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The advantages of a group embryo culture approach for women of advanced age: A prospective study from a Tunisian reproductive center

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ABSTRACT

Objective: To evaluate the impact of a group embryo culture approach for women of advanced age.**Methods:** In this prospective study, two embryo culturing strategies—group and individual—were compared in women who were older (≥ 35 years) and younger (< 35 years). Data was collected from 450 embryos over 291 intracytoplasmic sperm injection (ICSI) cycles performed on 291 patients. A grouped embryo culture group and an individual embryo culture group were generated by selecting the zygotes into two groups based on the type of culture that was used. The main outcomes compared between the groups were the pregnancy and the live birth rates.**Results:** The pregnancy, and live births rates were significantly higher when group culture was carried out in an advanced-aged women group (41.5% *vs.* 25.0%, $P=0.04$; 14.3% *vs.* 6.6%, $P=0.04$; respectively). Similarly, the cleavage rate was better in the same group (98.6% *vs.* 91.2%, $P=0.001$). However, in the group of young women, there were not significant differences in these rates.**Conclusions:** Group embryo culturing seems to be a promising strategy to improve embryo development and live births for patients over 35 years of age.**KEYWORDS:** Group embryo-culture; Individual embryo-culture; Embryo quality; Human IVF; Maternal advanced-age

1. Introduction

Intracytoplasmic sperm injection (ICSI) is the most widely used assisted reproductive technology technique. Despite efforts to increase its efficiency, pregnancy and live birth rates continue to fall short of expectations, especially for older women[1]. Besides aneuploidy, age-dependent impacts on embryo competence may also result from other embryonic variables. Some of the adverse

effects could be explained by differences in the euploid embryos' metabolism, gene expression, and epigenetic status[2].

Up to now, there is no agreement, regarding the strategies that should be used to enhance ICSI results for advanced maternal age. The only way to detect chromosomal abnormalities is through pre-implantation genetic testing for aneuploidies (PGT-A); however, few *in-vitro* fertilization (IVF) centers use this outdated procedure[3]. This has most likely, in some way, taken the focus off of other age-related issues that could be connected—independent of ploidy—to a fall in women's fertility. Nevertheless, the most important and limited

Key Points

Question: Is the group embryo culture approach beneficial for women of advanced age?**Findings:** This prospective comparative study included 291 intracytoplasmic sperm injection (ICSI) cycles. A grouped embryo culture group and an individual embryo culture group were compared and found that in an advanced-aged women group, the pregnancy, and live births rates were significantly higher in the embryo culture group.**Meaning:** Group embryo culturing seems to be a promising strategy to improve embryo development and live births for older women.✉ To whom correspondence may be addressed. E-mail: olfa.bahri@fmt.utm.tn

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step for ICSI is the embryonic culture; in fact, to improve embryonic development, it is important to create a suitable environment closest to the embryo's natural state[4]. However, embryonic culture can be influenced by different parameters, such as incubators and laboratory equipment used, the composition of culture media, methods and duration of culture, and the addition of nutrients or growth factors to the culture environment[5].

Various approaches to enhance embryo culture have been investigated in the past few years, including individual and group culture. Traditionally, every embryo is cultivated in a different drop of media in an individual culture. While each stage of development may be tracked separately, embryos can only alter their surroundings through autocrine signaling. Since these factors are known to have a significant impact on the quality of the embryo and its endometrial implantation, they cannot benefit from the growth factors provided by the nearby ones[6,7]. A grouped culture consists of a pooled culture of embryos in small groups within the same drop of culture medium. This alternative to conventional individual culture allows the exchange of signals and communication between embryos and then improves the quality of the embryos by providing a suitable environment of metabolites and nutrients[6,8]. Some authors showed no clinical benefit of group culture compared to individual culture[9]. Others thought that group culture has negative effects on embryonic development since it may enable substances from the metabolism of the embryos to accumulate to an inappropriate concentration[10,11]. The objective of this study was to evaluate the two embryo culture techniques—single culture and group culture—in order to determine which produces the highest embryo quality and to know if this approach is more efficient for advanced-age women.

2. Methods

2.1. Study design and participants

This prospective study was conducted over 13 months by our IVF Unit. It included all infertile couples for whom ICSI cycles were performed during this period. The inclusion criteria for participants were a female partner aged less than 42 years, couples monitored at our unit and at least two fertilized oocytes were detected on Day 1 after ICSI. The exclusion criteria were cycles in which fewer than 2 fertilized oocytes were observed on Day 1.

2.2. Procedures

2.2.1. Ovarian stimulation

Two types of protocols were used: i) a long-agonist protocol for female less than 40 years old with follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E_2) levels less than 10 UI/L, 7 UI/L, and 50 pg/mL, respectively, at day 3, and

five to seven antral follicles uniformly distributed in each ovary by a transvaginal ultrasound; ii) a short agonist or antagonist protocol for poor responders (aged over 40 years, with poor hormonal levels and less than 5 antral follicles in both ovaries).

Ovulation and adjustment of doses of gonadotrophins were monitored by ultrasound and E_2 level. When satisfactory follicular maturity is reached, the final oocyte maturation was triggered by an injection of 5000 to 10000 units of human chorionic gonadotrophin (HCG) (Ovitrelle[®], Merck Serono, Tunisia). Transvaginal ultrasound-guided aspiration of follicles was then performed 36 h after HCG administration.

2.2.2. ICSI and embryo culture

Cumulus cells and corona around oocytes were removed by gentle pipetting following a short enzymatic digestion using hyaluronidase (10X, Vitrolife). A micromanipulation system (Narishige) and an inverted microscope type Nikon Eclipse TE 300 were used for microinjection. Oocytes were then placed in a culture medium type G1 (Vitrolife) and incubated at 37°C under 5% CO_2 . Fertilization, checked 18 to 22 h after microinjection, was confirmed by the detection of two pronuclei and the expulsion of the second polar body.

The type of culture used was chosen randomly according to the day of puncture; zygotes obtained on Monday, Wednesday, or Friday were cultured together in a 30 μ L medium G1 (Vitrolife[®]) droplet: a grouped embryo culture group. Those, obtained on Tuesday, Thursday, or Saturday, were cultured individually in a single drop of culture medium: the individual embryo culture group.

The embryonic culture was performed in the same drops until Day 3. A transfer to a second type of culture medium, the G2 (G-2TM PLUS, Vitrolife[®]), was performed on Day 3, and embryos were cultured as described previously until Day 5.

2.2.3. Outcome measures

The embryo quality was evaluated on Day 2 or Day 3 after ICSI, as recommended by the BLEFCO (Biologists at the Fertilisation and Egg Cryopreservation Research Laboratories) French classification. It was performed by assessment of the number and regularity of blastomers and the presence or absence of cytoplasmic fragments. Top embryo transfers were then carried out using a Frydman catheter (CDD, France). All women were supplemented with intravaginal progesterone (Utrogestan, International Besins, France) twice a day (400 mg/day) and with injected Progesterone[®] every three days.

Serum β -HCG level was performed 15 days after the transfer. Clinical pregnancy was confirmed by the presence of a gestational sac with fetal heart activity 4 weeks later. Pregnancies were followed up until delivery in the obstetric department of the hospital. The findings were presented as either ≥ 35 years old or < 35 years old for women.

2.3. Statistical analysis

The statistical analysis was performed using SPSS Version 21.0 software. The data were normally distributed, so the Student's *t*-test was used to compare averages (mean $\pm$ SD), and the *Chi*-squared test was used to compare qualitative variables (%). The difference was considered significant when $P<0.05$.

2.4. Ethics statement

This study was approved by the Institutional Review Board (ethics approval number: 13/24) and was undertaken with appropriate informed consent of participants.

3. Results

3.1. Demographic, clinical, biological and ovarian stimulation parameters

This study included 291 ICSI cycles performed in 291 patients and participants' average age was 34.1 years. Patients' demographics and

cycle performance are shown in Table 1. Age, the woman's hormonal profile, the semen parameters for partners, and the stimulation procedures employed were all comparable between the two groups, which were formed based on the culture method (Table 1). The number of oocytes collected and the oocyte maturity rate did not differ statistically between the two groups.

3.2. ICSI outcomes

The ICSI outcomes for the two groups are shown on Table 2; the individual embryo culture group had a decreased fertilization rate (69.9% *vs.* 73.1%). When grouped embryo culture was used, the cleavage rate and the mean number of top embryos were the only two variables that were substantially higher (96.1% *vs.* 91.8%, $P=0.01$, and 1.2 *vs.* 0.9, $P=0.009$, respectively). We observed that, when maternal age was taken into account, there was no significant difference between the grouped embryo culture group and the individual embryo culture group when patients were young. However, in patients over 35 years, the grouped embryo culture group surpassed the individual embryo culture group in terms of both pregnancy rate ($P=0.04$) and live birth rate ($P=0.04$) (Table 3).

Table 1. Participants' demographics and cycle performance.

Parameters	All group		Advanced age women group	
	Group embryo culture (<i>n</i> =140)	Single embryo culture (<i>n</i> =151)	Group embryo culture (<i>n</i> = 68)	Single embryo culture (<i>n</i> =81)
Age, years				
Female	33.7 $\pm$ 4.2	34.5 $\pm$ 4.2	37.5 $\pm$ 4.2	37.4 $\pm$ 4.2
Male	39.6 $\pm$ 5.7	40.8 $\pm$ 5.7	42.4 $\pm$ 6.2	41.9 $\pm$ 5.9
Cause of infertility, %				
Female	31.1	33.2	35.5	32.1
Male	28.3	29.8	24.5	30.8
Mixed	19.5	21.9	16.8	23.4
Unexplained	21.1	15.1	22.9	13.6
Hormonal levels				
FSH, UI/L	7.3 $\pm$ 2.7	7.3 $\pm$ 2.7	8.3 $\pm$ 3.1	8.4 $\pm$ 3.1
LH, UI/L	4.8 $\pm$ 2.7	5.0 $\pm$ 2.7	4.8 $\pm$ 2.7	4.8 $\pm$ 2.7
Rank of attempt, <i>n</i>				
1st	85	93	34	44
2nd	28	35	15	17
3rd or more	27	22	19	20
Stimulation protocols,%				
Long agonist	55.7	47.0	50.0	39.5
Short agonist	7.1	11.2	11.7	16.3
Antagonist cycle	34.2	38.4	36.7	41.9
Others protocols	2.8	3.3	1.4	2.1
Gonadotrophine doses, UI	2522.0 $\pm$ 816.0	2488.0 $\pm$ 816.0	2783.2 $\pm$ 817.0	2617.4 $\pm$ 817.0
Endometrial thickness, mm	10.1 $\pm$ 2.3	9.7 $\pm$ 2.4	9.7 $\pm$ 2.3	9.6 $\pm$ 2.3
Seminal parameters				
Sperm concentration, M/mL	43.9 $\pm$ 57.7	49.9 $\pm$ 57.8	49.3 $\pm$ 57.0	43.4 $\pm$ 55.2
Progressive mobility, %	23.4 $\pm$ 17.0	25.6 $\pm$ 17.6	23.8 $\pm$ 17.0	23.1 $\pm$ 17.0
Cycle performance				
Number of cumulus-oocyte complex (COC) collected	7.3 $\pm$ 4.4	7.3 $\pm$ 4.4	6.1 $\pm$ 4.1	6.6 $\pm$ 4.1
Oocyte maturity rate, %	73.5 $\pm$ 18.0	74.1 $\pm$ 18.0	75.8 $\pm$ 18.0	74.5 $\pm$ 18.0

Note: 1) In the all group, there is no significance between the group embryo culture and the single embryo culture, $P>0.05$; 2) In the advanced age women group, there is no significance between the group embryo culture and the single embryo culture, $P>0.05$.

Table 2. Evaluation of intracytoplasmic sperm injection (ICSI) outcomes according to type of embryos culture.

Parameters	Group embryo culture (n=140)	Single embryo culture (n=151)	P-value
Fertilization rate, %	73.1±19.0	69.9±19.0	ns
Cleavage rate, %	96.1±11.9	91.8±17.0	0.01
Mean number of "Top" embryos/cycle	1.2±1.1	0.9±1.1	0.009
Mean number of embryos transferred	2.3±0.5	2.3±0.5	ns
Pregnancy rate/cycle, %	35.00	29.13	ns
Pregnancy rate/transfer, %	36.56	29.72	ns
Pregnancy cumulative rate, %	36.42	29.13	ns
Implantation rate, %	22.14	18.02	ns
Rate of spontaneous miscarriage, %	18.36	15.90	ns
Rate of late miscarriage, %	8.16	9.09	ns
Rate of live births, %	14.87	12.61	ns

ns: non-significance, $P>0.05$.

Table 3. Evaluation of intracytoplasmic sperm injection (ICSI) outcomes in the two groups according to female age.

Parameters	Young patients (<35 years) (n=142)		P-value	Advanced maternal age (≥35 years) (n=149)		P-value
	Single culture (n=70)	Group culture (n=72)		Single culture (n=81)	Group culture (n=68)	
Mean number of embryos obtained	3.12±1.11	3.38±1.20	ns	2.81±0.70	3.07±1.04	ns
Cleavage rate, %	92.5	93.5	ns	91.2	98.6	0.001
Rate of "Top" embryos, %	28.0	32.0	ns	34.7	40.6	ns
Blastulation rate, %	15.0	20.6	ns	26.7	23.9	ns
Pregnancy rate/transfer, %	35.2	29.4	ns	25.0	41.5	0.04
Implantation rate, %	22.8	19.5	ns	13.5	22.5	0.05
Live birth rate, %	10.0	16.8	ns	6.6	14.3	0.04

ns: non-significance, $P>0.05$.

4. Discussion

Our research shows that the grouped embryo culture approach increases the growth and the quality of the embryo and the live birth rate, particularly for women with advanced age. Pregnancy and birth rates did not change significantly between the two embryo culture methods for the group of young women (less than 35 years old).

Because of its apparent advantages, grouped embryo culture has been used in several labs worldwide. The presence of "survival factors" generated by the embryo in the culture media is known to promote mitosis[8]. These factors are released in the early phases of cleavage (the two-cell stage); they protect embryo from apoptosis and allow it to continue growing[12]. Different studies conducted *in vitro* supported this hypothesis; it was shown that inhibition of the transcriptional pathway of guanosine triphosphate (GTP)-binding protein by injecting a mutant plasmid into zygotes in culture prevents their evolution to the two-cell stage[12]. Grouped embryo culture has an advantage over an individual culture because embryonic growth factors can function in both an autocrine and paracrine manner[12,13]. Additionally, it was demonstrated that the implantation rate for group culture can be affected by cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), leukemia inhibitor factor (LIF), growth factors [insulin growth factor (IGF I and II), transforming growth factor (TGF α and β 1)], mitogenic factors [epidermal growth factor (EGF)], and platelet-activating factor (PAF)[14]. According to a review of the literature by Babayev

et al, there are genetic, epigenetic, transcriptomic, proteomic, and metabolomic changes in the cumulus cells and follicular fluid as well as age-related changes in the local oocyte microenvironment of antral follicles[15]. To better define potential mechanisms for the effect of maternal aging on fertility and potential biomarkers for evaluating the quality of blastocysts and improving pregnancy outcome rates in IVF patients, Tedeschi *et al* examined the proteomic profile of blastocoel fluid in women under the age of 37 and women over the age of 37[16]. Using a proteomic technique, they came to the conclusion that the two groups differed in the expression of 109 proteins. The main perturbed networks refer to fertilization and embryo implantation, as well as nucleic acid interactions[16]. It seems reasonable to experiment with adding growth factors to the cultural medium in order to correct these changes. In fact, Chu *et al* showed that adding GM-CSF at a dose of 0.6 ng/mL considerably increased the rate of blastocyte production and cleavage in patients older than 38 years[17]. This cultural strategy seems to be an excellent substitute method for enhancing IVF outcomes in older women. However, few researches evaluated pregnancy and live birth rates following grouped embryo culture, and the majority of them neglected the age of the women[18,19]. Almagor *et al*'s investigation revealed a significant increase in pregnancy rates, with grouped embryo culture than individual embryo culture showing 45% and 25% pregnancy rates, respectively[20]. Grouped embryo culture was associated with significantly higher rates of pregnancy and births than the individual embryo culture group; cumulative pregnancy rates were higher in the

grouped embryo culture group, as reported by Restelli *et al*[21]. Ebner *et al*, however, found no difference between the grouped embryo culture and individual embryo culture groups[22]. In contrast, some researchers suggested that group culture may deplete the nutrients needed for embryonic growth and also overload this medium with free radicals and ammonia, among other embryonic metabolites[11].

In addition to the transfer day, the quality of the embryos during group culture has been shown to be significantly influenced by embryo density[23,24].

The study has some limitations. The results only took into account fresh cycles, and we did not compare the cumulative pregnancy and birth rates between the two groups.

In conclusion, grouped embryo culture seems to be a good strategy for enhancing IVF outcomes in women with advanced age. More research is required to support the secretome's role in embryonic development and to validate its advantages.

Conflict of interest statement

The authors declare no conflicts of interest.

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Authors' contributions

Khadija Kacem Berjeb and Sana Chtourou conceived and designed the study. Mounir Ben Mefteh and Marouen Braham conducted the oocytes pick up; Linda Debbabi, Sana Chtourou and Khadija Kacem Berjeb contributed to the interpretation of data; Khadija Kacem Berjeb, Sana Chtourou, Maha Bouyahya and Neila Ben Othman drafted the manuscript; Anis Fadhlou, Fethi Zhioua, Nozha Chakroun and Olfa Bahri provided expertise throughout the study and contributed to the critical revision of the manuscript.

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