 4 weeks post-transfer, a gestational sac and fetal viability were detected, indicating an ongoing pregnancy.

Statistical analysis:

All data were organized in SPSS version 27. Nominal data were presented as counts and percentages and analyzed using the chi-square test. Data that followed normal distribution were presented as numbers and percentages and analyzed using the student t-test; significance was established at a p-value of less than 0.05.

Results:

The study involved a total of 100 women. The average maternal age was 26.6 ± 5.6 years, with a mean BMI of 27.3 ± 1.4 kg/m². The average paternal age was 33.1 ± 4.6 years, and the mean BMI was 29.1 ± 2.6 kg/m². The average levels of Anti-Müllerian Hormone (AMH) and Follicle-Stimulating Hormone (FSH) were within normal ranges, with AMH at 2.08 ± 0.64 ng/mL and FSH at 5.68 ± 1.4 IU/L. The average sperm concentration among the participants' husbands was 39.4 ± 17.2 (10^6/ml). The average overall motility rate was 49.5 ± 14.4%, with a mean proportion of normal morphology sperm of 1.1 ± 0.3%.

A total of 2,446 oocytes were obtained from all patients, with an average of 24.5 ± 11.8 per patient.

Of the total collected oocytes, 1,688 were at the metaphase II (MII) stage, accounting for 69%. Each patient had an average of 16 ± 6.44 MII oocytes (Table 1).

Each patient's oocytes were cultivated in either global or cytokine-supplemented media, and no significant variations were found as regards the number of cultured oocytes among the groups. Similarly, no significant differences were found as regards fertilized ova count, fertilized ova mean values, or total fertilization rate.

However, oocytes cultivated in cytokine-supplemented media had significantly higher cleavage, top cleavage, and blastocyst formation rates than those cultured in global media (p=0.04, p=0.03, and p=0.03, respectively). The cytokine-supplemented media group had significantly greater numbers and percentages of cleaved oocytes, top-cleaved oocytes, and blastocysts (Table 2).

Patients were randomized into two groups:

- **Global media group**: 48 patients, in whom embryos were transferred from global media-cultured oocytes.

- **Cytokine-supplemented media group**: 52 patients, in whom embryos were transferred from cytokine-supplemented media-cultured oocytes.

No differences were present between the 2 groups as regards maternal age, BMI, paternal age, or BMI. AMH and FSH levels were also comparable between the groups. Additionally, semen characteristics showed no significant differences.

The global media group had a higher mean No. of collected oocytes and- M.II oocytes than the cytokine-supplemented media group. These differences were statistically significant (p=0.017 and p<0.001, respectively). However, there was no significant variation among both groups in the total number of MII oocytes obtained from all patients in each group, as well as the percentage of MII oocytes to the total collected oocytes (Table 3).

The global media group had a significantly lower number of collected and fertilized oocytes compared to the cytokine-supplemented media group. On the other hand, the rates of fertilization did not differ significantly between the groups.

Statistically significant differences were seen in the **number of cleaved oocytes**, **top-cleaved oocytes**, and **blastocysts** between the global media group and the cytokine-supplemented media gps. In addition, there were statistically significant differences between the two groups, with the cytokine-supplemented media group showing a significantly higher **cleavage rate**, **top cleavage rate**, and **blastocyst formation rate** (Table 4).

The cytokine-supplemented media group had a significantly higher No. of transferred embryos than the global media group (p=0.037). Additionally, the pregnancy rate was significantly higher in the cytokine-supplemented media gps (p=0.04). No differences were reported between the 2 gps regarding the day of transfer or the percentage of high-quality embryos (Table 5).

Table (1): Demographics, baseline characteristics, hormonal assay, semen analysis characteristics, and characters of collected oocytes:

	Total cohort (n= 100) Mean ± SD
Maternal age (years)	26.6 ± 5.6
Maternal body mass index (Kg/m^2)	27.3 ± 1.4
Paternal age (years)	33.1 ± 4.6
Paternal body mass index (Kg/m^2)	29.1 ± 2.6
Anti- Mullerian Hormone	2.08 ± 0.64
Follicle-stimulating Hormone	5.68 ± 1.4
Concentration (*10^6)/ml	39.4 ± 17.2
Total motility (%)	49.5 ± 14.4
Normal morphology (%)	1.1 ± 0.3
Collected oocytes	24.5 ± 11.8
Total number of collected oocytes	2446
MII oocytes	16 ± 6.44
Total number of MII oocytes (% of collected)	1688 (69%)

Table (2): The outcome of oocyte culture in global media and cytokine-supplemented media:

	Global Media Mean ± SD	Cytokine supplemented media Mean ± SD	(t)	P value
Cultured oocytes	8.6 ± 3.5	8.27 ± 2.65	1.47	0.14
Total cultured oocytes	861	827		
Fertilized oocytes	7.23 ± 3.2	6.9 ± 2.5	1.3	0.19
Fertilization rate (%)	83.6 ± 8.5	83.5 ± 6.76	0.11	0.9
Total number of fertilized ova No. (%)	723 (83.9%)	695 (84.03%)	X^2= 0.01	0.97
Cleaved oocytes	6.1 ± 3	5.69 ± 2.4	1.79	0.076
Cleavage rate (%)	82.3 ± 11	85.7 ± 12.4	-1.25	***0.04***
Total number of cleaved ova No. (%)	607 (83.9%)	650 (93.5%)	X^2= 32.2	***<0.001***
Top cleaved oocytes	3.12 ± 1.4	3.5 ± 1.5	-1.8	0.06
Top cleavage rate (%)	42.7 ± 21.8	49.1 ± 20	-2.1	***0.03***
Total number of top cleaved ova No. (%)	312 (43.2%)	355 (51.07%)	X^2= 8.9	***0.002***
Blastocyst formation	2.42 ± 1.2	2.7 ± 1.2	-0.93	0.07
Blastocyst formation rate (%)	37.4 ± 16.2	42.1 ± 14.4	-2.2	***0.03***
Total number of blastocysts No. (%)	58 (39.7%)	79 (47.8%)	X^2= 4.5	***0.03***

(t) Student t- test; (X^2) Chi square test; Level of significance < 0.05

Table (3): Demographics, baseline characteristics, hormonal assay, semen analysis characteristics, and characters of collected oocytes differences between global media and cytokine groups:

	Global Media (n= 48) Mean ± SD	Cytokine-supplemented media (n= 52) Mean ± SD	(t)	P value
Maternal age (years)	26.2 ± 5.9	26.9 ± 5.3	-0.56	0.57
Maternal BMI (Kg/m^2)	26.4 ± 2.3	27.1 ± 1.9	-0.34	0.4
Paternal age (years)	32.9 ± 4.9	33.2 ± 4.5	-0.3	0.76
Paternal BMI (Kg/m^2)	28.8 ± 4.3	29.3 ± 2.8	-0.14	0.6
Anti- Mullerian Hormone	2.1 ± 0.64	2.1 ± 0.6	-0.1	0.92
Follicle-stimulating Hormone	5.6 ± 1.4	5.7 ± 1.5	-0.29	0.77
Sperm Concentration ($*10^6$)/ml	40.63 ± 17.8	38.2 ± 16.7	0.7	0.47
Total motility (%)	51.6 ± 15.3	47.5 ± 13.4	1.4	0.16
Normal morphology (%)	1.1 ± 0.31	1.1 ± 0.2	0.13	0.89
Collected oocytes	27.4 ± 13.1	21.8 ± 9.8	2.4	***0.017***
Total number of collected oocytes	1314	1132		
MII oocytes	18.2 ± 7.1	14.02 ± 5.1	3.4	***<0.001***
Total number of MII oocytes (% of collected)	874 (66.5%)	729 (64.4%)	X^2= 0.4	0.27

(t) Student t- test; (X^2) Chi square test; Level of significance < 0.05

Table (4): Comparison between embryos transferred from global or cytokine-mediated cultures as regards oocyte development characters:

	Global Media (n= 48) Mean ± SD	Cytokine supplemented media (n= 52) Mean ± SD	(t)	P value
Cultured oocytes	7.31 ± 2.1	9.9 ± 4.13	-4.03	***<0.001***
Total cultured oocytes	373	479		
Fertilized oocytes	6.1 ± 2.1	8.4 ± 3.7	-3.76	***<0.001***
Fertilization rate (%)	83.2 ± 7.12	83.9 ± 9.8	-0.4	0.69
Total number of fertilized ova No. (%)	313 (83.9%)	403 (84.1%)	X^2= -0.007	0.9
Cleaved oocytes	4.9 ± 1.9	7.08 ± 3.5	3.36	***0.001***
Cleavage rate (%)	82 ± 8.4	85.6 ± 13.3	-2.1	***0.04***
Total number of cleaved ova No. (%)	252 (80.5%)	340 (84.4%)	X^2= -5.3	***0.02***
Top cleaved oocytes	2.7 ±0.3	3.54 ± 0.4	-2.1	***0.03***
Top cleavage rate (%)	41.8 ± 20.4	45.3 ± 20.7	-2.5	***0.02***
Total number of top cleaved ova No. (%)	141 (45%)	215 (53.3%)	X^2= -4.8	***0.03***
Blastocyst formation	1.88 ± 0.9	3.25 ± 1.3	-3.2	***0.003***
Blastocyst formation rate (%)	32.9 ± 13.1	47.3 ± 12.2	-2.9	***0.007***
Total number of blastocysts No. (%)	30 (35.7%)	43 (51.2%)	X^2= -4.1	***0.04***

(t) Student t- test; (X^2) Chi-square test; Level of significance < 0.05

Table (5): Comparison between embryos transferred from global or cytokine-mediated cultures as regards pregnancy outcome:

	Global Media (n= 48) Mean ± SD	Cytokine supplemented media (n= 52) Mean ± SD	(t)	P value
Number of transferred embryos	2.4 ± 0.5	2.9 ± 0.5	-2.1	***0.037***
Quality No. (%) - Good - Fair	 38 (79.2%) 10 (20.8%)	 41 (78.8%) 11 (21.2%)	X^2= 0.001	0.9
Day of transfer	4.1 ± 0.5	4.2 ± 0.7	-0.05	0.9
Pregnancy No. (%)	16 (33.3%)	29 (55.7%)	X^2= 3.9	***0.04***

(t) Student t- test; (X^2) Chi square test; Level of significance < 0.05

Discussion:

The intricate relationships between the endometrium, human embryo development, and implantation are major factors in mammalian reproduction [7]. For embryonic implantation, chemokines at the maternal-fetal contact are essential [8]. Although the majority of embryo culture media still contain proteins, such as globulin-enriched preparations or human serum albumin, non-protein macromolecules and recombinant protein products are becoming more and more popular as viable substitutes for proteins for manipulating gametes and embryos [9].

There was little data before this study regarding the safety and clinical effectiveness of cytokine-enriched medium for the culture of human embryos. The purpose of this study was to assess how the addition of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) to culture media affected the results of intracytoplasmic sperm injection (ICSI). We carried out a study with 100 patients to address this. Either global media or media enriched with GM-CSF were used to cultivate the recovered oocytes. Following this, 48 patients received embryo transfers from global media, while 52 patients received embryo transfers from GM-CSF media.

The main findings of this study highlight that supplementation of culture media with Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) significantly enhances several key metrics compared to global media. Specifically, GM-CSF-enriched media were associated with increased cleavage rates, top cleavage rates, blastocyst formation rates, and pregnancy rates.

GM-CSF is an important mediator in the early stages of pregnancy when neutrophils and macrophages are recruited and activated. Its effects include the stimulation of granulocytes, mononuclear phagocytes, and dendritic cells. Essential processes such as cytotoxicity, phagocytosis, antigen presentation, and cytokine release are supported by GM-CSF, which also regulate their migration into tissues and impact their activity on-site. By facilitating cell proliferation, blastocyst development, hatching, and implantation, these functions contribute to its role in embryonic development, growth, and viability [10].

The observed improvements in embryo development and pregnancy outcomes with GM-CSF supplementation may be attributed to these multifaceted actions. By enhancing the microenvironment in which embryos develop, GM-CSF supports critical stages of embryo maturation and implantation, potentially leading to better clinical outcomes.

Overall, the findings suggest that GM-CSF-enriched culture media could offer significant benefits in assisted reproductive technologies, particularly in optimizing embryo culture conditions and improving the likelihood of successful implantation and pregnancy.

In this study, patients were randomly assigned to two groups: the global media group, consisting of 48 patients, and the cytokine-supplemented media group, consisting of 52 patients. The demographic and clinical characteristics of both groups were well-matched. The mean age of the women was 26.6 ± 5.6 years, while the mean paternal age was 33.1 ± 4.6 years. The mean maternal Body Mass Index (BMI) was 27.3 ± 1.4 kg/m², and the mean paternal BMI was 29.1 ± 2.6 kg/m². Both groups exhibited comparable maternal and paternal ages as well as BMIs. Additionally, levels of Anti-Müllerian Hormone (AMH), Follicle-Stimulating Hormone (FSH), and semen characteristics were similar between the two groups.

These findings align with results from several previous studies that investigated the impact of cytokine-enriched culture media. For example, Ziebe et al., Huang et al, Fawzy et al., Lee et al., and

Sipahi et al., reported that the inclusion of cytokines in culture media did not result in significant differences in patient age, BMI, hormonal profiles, or semen characteristics between cytokine-enriched and control groups. This consistency across studies supports the validity of our results and indicates that the observed effects are likely due to cytokine supplementation rather than demographic or baseline clinical differences [4, 10-13].

Overall, the absence of significant differences in these baseline characteristics reinforces the robustness of our findings, suggesting that the improvements in embryo development and pregnancy outcomes associated with GM-CSF supplementation are attributable to the cytokine's effects rather than variations in patient demographics or initial clinical parameters.

We noticed in the current research, that the cytokine-supplemented media group had a significantly lower number of collected oocytes and MII oocytes than the global media group. The cytokine-supplemented group had (2.9 ± 0.5) embryos transferred, which was significantly more than the global media group's (2.4 ± 0.5) embryos, with a p-value of 0.037. Despite this, there was no statistically significant difference in the quality of the embryos that were transferred between the groups.

Our findings are consistent with those of Sjöblom et al., who found that the number of transferred embryos was significantly higher in the GM-CSF group compared to the control group (p=0.03) [14]. Conversely, Fawzy et al., and Sipahi et al., found that there were no statistically significant differences between cytokine-enriched and control groups in terms of the number of retrieved oocytes, MII oocytes, or transferred embryos [4, 10]. Huang et al., also found that there were no statistically significant changes between IL-18 enriched media and the control groups in terms of total number of retrieved oocytes, MII oocytes, or embryo transferred number [12].

Regarding the number of cultured oocytes, our study found that cytokine-supplemented media was more effective, with a significantly higher number of cultured oocytes in the cytokine group (9.9 ± 4.13) compared to the global media group (7.3 ± 2.1) ($p<0.001$). The global media group had a lower number of fertilized oocytes (6.1 ± 2.1) compared to the cytokine group (8.4 ± 3.7). However, the rates of fertilization did not differ significantly among these groups.

These results align with findings from Ziebe et al. and Sipahi et al., who did not find significant differences in fertilization rates between GM-CSF enriched media and global media [10,11]. However, when comparing the cytokine and control groups for cultured oocytes or fertilization rates, Fawzy et al., found no statistically significant differences [4]. Huang et al., found that the IL-8 group, in contrast to the control group, had a significantly higher fertilization rate (p=0.02) [12].

The results of the present study demonstrated that the number of cleaved oocytes and cleavage rates was significantly higher among the cytokine group (7.08 ± 3.5 cleaved oocytes; 85.6 ± 13.3%) than the global media group (4.9 ± 1.9 cleaved oocytes; 82 ± 8.4%) showing statistically significant differences ***(p= 0.001; 0.04)***. Another significant finding in the present study is that the number of top cleaved oocytes and top cleavage rates was significantly higher among the cytokine group (3.54 ± 0.4 top cleaved oocytes; 45.3 ± 20.7%) than the global media group (2.7 ± 0.3 top cleaved oocytes; 41.8 ± 20.4%) showing statistically significant differences ***(p= 0.03; 0.02)***.

The findings of the present study are in line with those of previous research, highlighting the impact of cytokine-supplemented media on embryo development. Lee et al., in a study involving 45 patients, reported significantly higher levels of cytokines—particularly CCL27, CXCL12, and CCL15—in the top cleaved group [13]. This

suggests a correlation between cytokine levels and enhanced cleavage potential. Similarly, Sipahi et al., showed that both cleavage and top cleavage rates were significantly elevated in the cytokine-supplemented group in comparison with the control group, especially among patients over 38 years of age [10]. This underscores the beneficial effects of cytokine enrichment, particularly in populations with reduced reproductive potential.

In addition, Ziebe et al., supported the role of GM-CSF in embryo culture, reporting that GM-CSF-enriched media accelerates embryo development, increases the number of early cleaved embryos that reach the blastocyst stage, and enhances the viability of the inner cell mass by reducing apoptosis in cultured human embryos [11]. These results align with our findings, where the cytokine-supplemented media group demonstrated significantly higher cleavage rates, top cleavage rates, and blastocyst formation rates compared to the global media group.

However, some studies have yielded conflicting results. Fawzy et al., and Ziebe et al., in separate studies, found no statistically significant differences in cleavage rate or top cleavage rate between the cytokine-enriched and control groups [4, 11]. These discrepancies could be attributed to differences in study design, patient populations, or the specific cytokines used in culture media.

The present study demonstrated that the number and rate of blastocyst formation were significantly higher in the cytokine-supplemented group (3.25 ± 1.3 blastocysts; 47.3 ± 12.2%) compared to the global media group (1.88 ± 0.9 blastocysts; 32.9 ± 13.1%), with statistically significant differences (p= 0.003; 0.007). These findings are consistent with those reported by Fawzy et al., who found that the cytokine-enriched media led to improved blastocyst formation compared to the control group (48% vs. 36%; p= 0.001) [4]. Similarly, Sjöblom et al., in a study of 374 patients, demonstrated that the blastocyst formation rate was significantly higher in the GM-CSF group compared to the control group [14]. This improvement in blastocyst development could be attributed to the synergistic effect of the selected cytokines, which may activate pathways in the embryo that mimic in vivo development. The presence of more viable embryos in the cytokine group resulted in a higher number of useable blastocysts, thereby enhancing the cumulative clinical outcomes and potentially improving the cost-effectiveness of a single IVF attempt, as noted by Robertson et al. [15].

In this study, the cytokine group (55.7%) had significantly elevated pregnancy rates compared to the global media group (33.3%) (p= 0.04). This result is consistent with the finding in the work of Fawzy et al. (2019), who found that the cytokine group had an elevated level of ongoing pregnancy rate of transferred blastocysts compared to the control group (47% vs. 36%; p= 0.012), but not significant differences in chemical pregnancy rates were found. Furthermore, Sequeira et al., discovered that higher concentrations of interleukin (IL)-1β in embryo culture media were that are associated with positive pregnancy outcomes. This result provides additional evidence that cytokines play a significant role in effectively enhancing clinical outcomes [16]. In a similar way, Huang et al., also found that IL-8 concentration greater than 0.16 pg/mL in human embryo culture media increased the rates of pregnancy and implantation. They also found that 54.2% of patients in the IL-18 enriched culture group were positive for chemical pregnancy, whereas 40.8% of patients in the control group were positive for chemical pregnancy (p= 0.02), even though clinical pregnancy rates were comparable [12].

However, some studies reported different results. Lee et al., who evaluated cytokine concentrations in culture media of pregnant and non-pregnant patients after ICSI, did not find significant differences in cytokine levels related to pregnancy status. Nevertheless, extremely low levels of CCL15 (<16.9

pg/mL) were associated with significantly lower pregnancy rates, indicating the potential impact of specific cytokines on reproductive outcomes [13]. Additionally, Ziebe et al., who compared GM-CSF-enriched media to global media, found no statistically significant difference in pregnancy rates between the GM-CSF group (37.9%) and the control group (73.3%) (p= 0.46) [11]. Similarly, Sipahi et al., reported comparable pregnancy rates between the cytokine group (33.3%) and the control group (32.5%) in a study of 119 patients, in contrast to the findings of the current study [10].

These varied outcomes underscore the complexity of cytokine interactions within the embryo environment. While the majority of studies, including the present one, demonstrate a positive impact of cytokine-enriched media on embryo development and pregnancy outcomes, there remains a need for further research to fully understand the mechanisms and identify which cytokines contribute most to these improvements. Differences in patient populations, cytokine types, and study designs may explain the discrepancies observed in various studies.

This study had several limitations that should be acknowledged. Firstly, cumulative live birth was not considered as a primary endpoint, which could have provided a more comprehensive assessment of the clinical outcomes associated with cytokine-enriched media. Additionally, we did not perform subgroup comparisons between patients with good and poor outcomes, which could have led to a potential type I statistical error, limiting the robustness of the findings.

It should be noted that this study has multiple limitations. The absence of long-term follow-up on the offspring was another limit, as evaluated in previous studies like that of Sjöblom et al., where postnatal outcomes were considered [14]. The absence of this data in our study prevents us from assessing the potential long-term benefits or risks associated with the use of cytokine-enriched culture media.

Lastly, a key limitation is the study's small sample size. The number of patients included in the study depended on their availability, which may reduce the generalizability of the results. A larger cohort could have strengthened the statistical power and provided more definitive conclusions regarding the effects of cytokine-enriched culture media on ICSI outcomes.

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