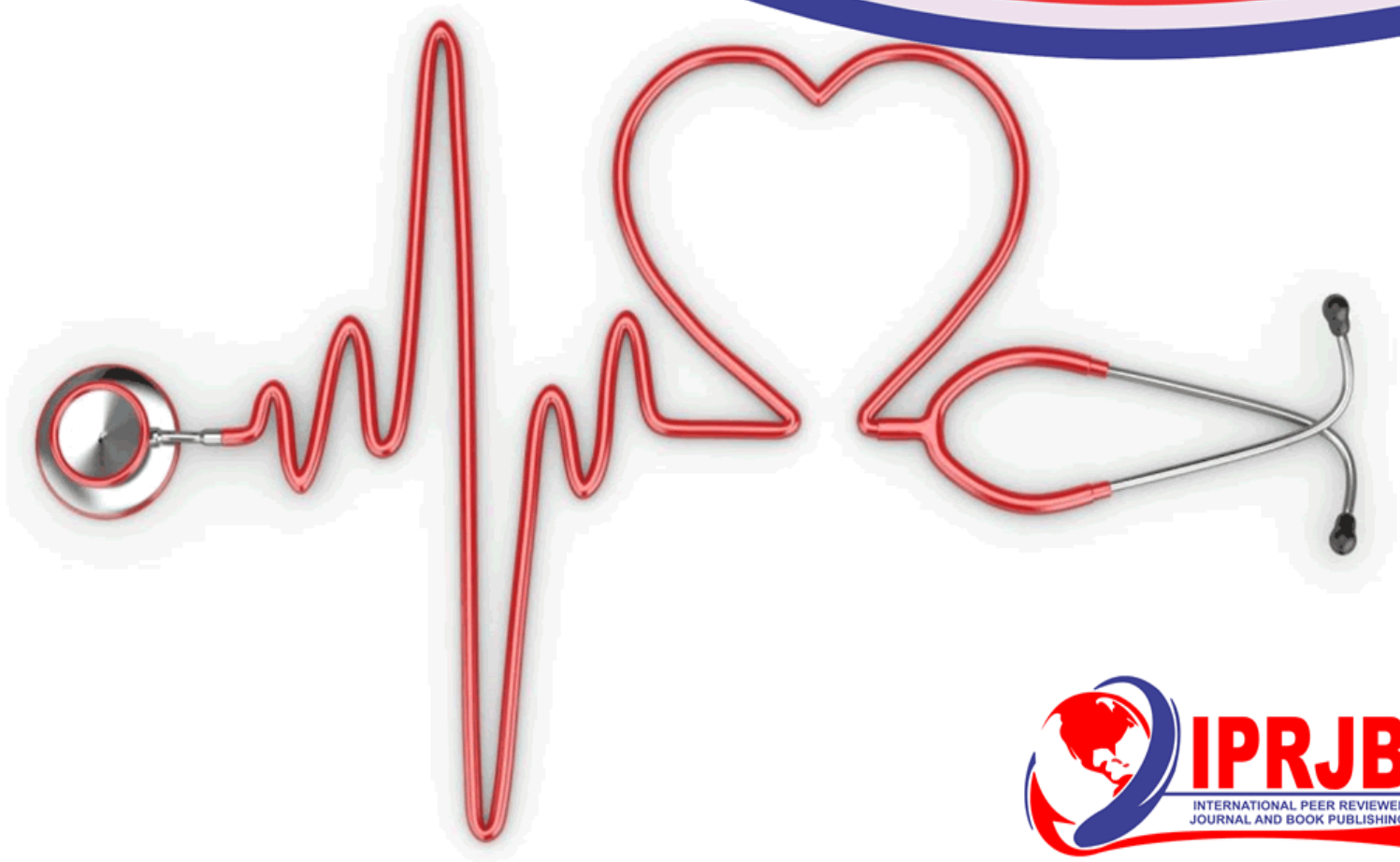


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EMBRYO GLUE AND CLINICAL PREGNANCY RATES IN ICSI EMBRYO TRANSFER CYCLES: A PROSPECTIVE STUDY

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Abstract

Purpose: One of the reasons for failed implantation after transferring good quality embryos in an intracytoplasmic sperm injection cycle is the failure of creation a viscid layer between the embryo and the endometrium. Many modifications have been made in embryo transfer medium to improve implantation and increase pregnancy rates such as adding albumin as a source of energy and adding hyaluronic acid in high concentrations such as in Embryo Glue medium: a human embryo transfer medium. To investigate whether the use of Embryo Glue had any effect on clinical pregnancy rates in intracytoplasmic sperm injection-fresh embryo transfer cycles.

Methods: A prospective study included one hundred and twenty-eight infertile Iraqi women who were selected and subjected to a stimulation protocol in an intracytoplasmic sperm injection-fresh embryo transfer cycle. All patients were considered to be eligible for embryo transfer (no visible causes could prevent implantation) and only good quality embryos were transferred to them. Those women were divided randomly into two groups according to type of embryo transfer medium: group A: Embryo Glue medium. group B: Conventional medium. Then group A was subdivided according to age into: AI (34 women with age < 35 years and represented 50.7%) AII (33 women with age ≥ 35 years and represented 49.3%) While group B was subdivided into: BI (41 women with age < 35 years and represented 67.3%) BII (20 women with age ≥ 35 years and represented 32.7%).

Results: Although there was no significant difference between all groups in causes of infertility, the pregnancy rate was significantly higher in subgroup AII (18 pregnant from 33 women) while only 5 patients became pregnant from 20 patients in subgroup BII. In all women no more than four good quality embryos were transferred, and when total number of transferred embryos was significantly more in group B than group A ($P=0.013$), the significant increase in pregnancy rates was only observed in subgroup AII ($P=0.048$). Even though a highly significant difference in number of repeated implantation failure was in group A than group B ($P=0.027$), the pregnancy rates were significantly higher in group A ($P=0.038$).

Conclusion: This study concluded that using Embryo Glue has a beneficial effect on old women and increase pregnancy rates, also it has a positive effect on pregnancy rates in repeated implantation failure and increases pregnancy rates even if the women is old.

Keywords: Embryo Transfer, Failed IVF Trials, Implantation Failure, Infertility, Embryo Glue, ICSI, IVF

INTRODUCTION

The basic outcome that determines successful intracytoplasmic sperm injection (ICSI) cycles is a positive pregnancy and a successful embryo implantation⁽¹⁾. Implantation represents a critical step that involves an exceptional biological communication between two performers: the embryo and the endometrium. This communication leads the first to invade and embed in the second during a specific time which is known as a window of implantation (WOI) and this needs: a receptive endometrium, a functional healthy embryo and a synchronized dialogue between them^(1, 2). Methods that assess endometrial receptivity (ER) directly are rare as we lack clinical tools, instruments or any means to diagnose and treat causes of implantation failure (IF) after successful embryo transfer (ET). Although many potential biochemical markers of ER were investigated, but there is no a clinically available basis^(3, 4). Methods that are used to improve implantation were only on an experimental basis. Over the years experiences by infertility specialists, efforts of scientists and researchers in medical companies and the desire to increase pregnancy rates (PRs) lead to present several advances such as improving stimulation protocols and choosing an optimal protocol for each case, presenting improvements in incubators, devices, tools, culture media and instruments, all these efforts lead to increase fertilization rates, cleavage rates, number of good quality embryos and improve PRs, but a successful implantation is still represent a very difficult step in ICSI cycles^(1, 4, 5). Transfer of good quality embryos in these cycles results in about 40-60% successful implantation depending on their developmental stage, female's age, infertility causes and the ICSI team. Traditionally, the medium that is used for ET has been similar or identical to culture medium that is used for growing the embryos^(2, 5). Although several improvements were made in culture media, and various sequential media have been developed that mimic the in vivo environment, the role and composition of ET medium were not studied extensively as other media, but some evidence has been presented concerning the positive effect of hyaluronic acid (HA); a naturally existing macromolecule abundant in human fluid secretions and extracellular matrix of the reproductive tract on in vitro embryo development and implantation. Considering the chemical properties of HA, one may hypothesize that it may have a role in assisting the embryo–endometrial interaction during the early phases of implantation^(6, 7, 8). This study was inspired by many researches who claimed that using Embryo Glue (EG) which contains a high concentration of HA has a beneficial effect on PRs, so it was dedicated to investigate this effect for the first time in Iraq, although this medium has being used in many infertility centers around the world since 2003, only 2-4 centers in Iraq use it due to its expensive price and culture media that are used in most Iraqi infertility centers need 5% CO₂ which has an affordable price for Iraqi culture while EG needs 6% CO₂ that means it should be incubated in separated CO₂ incubator which cause more efforts and more incubators.

MATERIALS AND METHODS

A. *Type of study, Site and Duration*

A prospective study was performed by the medical team in the Specialist Fertility Department/ Al-Bonook hospital in Baghdad/Iraq from October 2016 to May 2017.

B. *Subjects*

One hundred and twenty-eight infertile Iraqi women were selected and subjected to an IVF stimulation program in a fresh ICSI-ET cycle, and written agreement was obtained from all patients to participate in the study. The enrolled women were only those who considered having a normal hormonal baseline and normal uterus shape.

C. *Controlled Ovarian Hyperstimulation (COH)*

This study included two protocols: agonist and antagonist. The first was began from the mid-luteal phase before the cycle by daily given a subcutaneous injection of gonadotropin-releasing hormone GnRH Decapeptyl 0.1mg (Decapeptyl® 0.1mg, Ferring GmbH Germany) to suppress the pituitary gland and preventing luteinizing hormone (LH) surge in patients with age < 35 years who had a good ovarian reserve and with cases of male factor, then from D2 of ICSI-ET cycle subcutaneous injections of gonadotropins (GONAL-f® EMD Serono, Inc. Merck KGaA, Darmstadt, Germany) was given also daily and the beginning dose was between 150 and 375 units of Gonal-F per day, the dose was adjusted as the stimulation progressed. While antagonist protocol was given for patients with advanced age (≥ 35 years), predicted poor responder and polycystic ovary syndrome (PCOS). Gonadotropins (GONAL-f® EMD Serono, Inc. Merck KGaA, Darmstadt, Germany, Pergoveris® (r-hFSH 150IU and r-hLH 75IU), MENOSON® Ferring Pharmaceuticals, Switzerland and Merional IBSA Farmaceutici Italia Srl) were administrated from D2 of ICSI-ET cycle. Follow up was done by following estrogen level measurements and ultrasound (U/S) assessment during stimulation then when follicles size reached 14 mm, antagonist (Cetrotide® cetrorelix acetate for injection Serono, Inc. Merck KGaA, Darmstadt, Germany) was given daily till administration decision of human chorionic gonadotropin (HCG) injection. In both protocols the decision of giving HCG (Ovitrelle® solution for injection in prefilled pen Serono, Inc. Merck KGaA, Darmstadt, Germany) was dependent on 4 parameters; at least 4 leading follicles reached to 16 mm or more under U/S, estradiol (E2) levels ≥ 1500 IU, progesterone (P4) levels under ≤ 1.3 ng/mL and endometrium thickness was ≥ 7 mm under U/S.

D. *Ova Pickup, ICSI and ET*

After 34-35 hours of HCG administration the ova pickup (OPU) was done under U/S guidance. Retrieved oocytes were fertilized by ICSI technique. Embryos were cultured in sequential media (our conventional medium) using Gain medium (GAIN™ MEDIUM, FertiCult™, Belgium) either the early or blastocyst stage depending on the division phase. Good quality embryos were transferred by using one type of embryo transfer catheter (Cook Medical Incorporated, Australia) with the same embryo loading procedure. All procedures of OPU-ICSI-ET were done by two embryologists and three gynecologists. The patients were divided randomly into two groups according to type of embryo transfer medium: group A: EG medium, group B: Conventional medium. Each group were subdivided according to age into: group AI (34 women with age < 35 years and represented 50.7%), group AII (33 women with age ≥ 35 years and represented 49.3%), group BI (41 women with age < 35 years and represented 67.3%) and group BII (20 women with age ≥ 35 years and represented 32.7%). The decision of embryos number to be transferred and time of ET was dependent on female's age, number of good quality embryos, this was made by collaboration between the physician, embryologist and the treated patient's agreement. On the day of ET (D2, D3, D4 or D5) the good quality embryos were placed in either preincubated EG for group A or in fresh preincubated conventional medium (group B) prior to transfer. Before this step, EG was incubated in another CO₂ incubator for culture for at least 4 hours, while conventional medium was incubated for overnight. Embryos in group A were incubated in EG for (10-30) minutes before ET, while embryos in group B were incubated early on the day of ET.

E. *Patient's follow up*

Luteal support was initiated after the day of OPU by given 400mg intravaginal progesterone pessaries (Cyclogest® 200mg pessary Progesterone, UK) and sustained for up 14 days after

ET, if the pregnancy was assured then it was continued until 12 weeks of gestation. Pregnancy was detected by doing β -HCG, the normal range for β -HCG standard was between (2-1500) mIU/mL. When the result was < 2 mIU/mL negative pregnancy was confirmed, but when the result was above, the test was repeated weekly until the level of HCG was raised >1500 with intravaginal U/S in order to follow up the pregnancy. Positive embryonic cardiac activity proved positive clinical pregnancy.

Statistical analysis

Descriptive data such as mean and standard deviation (SD) were calculated and the mean values of normally distributed data were compared by using the unpaired t-test. Categorical data were presented as frequencies, and the differences in frequencies between the groups of this study were analyzed by using Fisher's exact test and Chi square test. For abnormal distributed data between patients with RIF, Mann Whitney U test was used to compare PRs between them and. P value < 0.05 was considered significant.

RESULTS

Table 1 represents the comparison between A and B which includes patients' data (demographic, stimulation cycles and their OPU-ICSI-ET data). No significant difference in demographic data was found between them, but the number of repeated implantation failure (RIF) was significantly higher in A ($P= 0.027$) than B. Also, comparisons of follicles number by U/S after stimulation cycle and before OPU showed that significant differences were noticed in number of follicles with size of (15-17) mm in B and $P= 0.022$. A high number of oocytes retrieved was noticed in B (12.41+5.82) than A (10.57+6.02), while the number of abnormal oocytes retrieved from patients of A were more (1.25+1.75) than those retrieved from patients of B (1.15+1.59), but not significant. The only significant difference was found to be much higher in number of mature retrieved oocytes (MII) and number of transferred embryos in patients of B than the A, the $p=$ (0.004 for MII oocytes and 0.013 for number of transferred embryos). All patients had different causes of infertility and they were compared between both groups as displayed in figure 1 and $P= 0.702$ which showed no statistical differences. There were 36 pregnant women in A and only 30 women became pregnant in B ($P= 0.724$). A comparison between young women in both groups showed no significant difference as $P= 0.640$ (25 pregnant women from 41 in B and 18 pregnant from women from 34 in A), while a highly significant difference was found in pregnancy rates in women with advanced age (18 pregnant from 33 women in A and only 5 pregnant women from 20 in B), $P=$ was 0.048. Table 2 illustrated a comparison of PRs for all patients to evaluate number of failed ICSI trials and the pregnancy outcomes in both groups. Group A consisted of 67 patients, 36 of them became pregnant, 15 of them had at least one failed ICSI trial (the range number of failed ICSI trials in A was between no previous trial to 9 failed trials), while group B included 61 patients, 30 of them became pregnant and only 5 patients who had at least two failed ICSI trial became pregnant (the range number of failed ICSI trials in B was between no previous trial to 7 failed trials), this comparison showed that there was a highly significant increase in PRs in those patients with previous failed ICSI trials or what is known as patients with RIF after using EG ($P= 0.038$).

Table 1 Demographic data, Stimulation Cycles data, OPU-ICSI-ET data

Parameter	Group A Mean±SD N=67	Group B Mean±SD N=61	P value
Age (year)	33.79+5.67	32.0+5.51	0.072
Duration of infertility (year)	7.87+4.27	7.38+3.62	0.485
RIF (trials)	1.01+1.8	0.41+1.22	0.027*
FSH (mIU/mL)	5.66+1.71	5.56+1.61	0.737
LH (mIU/mL)	3.83+1.43	3.89+1.51	0.816
PRL (ng/mL)	17.92+7.39	19.27+6.73	0.282
stimulation protocol	Agonist	40	39
	Antagonist	27	22
Number of stimulated follicles under U/S	10.9+6.29	12.64+6.15	0.115
Number of follicles with size> 17.5 mm	1.82+1.06	2.15+1.01	0.077
Endometrium thickness (mm) before HCG injection	8.2+0.85	8.28+0.76	0.550
Oocytes retrieved	10.57+6.02	12.41+5.82	0.081
Mature oocyte in MII	7.06+4.21	8.15+3.54	0.115
Endometrium thickness at embryo transfer day	10.97+1.14	10.96+1.06	0.949
Number of transferred embryos	3.45+0.68	3.7+0.46	0.013*

N=number of patients, RIF= repeated implantation failure, N=number of patients, FSH=follicle stimulation hormone, LH= luteinizing hormone, E2=estradiol, PRL=prolactin, HCG= human chorionic hormone, mm=millimeter, U/S=ultrasound, MI=oocyte in metaphase one, MII= oocyte in metaphase two

Table 2: Comparison of number of failed in vitro fertilization between of group A and group B by Mann Whitney U test

Parameter	Group A Median (Range) N=36	Group B Median (Range) N=30	P value
Positive pregnancy	0 (0-9)	0 (0-7)	0.038*
Negative pregnancy	0 (0-4)	0 (0-3)	0.246
Total No.	67	61	

N=number of patients

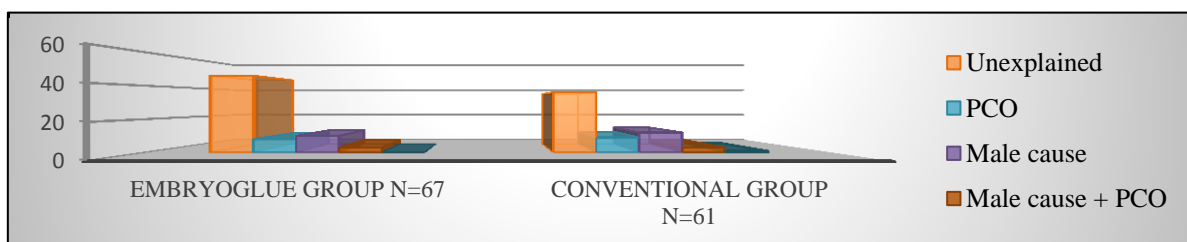


Fig. 1. causes of Infertility in this study

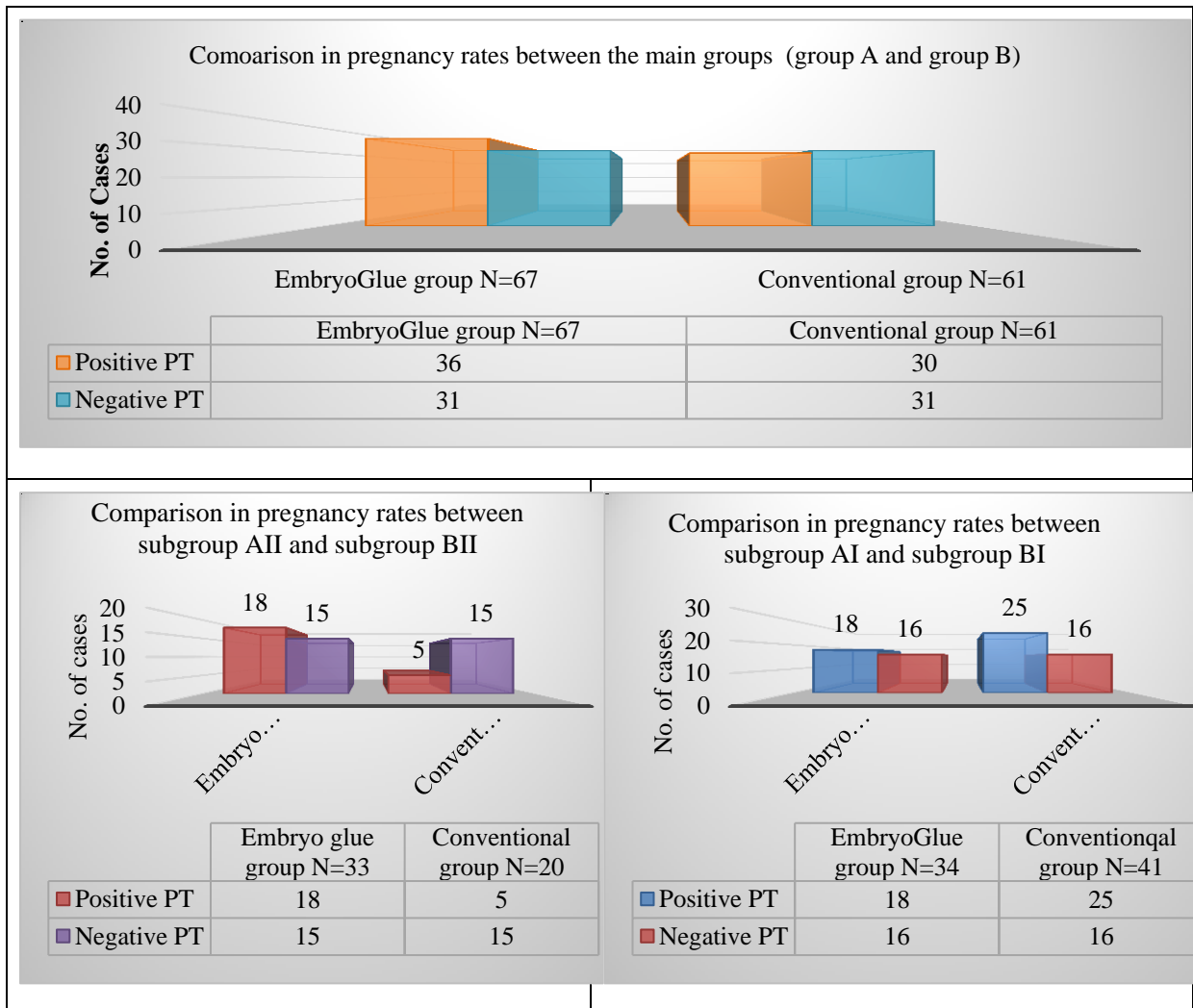


Fig. 2. Comparisons in pregnancy rates between main groups and subgroups

Discussion

Despite numerous improvements in infertility treatments and technologies, implantation is still representing the ‘black box’ of ART. IF may be due to embryonic or maternal factors which affect synchronization of embryo- uterine dialogue, but maternal factors are considered the most difficult ones to be diagnosed and inadequate ER is the major responsible cause for approximately two-thirds of IF, while embryonic factors are responsible for only one-third of these failures. This percentage could be minimized when embryos with good quality are transferred to the uterus ^(4, 9, 10). The comparisons showed no significant increase in PRs neither in all patients nor in the young, whereas in old patients the results were different. Although there was no significant difference in duration of infertility (the range was (2-19) years in A and (2-16) years in B, the PRs significantly increased in subgroup AII (figure 2) when 18 patients became pregnant from 33 patients versus 5 patients became pregnant from 20 patients in the conventional subgroup, $P= 0.048$. Only one study mentioned the duration of infertility as one of the patients' characteristics data and compared PRs. Their results were in

agreement with our results as they found that using EG significantly increased PRs in women ≥ 35 years and the mean duration of infertility in their study was 6.9 years⁽¹¹⁾.

According to clinical guidelines of National Collaborating Centre for Women's and Children's Health (NICE) revealed that 16 studies showed that there is a significant decrease in PRs when women is ≥ 35 years, but these data do not suggest any lower age limit for infertility treatment and ARTs⁽¹²⁾. The idea that a good quality embryo can be implanted anywhere comes from reports showing ectopic pregnancies in hysterectomized women. However, it is known that age affects ER so the embryo-uterine dialogue will not be perfect which lead to IF^(13, 14). In the guidelines mentioned above, another 13 studies demonstrated that PRs decrease with increased duration of infertility. According to Swift et al. the increase in duration of infertility (the range of infertility duration in their study was from 1-12 years) is associated with a significant decrease in success of pregnancy and live birth. Moreover 9 studies demonstrated that this success is significantly decreased in couples with primary type infertility^(12, 15). Studies that follow up PRs after continual ICSI attempts showed that the possibility of pregnancy per cycle tends to be stabilized in the first three attempts, and then it declines afterwards. Some specialists considered that after each failed trial, the PRs in subsequent trial decrease by as much as 57%, but the most remarkable decrease is noticed after the third trial^(16, 17). The stimulation data of all patients who entered COH were compared (table1). The significant differences between A and B were in number of stimulated follicles that had size of (15-17) mm which were significantly more in B than A.

However, PRs of all patients were higher in A(53.7%) than in B(49.1%), but not significant (figure 2) and this result agreed with Fancsovits *et al.* who used EG in ET for 290 patients and found 3% higher PRs in EG group (in their study the number of stimulated follicles between 15-17 mm was also significantly higher in the conventional group than EG group), though the difference was not significant only in old women^(18, 19) as in this study. One of the elements that increase the embryo activity during implantation after ET and increase PRs is the culture medium that is used for ET, especially the component that would increase the energy of the embryo. So, supplementation of proteins as a source of energy for embryo development and activity with vitamins and minerals has traditionally been performed. One of those energy sources is recombinant human albumin(rHA) that has been added to ET medium such as in EG medium^(20, 21, 22). Unique important optimization in the field of manufacture a good human ET medium is adding macromolecule such as HA which interacts in autocrine and paracrine manner and it is involved in embryo development, migration, adhesion, proliferation, differentiation of cells during implantation^(20, 21). In 2003, evidence had been given by Martin *et al.* indicated changes in HA distribution in mouse uterus during WOI suggesting that it may have an important role in the process of endometrial decidualization and embryo implantation⁽²³⁾. According to Babayan *et al.*, the production of HA reduces in follicular fluid women with IF or with an endocrine disorder, but women with successful implantation following ET have higher levels of HA in their follicular fluid⁽²⁴⁾. The reason of adding high concentrations of HA to ET medium is that several studies have shown that HA can modulate itself and regulates many biological events as inflammation, cellular migration, angiogenesis, tissue organization and embryonic development and the concentration of this macromolecule increases in the fallopian tubes and the uterus during implantation.

According to recent studies that focus on studying HA effect during ET, it has been found that the presence of high concentration of HA in ET medium has several advantages for ET step in IVF. It facilitates the formation of a viscid bridge between the embryo and the uterine

lining, prevents embryo ejection, supports embryo implantation and enhances PRs, that's why HA is now known as an implantation enhancing-molecule^(20, 22,25,26). According to Cochrane update review in 2014 that comprises analysis of 17 randomized controlled trials for 3898 patients from different ICSI centers' using ET medium with adherent compounds such as EG has evidence with moderate-quality of improved PRs and live-birth rates. This medium is considered to have a great value especially for patients with RIF, while in Cochrane previous review in 2010 for 3698 participants only improvements have been made in PRs with no improvements in live birth rates, however, not all centers gave their selection criteria⁽²⁷⁾. The study's results agreed with the update of Cochrane review in 2014 of using EG in ET has beneficial effect in patients with RIF and PRs were significantly higher in EG group as mentioned in table 2. According to Simon *et al.* the addition of HA and rHA to ET medium improves apposition and attachment of embryos after ET which are the key steps in implantation and this medium has a viscid-thick texture which promotes easy handling, facilitating embryos loading, and by virtue of apposition. It prevents expulsion of embryos from uterine cavity post ET and increases PRs in RIF patients from 10-32%⁽²⁸⁾.

In this study, using EG eased embryos loading in ET catheter and minimized ET time, no embryo expulsion from uterine cavity post ET occurred in this study and its agreed with the study above that using EG in ET increases PRs in patients with RIF as noticed in table 2. The increase was from 49.1% to 53.7% after using EG in RIF. Regardless of whether couples who registered in ICSI cycle are young or not, patients of most ICSI centers in Middle East especially Muslim people usually request to transfer most of their embryos, many infertility centers transfer at least four embryos to increase PRs, or sometimes patients refuse to pay money for freezing their extra embryos and of course lack of medical insurance coverage makes it difficult for them to afford repeating these cycles. According to Saudi Arabia guidelines of good practice in these centers, number of transferred embryos varies from patient to patient depending on doctor's decision in order to increase implantation and PRs with the caution of increasing chances of abortion^(29,30,31,32).

Number of transferred embryos were significantly more in B than A (table 1). All transferred embryos in all groups had grade one assessment, but no more than four embryos were transferred in all groups of this study. Although pregnant women in A included women of different age were more, but not significantly, the PRs in old women in EG group were significantly higher (figure 2). In all studies^(19,20,33), the number of transferred embryos was equal between the EG and the conventional groups^(19,20,33). In this study, all patients were informed with the risk of multiple pregnancies but they request high order multiple pregnancy (HOMP) in order to deliver more than one child in the same pregnancy and to prevent paying extra money for freezing their extra embryos, but the decision of number of transferred embryos (the range of number of transferred embryos in this study was between 1-4) was made by doctor, embryologist and the couples. In contrast to the results of this study, a study in 2003 showed that there was improvement in PRs with HA enriched transfer medium, but the difference was not significant may due to the small size of the sample (only eighty patients who were < 37 years) with removing of rHA from transfer medium and no criteria for patients' selection may affect the results⁽²⁸⁾. In 2006 Loutradi *et al.* showed no improvements in PRs after using EG in 402 cycles with no specific criteria in patients' selection (uterine abnormalities, polyp and endometriosis were included as causes of infertility in those patients). In 2007 a prospective randomized study for 279 patients with transferring only one blastocyst on day 5 after ICSI and all women aged <37 years, but no other criteria for selection, PRs were close^(34,35). In 2016 Chun *et al.* demonstrated that using EG (they performed a blind ET without using U/S)

for 82 patients with no specific criteria for inclusion and exclusion of their patients and there was not any assessment for uterine endometrium status such as leiomyoma, polyps, and other uterine abnormalities didn't appear to have any significant effect on PRs in patients with RIF⁽³³⁾.

When patients with RIF were compared in this study (table 2), PRs were significantly higher in EG group, this result is in agreement with few other studies such as a study done by Urman *et al.*, who demonstrated that the use of EG increased PRs, not only in women with RIF but in general in all age groups especially the study population was big. It included 1282 patients with RIF and all of these patients were registered according to specific selection criteria so they were eligible to ET procedure (no anatomical abnormalities in the uterus), PRs increased after using EG in old women, in women with RIF, and in women with poor-quality embryos (grade two and grade three); however, the difference in PRs did not reach statistical significance in young women⁽¹¹⁾, but their study included some patients with endometriosis as a cause of infertility, and did not mention the stage or percentage of endometriosis in each group which could be a cause of IF in some patients. Results of this study are in agreement with a study for Friedler *et al.* for 101 patients with specific selection criteria such as excluding all patients who had an excessive BMI of >29 kg/m², uterine malformation, evidence of low ovarian response in previous treatment cycles with less than four oocytes retrieved, elevated baseline (day 3) FSH (>12 IU/L), the patients' selection was with more than four RIF, the PRs in EG group were significantly higher than the other group⁽²²⁾. Another large randomized trial was in 2006 for 815 patients who were suitable for ET with no uterine abnormalities showed improvements in PRs⁽³⁶⁾. In 2009 a study by Sifer C *et al.* with the same selection criteria of this study stated the same results and they recommended using EG in ET for women over 35 years or who had RIF⁽³⁷⁾.

CONCLUSION

Usually, PRs for women ≥ 35 is low and not encouraging, but this study found that using EG increases PRs and makes them double in old women than the usual results without using EG and it is possible to conclude that EG has a positive effect on PRs in cases with RIF even if the age of women is high and increases PRs.

Ethical approval

This study was approved by the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University.

Conflict of interest

No financial conflict of interest existed with any commercial entity whose products are described, reviewed, evaluated and compared in this study.

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