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Effects of Cytokines Supplementation in human Embryo Culture Medium in patients with previous recurrent pregnancy loss.

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Abstract: Several studies in both animals and humans indicate that supplementation of in vitro culture media with cytokines and growth factors can improve pre-implantation embryo development, blastocyst formation rates, ongoing pregnancy rates and reduce pregnancy loss as well. Objective*:* This study was designed to evaluate the impact of adding combination of three cytokines (granulocyte-macrophage colony-stimulating factor GM-CSF, heparin-binding epidermal growth factor-like growth factor HB-EGF, and leukemia inhibitory factor LIF) into human embryo in vitro culture medium on preimplantation embryo development, embryo quality and pregnancy outcomes after intracytoplasmic sperm injection (ICSI) in patient with history of recurrent pregnancy loss (RPL).

Study design, size, duration: This is a subgroup analysis of a prospective RCT (trial registration no:NCT04547699) including 136 women with a previous history of recurrent pregnancy loss (RPL) and treated by ICSI for other indications between September 2019 and August 2021 at Mansoura Integrated Fertility Center (MIFC), Mansoura, Egypt; and Rahem Fertility Center (RFC), Zagazig, Egypt.

Patients were randomized, and the culture medium were supplemented with three main protocols; group A, culture media prepared with the standard single step culture media (SSCM) plus 5 mg/ml HSA without CYK supplementation, group B prepared with (SSCM) supplemented by cytokines plus high protein (HSA) concentration (5 mg/ml) and the third group C, supplemented by cytokines plus low protein concentration (2.5 mg/ml HSA).

All women were followed-up to primary outcome, Ongoing pregnancy ≥ 12 weeks of gestation which was 25.5% (12/47) in group A of standard single step culture media (SSCM) combined with 5mg/ml HSA, without CYK supplementation, 39.1% (18/46) in group B prepared with (SSCM) supplemented by CYK plus (5mg/ml) HSA, and 51.1% (22/43) in the group C, (SSCM) supplemented by CYK plus (2.5mg/ml) HSA, giving a significant difference in group C when compared with group A (P =0.048). while comparable differences between grouops A vs B and B vs C, p = 0.29 and 0.18 respectively. Pregnancy loss rate (PLR) was significantlly lower in group C when compared with group A, p =0.009. PLR was comparable between group A vs B (p =0.186) and B vs C (p =0.104). Our results indicated that, integration of cytokines and growth factors combined with low HSA concentration into ICSI culture media has significant benefical effect on cycle outcome in cases with history of RPL.

keywords: ICSI, IVF, Cytokines, medium, infertility.

1.Introduction

Recurrent pregnancy loss (RPL), or the loss of two or more clinical pregnancies, is one of the most prominent and major problems that affects 2% of infertile couples (1). RPL has several etiologies, including genetic, immune, and uterine abnormalities; however, in 50% of

cases, the cause is unknown. As current treatment protocols rely on diagnostic testing and treatment of known causes (e.g., pre-implantation genetic screening PGT-A to ensure euploid embryo transfer and surgical correction of uterine abnormalities), the most effective way to treat unknown cases of recurrent pregnancy loss is unclear (2,3).

Recent studies have explored potential immune-related explanations for recurrent pregnancy loss. During pregnancy, the fetus acts as a semi-allogenic graft, where both parent contribute in genetic development (4). As response, the fetus contains paternal antigens foreign to the maternal immune system, for this successful pregnancy requires the suppression of the maternal immune response. Despite this, the immune system must also preserve an adequate level of malleability to protect against infection. This control of the immune system likely stems from various mechanisms. For example, during pregnancy, the immune system shifts from less support mechanism through the T helper type 1 (TH1) inflammatory immune response to the more targeted response through the T helper type 2 (TH2) immune response, the latter of which is a more targeted system that better supports fetal development (5). In women with recurrent pregnancy loss, several studies have observed that the TH1 to TH2 cytokine ratio remains high during pregnancy (6), these results suggest that cytokine levels may play important roles in the success of pregnancies. Cytokines include a group of proteins which are responsible for several cellular functions, like cellular proliferation and differentiation. They play an important role in regenerative and inflammatory–like processes which are verified at every menstrual cycle in human endometrium and are involved also in specific events in reproduction, ovulation, and implantation (7). integration of GM-CSF into embryo culture medium after intra cytoplasmic sperm injection (ICSI) improves blastulation rates, ongoing pregnancy rates, and reduces pregnancy loss rates.(8–11), which was confirmed by Ziebe et al, which has potentially great benefits for RPL patients when culture media were supplemented by GM-CSF plus low human serum albumin (HSA) concentration (12). Furthermore Fawzy et al (13), demonstrated a benefit to ongoing implantation rates at 12 weeks of gestation and live birth rates when culture media were supplemented by a combination of cytokines and growth factors (GM-CSF, HB-EGF, LIF).

In the current study a combination of growth factors (GM-CSF, HB-EGF, LIF) was supplemented to the human embryo in vitro culture media with high and low (HSA) concentrations, to evaluate the subsequent effect on embryo development and ongoing pregnancy outcome in RPL patients after fresh embryo transfer cycles (ICSI-ET).

2. Materials and methods

This study is a part of a multi-center study, that was prospectively, randomized design (ClinicalTrials.govidentifier: NCT04547699) conducted in Mansoura Integrated Fertility Center (MIFC), Mansoura, and Rahem Fertility Center (RFC), Zagazig, Egypt, between September 2019 and August 2021.

Out of 1095 patients, 700 fulfilled the inclusion criteria and were included in the whole study (fig- 1). Informed consent from participants before implementation of the study and hospital ethics approval were obtained for this study, patients were included based on the following inclusion criteria: Patients who fitted the medical definition of infertility “One year of unprotected intercourse but not pregnant”, women between 18 and 38 years of age. Patients with a history of recurrent pregnancy loss were included.

Exclusion criteria: History of ovarian or adnexal surgery, Suspicious findings of ovarian malignancy, Presence of endocrine disorders such as diabetes mellitus, hyper-prolactinemia, thyroid dysfunction, congenital adrenal hyperplasia, Cushing’s syndrome, and adrenal insufficiency, Patient with poor ovarian response, defined as less than two MII oocytes were retrieved and history of male Globozoospermia.

After receiving informed written consent under ultrasound guidance. Retrieved oocytes

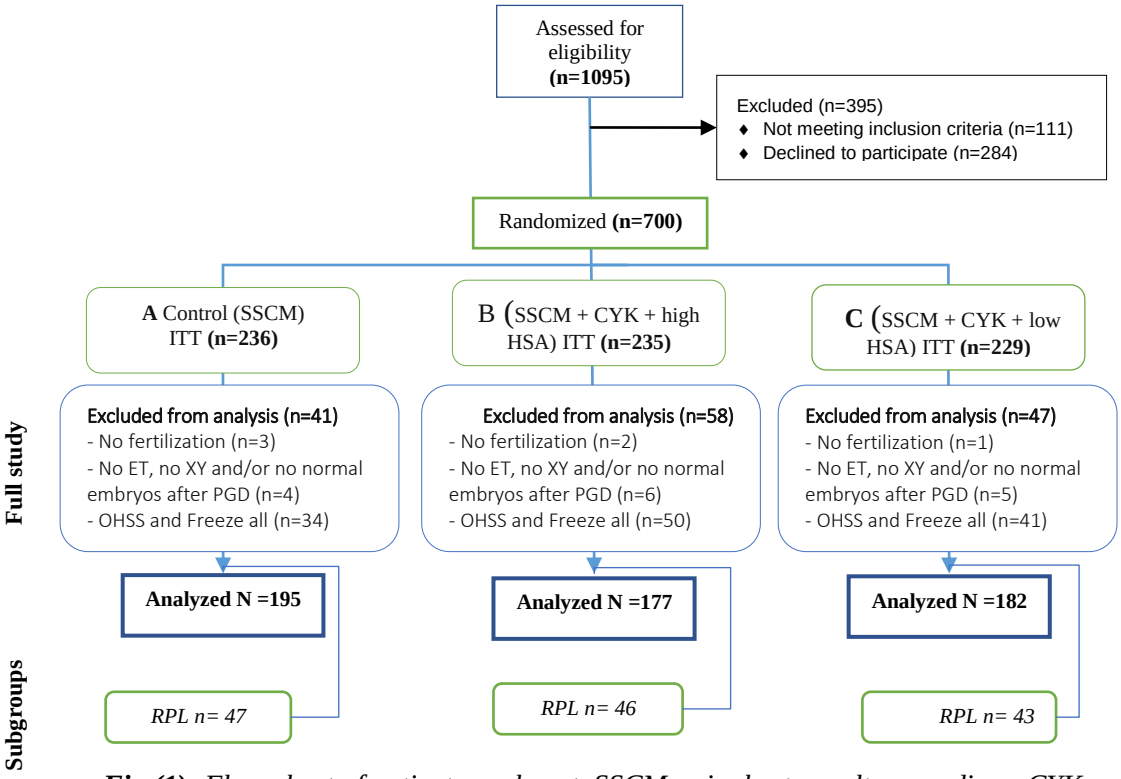


Fig (1): *Flow chart of patient enrolment. SSCM= single step culture medium; CYK = cytokines; LGPS = Life global protein supplement; ITT=Intention-to-treat; ET= Embryo transfer; OHSS = ovarian hyper stimulation syndrome; RPL= recurrent pregnancy loss.*

from participants. We randomized patients according to an opaque enveloped block method, indicating the treatment group. Allocation was randomly placed in opaque sequentially numbered envelopes by a person otherwise, uninvolved in the trial. An embryologist selects the next envelope in the sequence the day before oocyte retrieval to allow for preparation of culture medium. Culture dishes were prepared with patient identifiers and media type recorded by a data entry person. Clinicians, embryologists, and participants in the two centers had no access to allocate randomized patients.

Embryologists who were not involved in oocyte and embryo handling, supplemented the culture medium with cytokines and prepared the culture dishes according to random allocation numbers in the opaque envelopes provided in one of the three groups A, B, or C. Controlled ovarian stimulation was achieved using an antagonist/ agonist protocol, with doses tailored to patients. 36 hours following hCG administration, oocytes were retrieved were incubated for two hours prior to cumulus-corona denudation and morphological assessment, mature oocytes were then inseminated by Intracytoplasmic Sperm Injection (ICSI) and cultured according to randomization protocol.

Approximately 18 hours later, fertilization of inseminated oocytes was confirmed by the presence of two pro-nuclei (2PN). Embryos were cultured uninterruptedly until day 5 or 6.

Embryos were transferred on day 2, 3, 4,5 or 6 after oocytes retrieved, the day of the transfer depends on the number and quality of generated embryos on day 2 (number of top-quality embryos <4, ET on D2/3 and ≥4, ET D5/6). The embryos were assessed, and 1-3 embryos were selected for transfer ET, using labotect embryo transfer catheter (Labotect; Germany). The media used for transfer were pre-supplemented according to the same protocol for each participant A, B or C.

A subgroup of 136 patients were identified as RPL patients who received culture media among other cases in the 3 groups:

Group (A) control, (n=47), culture media were prepared with the standard single step culture media (SSCM; LifeGlobal, Belgium) combined with 5mg/ml HSA (LGPS; LifeGlobal, Belgium) without CYK supplementation.

Group B (n=46), prepared with (SSCM; LifeGlobal, Belgium) supplemented by cytokines (GM-CSF, HB-EGF and LIF) plus high HSA concentration 5mg/ml (LGPS; LifeGlobal, Belgium).

Group C (n=43), prepared with (SSCM; LifeGlobal, Belgium) supplemented by cytokines (GM-CSF, HB-EGF and LIF) plus low HSA concentration 2.5mg/ml (LGPS; LifeGlobal, Belgium). All conditions were monitored and calibrated to achieve the standard in vitro condition for pre-implantation embryo development, all culture media were equilibrated at 37˚C temperature, 5% O_2, 5-6% CO_2 concentration to achieve pH 7.27-7.29 as close as possible**.** The primary outcome of ongoing clinical pregnancy was assessed via ultra-sound examination at 12 weeks of gestation, the secondary outcome measures were: (a) Fertilization rate defined as fertilized oocytes with two pronuclei (2PN) per number of MII oocytes. (b) High-quality embryos day 3, defined as embryos with seven or eight blastomeres of stage-proper sizes, and <10% fragmentation by volume, embryo assessment was performed according to the Istanbul consensus workshop 2011.

(c) Blastocyst formation rate, defined as differentiated blastocyst on day 5/6 per number of 2PN zygotes in the same cycle.

(d) Biochemical pregnancy rate, Positive serum hCG at ≥14 days after ET per number of randomized women.

(e) Clinical pregnancy rate defined as the number of clinical pregnancies divided by the number of embryo transfer cycles.

(f) Implantation rate (IR), defined as the number of gestational sacs determined by ultrasound per number of embryos transferred.

(g) Pregnancy loss rate (PLR), defined as pregnancy loss before 12 weeks of gestational age divided by the total number of clinical pregnancies.(14)

(h) Embryo utilization rate, defined as the number of embryos utilized (transferred or cryopreserved) per number of 2PN zygotes in the same cycle.

Statistical analysis was performed using SPSS (ver. 26, IBM). Efficacy analysis was based on completed cycle principle; data were expressed as mean ± SD of quantitative variables or percentage (%) of qualitative ones. Data between the three groups were analyzed by Student's t-test for quantitative variables and χ^2 tests for qualitative ones. P-value of < 0.05 was considered statistically significant, the relative risks (RRs) with 95% CIs were calculated for the primary and secondary outcomes. Comparison between each 2 groups was done using Fisher Exact test after applying Bonferroni adjustment for multiple comparisons.

3. Results and Discussion

In total 136 women identified with previous history of recurrent pregnancy loss RPL, were randomized to have their oocytes fertilized and embryo cultured and transferred in control group SSCM (n= 47), SSCM supplemented with cytokines plus high HSA concentration (n= 46) or SSCM supplemented with cytokines plus low HSA (n= 43). The study groups were comparable regarding their baseline characteristics. Age, BMI, and infertility diagnosis (Table- I).

Table-1 Patient's baseline clinical features

Characteristic	Group A (n=47)	Group B (n=46)	Group C (n=43)	P
Age (y)	28.1 ± 5.5	28.3 ± 5.2	28.7 ± 5.3	NS[a]
BMI	31.1 ± 4.1	30.8 ± 4.1	31.2 ± 3.9	
Causes of infertility [n (%)]				
Azoospermia	1 (2)	3 (7)	1 (2)	NS[b]
Sever OAT	9 (19)	8 (17)	7 (16)	
Sex selection	3 (6)	2 (4)	2 (5)	
Combined male and female.	2 (4)	1 (2)	3 (7)	
Unexplained	21 (45)	17 (37)	17(40)	
PCO	4 (9%)	3 (7)	5 (12)	
Endometriosis	0	1 (2)	2 (5)	
Tubal factor	1 (2)	2 (4)	1 (2)	
Combined female factor	6 (13)	9 (20)	5 (12)	

Note: plus-minus values are mean ± SD, BMI= body mass index, Sever OAT= sever oligoathenoteratozoospermia, PCO= poly cystic

ovary, NS= not significant. [a]ANOVA test. [b] χ^2 - test.

Table-II Hormonal and laboratory parameters in the study groups

Parameters	**Group An=47**	**Group B n=46**	**Group C n=43**	**P**
Basal FSH (mIU/mL)	5.4 ± 3.2	5.3 ± 2.9	5.5 ± 3.1	NS[a]
Antimüllerian hormone (ng/mL)	5.2 ± 3.3	5.0 ± 3.7	5.4 ± 3.8	
Endometrial thickness at hCG day (mm)	9.8 ± 2.1	10 ± 1.8	9.5 ± 2.4	
Stimulation protocols [n (%)]				
Long agonist	25 (53)	21 (46)	23 (53)	NS[b]
Antagonist	22 (47)	25 (54)	20 (47)	
Cycles characteristics (Mean ± SD)				
No. of oocyte retrieved	14.6 ± 7.2	15.0 ± 8.6	14.5 ± 7.9	NS[b]
No. of MII oocytes	9.8 ± 5.5	10 ± 6.5	9.7 ± 5.6	
No. of fertilized oocytes.	7.6 ± 4.7	8.2 ± 5.8	8.1 ± 5.02	
Freeze all pp				
Laboratory data per randomized women (mean)[b]				
Good embryos day 2/3	4.8	5.7	5.6	0.05
Fair embryos day 2/3	2.2	2.1	2.03	0.52
Poor embryos day 2/3	0.53	0.38	0.44	0.27
Mean no. of fresh ET	1.95	1.79	1.8	0.20

Note: plus-minus values are mean ± SD, MII= metaphase II oocytes, ET= embryo transfer, NS= not significant. [a]ANOVA test, [b] χ^2 -test

Table-III clinical and laboratory Outcomes per embryo transfer cycles (n =136).

Parameters	**Group A(n=47)**	**Group B (n=46)**	**Group C(n=43)**	**P value(χ2 test)**
primary outcome [n (%)]				
Ongoing pregnancy rate	12/47(25.5)	18/46(39.1)	22/43(51.1)	**0.043[a]**
Secondary outcomes [n (%)]				
Fertilization rate	256/377 (68)	280/394 (71)	284/384 (74)	0.18
Biochemical pregnancy rate	24 (51.1)	23(50)	24(56)	0.84
Clinical pregnancy rate	21/47 (45)	22/46 (48)	23/43 (53)	0.700
Implantation rate	36/94(38)	36/92(39)	31/81(38)	0.990
Top quality embryos day 3	167 (63**)**	215 (70)	206 (69)	0.18
Blastocyst formation rate	108(42)	129(46**)**	133(47)	0.51
Utilization rate	115(45)	146(52)	156(55)	0.058
Pregnancy loss rate < 12 w	9/21 (43)	4/22(18)	1/23 (4)	0.007[b]

RPL, recurrent pregnancy loss (history of previous two or more clinical loss of pregnancy before 22 weeks of gestational age); PLR, pregnancy loss (<12 weeks of gestational age).

Fisher's Exact Test; [a]OPR (A vs. C; P= 0.048), (B vs. C; P= 0.29), (A vs B; P= 0.187),

[b]PLR (A vs. C; P= 0.009), (B vs. C; P= 0.186), (A vs B; P= 0.104)

Ovarian stimulation protocols, cycles characteristics, baseline levels of FSH, AMH, Endometrium thickness and progesterone at

trigger day were comparable between the study groups (Table II).

Good, fair, and poor embryos assessment according to the Istanbul consensus workshop 2011, were comparable in the three groups, mean number of transferred embryos were 1.95, 1.79, 1.81 in SSCM, cytokines plus high HSA and cytokines plus low HSA respectively (*p*= 0.205).

Clinical pregnancy rates were comparable in all studied groups (p =0.700).

ongoing pregnancy rate, 12/47 (25.5%), 18/46 (39.1%) and 22/43 (51.1%), in the groups A, B, C respectively (*p* =0.043) and pregnancy loss rates were 9/21(43%), 4/22 (18%), 1/23 (4%) (p= 0.007) respectively.

Comparison between each 2 groups was done using Fisher Exact test after applying Bonferroni adjustment for multiple comparisons revealed significantly higher OPR in group C which was subjected to culture in medium supplemented with CYK combined with low HSA (2.5mg/ml), when compared to control group (A), P =0.048.

No significant differences between groups B Vs C (P= 0.29); or between groups A Vs B (P= 0.187).

Significant lower rate of pregnancy loss (PLR) at 7-12 weeks of gestation in group C when compared with group A (A vs C, P= 0.009).

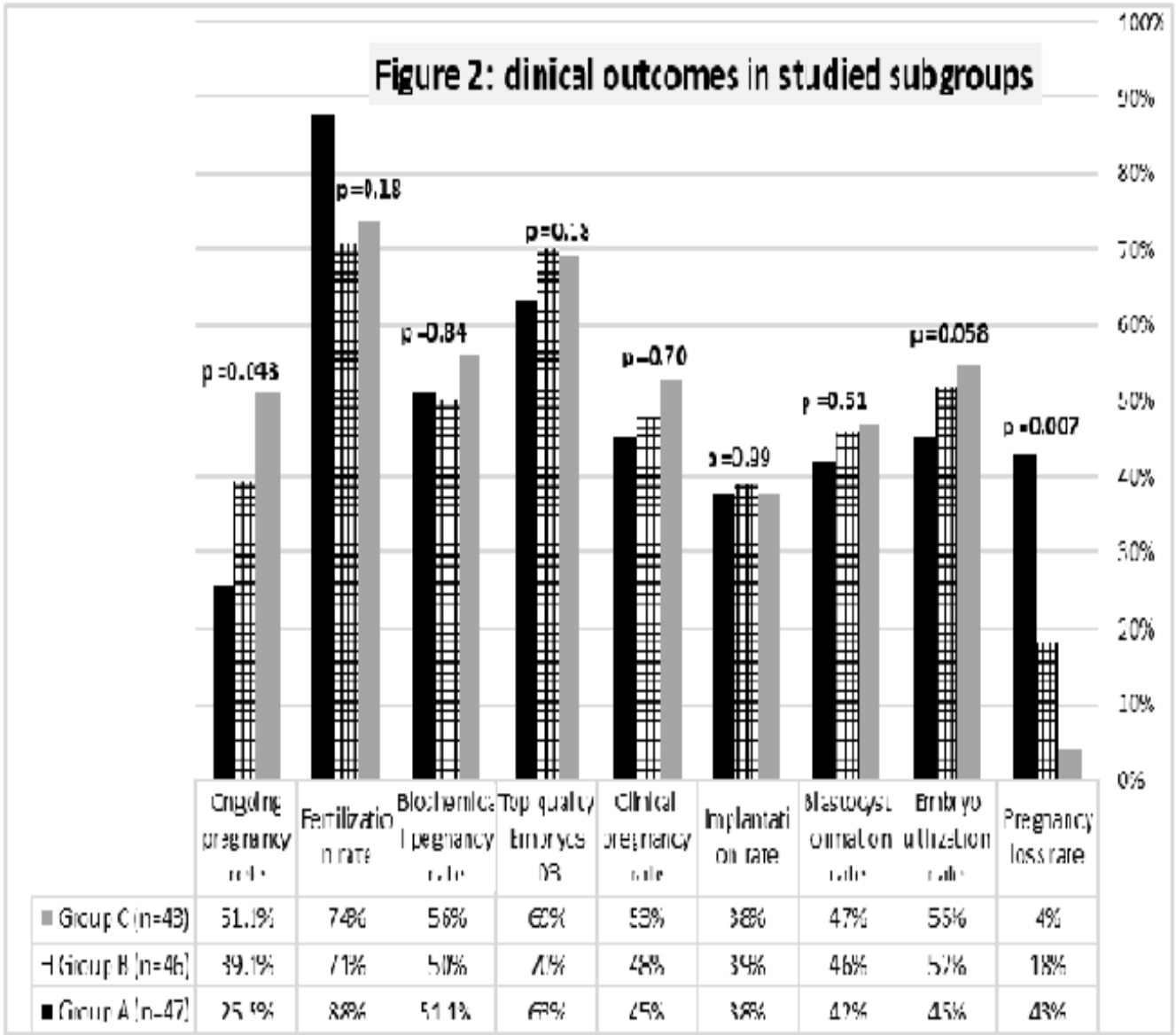
No significant differences in PLR between groups when compare B vs. C, (P= 0.186),or A vs B, (P= 0.104).

Biochemical pregnancy rate, embryo quality, blastulation rates, embryo utilization rates all were comparable in the studied groups (Table III).

In current study, two concentrations (5mg/ml and 2.5 mg/ml) of human serum albumin were used in our study to evaluate the cytokines effects in high and low HSA concentrations. Fawzy et al, 2019 (13) used recombinant human serum albumin (rHSA) to eliminates the introduction of unidentified cytokines with the HSA, whenever, added an extra cost to the IVF culture media without adding effectiveness.

Several studies used HSA in in vitro culture media supplementation (12,15,16).

Research in both human and animal studies demonstrated that, the benefit of adding cytokines to culture media were only observed when culture media contained low HSA



concentration 2 mg/ml (11,12). We employed low and high HSA concentrations with the CYK supplemented media (B and C), while the control group SSCM (A) not supplemented with low HSA concentration in all study, because suboptimal performance were evident in control group when supplemented with low HSA by previous

studies (12). Thus, we use SSCM with its commercially commonly used HSA concentration 5 mg/ml.

Our data demonstrated a significantly higher ongoing pregnancy rates among CYK supplemented groups in 136 patients who had previous RPL history only with low HSA concentration (Table III, Figure 2). These findings are consistent with Sfontouris et al. (17), who reported that the inclusion of GM-CSF in embryo culture media improved the pregnancy and implantation rates in patients

with multiple unsuccessful IVF attempts, these fining reported also by Ziebe, that the greatest benefit of GM-CSF culture medium was observed in women with a history of previous miscarriage, suggesting that, effect was more pronounced in this RPL subgroup (12).

To interpret the beneficial effects of CYK integration into IVF culture media in low HSA concentration only, a hypothesis refers to suboptimal and non-physiological conditions, i.e. the absence of protein (11), such that the benefit of adding cytokines was only apparent in culture medium containing the lower concentration of 2 mg/mL HSA (12).

In conclusion, our results indicate a significant benefit is obtained by supplementing SSCM with CYK and low concentration of HSA in ICSI patients with history of RPL, conducting a RCT evaluating the benefit of using CYK in RPL patients appears to be of value.

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