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ASSISTED REPRODUCTION TECHNOLOGIES

The strategy of group embryo culture based on pronuclear pattern on blastocyst development: a two center analysis

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Abstract
Purpose To compare two embryo grouping strategies.
Methods Retrospective time-course analysis in two different centres. Two culture protocols were used at the zygote stage: "Random Group" in which zygotes were randomly grouped and "Definite Group" in which zygotes were grouped based on pronuclear pattern. Embryo culture was extended to blastocyst stage. Primary and secondary outcomes were respectively the blastulation rate and the cumulative clinical pregnancy and implantation rates.
Result(s) A similar blastulation rate [42 and 41 % day (5+6) blastocysts] was obtained in the two groups. Conversely, after adjusting for baseline and cycle variables, cumulative pregnancy [adjusted Odds Ratio=2.10 (95%CI: 1.08–4.07)] and implantation [adjusted Odds Ratio=1.78 (95%CI: 1.06–2.97)] rates were significantly higher in the "Random Group" compared to the "Definite Group".
Conclusion(s) Two strategies of group culture gave similar results in terms of blastulation rate but the random grouping of zygotes improves pregnancy and implantation rates in IVF-cycles.

Capsule Embryo grouping strategy based on the random selection versus pronuclear pattern increases clinical pregnancy and implantation rates but not blastulation rate in fresh ART cycles.

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Keywords Blastocyst · Clinical pregnancy rate · Embryo grouping · Pronuclear morphology · Pronuclear pattern

Introduction

Despite the several breakthroughs achieved in Assisted Reproduction Technology (ART), the best culture conditions affecting embryo quality and success rates are still to be completely elucidated. Blastocyst development has been improved both in animals and in humans with the introduction of more complex tissue culture media strategies leading to improvements in blastocyst formation rate and quality [1, 2]. Over the years, these strategies have included the modification of aminoacid and energy substrate composition, the use of sequential culture media in order to take into account the metabolic needs of the embryo and the use of reduced volumes of culture medium to minimize the dilution of beneficial factors. More recently, more attention has being directed toward the physical requirements of preimplantation embryos including factors such as space, time, mechanical interactions, gradient diffusions and cell movement [3].

In this context, in several animal models, increased embryo density has been suggested to improve development, potentially through secretion of autocrine/paracrine factors [4]. Embryos can communicate through paracrine biomolecules that have been suggested to affect embryo homeostasis and growth. Thus, in litter-bearing species, the grouped embryo culture is a standard procedure. Conversely, this group effect or spacing effect in non-litter-bearing species such as the human has to be fully defined [5]. Many ART laboratories culture groups of embryos in small volumes to obtain this perceived benefit of concentrating growth-promoting autocrine/paracrine biomolecules. However, results from the studies that have investigated whether human embryos may take advantage from group culture remain controversial [6–9].

On the other hand, conclusions derived from the various studies are poorly comparable as conditions of embryo culture in terms of stage and quality of grouped embryos varied considerably. One must consider that embryos of different quality or stage of development may have either beneficial or detrimental influence on companion embryos in the same culture environment. Moreover, culture volumes, grouping periods, embryo density and grouping times were very different among the various studies.

In 2010, Ebner et al. published results from a prospective randomized comparison of single embryo culture and group culture on blastulation, implantation and pregnancy rates. Embryos were randomly grouped at the zygote stage and maintained in groups until the blastocyst stage. Group culture was shown to be superior in terms of compaction and blastulation rates and blastocyst quality as compared to individual culture. A tendency toward a higher cumulative clinical pregnancy and live birth rates was also observed [10].

As a further step to gain insight into the potential advantages of the embryo group culture, we have used a similar grouping approach from the zygote to the blastocyst stage to compare for the first time the grouping strategy based on the pronuclear pattern versus the random selection. This retrospective time-course analysis has been performed based on the results of IVF outcomes from two Italian ART centres.

Materials and methods

This retrospective time-course study implied the analysis of data from patients who underwent IVF/ICSI cycles at the Infertility Units of the Departments of Obstetrics and Gynecology of the "Fondazione Ospedale Maggiore Policlinico" and of the Scientific Institute San Raffaele between September 2012 and March 2013. Although the two units have no personnel in common, they interact strictly for quality control procedures and scientific purposes. After a common audit to evaluate clinical outcomes organized in July 2012, the lab personnel of the units decided to implement the group embryo culture. The time-course analysis of the outcomes was performed retrospectively and decision to publish the results taken thereafter.

The recruitment was limited to patients with at least six fertilized oocytes. All women undergoing IVF/ICSI cycles routinely provide informed consent for their clinical data and anonymised records to be used for research purposes in general. Local Institutional Review Board approvals for the use of clinical data for research studies were obtained.

Controlled ovarian stimulation was performed according to the standard clinical practice [11]. Either GnRH agonist or GnRH antagonist daily protocol was used for pituitary down-regulation and ovarian stimulation was carried out according to one of the following: a) recombinant FSH (rFSH) alone; b) rFSH combined with recombinant LH (rLH); c) highly purified human menopausal gonadotrophin alone (HP-hMG). Both initial dose and dose adjustments during treatment were chosen on a case-by-case basis according to patients' characteristics and response to gonadotrophins. Triggering of ovulation was performed with HP-human chorionic gonadotrophin (hCG) when one or more follicles had reached a diameter ≥17–18 mm. Serial determinations of serum oestrogen (E_2) and progesterone levels were performed during the treatment. The first (basal) determination was carried out before the beginning of gonadotrophin administration, while the last assessment of E_2 and progesterone levels was obtained on the day of hCG administration. Oocytes were retrieved after about 36 h after hCG administration.

ICSI and conventional IVF were performed in a standard way [11–13]. Cumulus-corona-oocyte complexes were collected, washed and allocated to fresh insemination or ICSI. For ICSI, denudation of the cumulus oophorus was performed by a brief exposure to hyaluronidase solution (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA). A fertilization check for two pronuclei took place16–18 h later. Two culture protocols were used at the zygote stage, namely:

1) "Random Group" (RanG) from September 2012 to November 2012, all regularly fertilized eggs were randomly grouped. After checking for pronuclear formation, randomization into culture drops was performed by splitting the zygotes under a binocular microscope not allowing for identification of pronuclear pattern. Three to four zygotes were grouped in 20 μl drops in a 60 mm Petri dish under mineral oil.
2) "Definite Group" (DefG) from December 2012 to March 2013, all regularly fertilized eggs were grouped based on the pronuclear pattern according to the Istanbul Consensus in which the pronuclear scoring of normal pronuclei includes those that are symmetrical (type 1) and those which are non-symmetrical (type 2). Thus, zygotes were grouped up to four per 20 μl drop [14].

In both groups, allocation to culture drops was made after discarding zygotes showing 0 or 1 nucleolar precursor body per pronucleus.

Embryos were cultured in sequential media (Sage In-Vitro Fertilization, Inc. Trumbull, CT, USA). For the next 2 days of preimplantation development, embryo quality was controlled for number and size of blastomeres, degree of fragmentation, multinucleation. An embryo was considered to be of top quality once it showed a stage-appropriate number of evenly sized blastomeres, complete absence of multinucleation and less than 10 % fragmentation. Strategy for embryo transfer was decided based on embryo evaluation on Day 3. In general, when on day 3, at least 3 top quality embryos or 4 top/good quality embryos were present, embryo culture was extended to undergo Day-5 blastocyst stage. At day 3, embryos were moved into fresh

20 μl drop media. Enrolled patients transferring embryos on day 3 were not included in final data analysis.

Blastocyst evaluation was performed on day 5 of in-vitro culture according to the Istanbul Consensus [14]. Blastocysts were given a score from 1 to 4 based on the degree of expansion and hatching status. The inner cell mass was scored as follows; 1: prominent, easily discernible, with many cells that are compacted and tightly adhered together; 2: easily discernible, with many cells that are loosely grouped together; 3: difficult to discern, with few cells. The trophectoderm was scored as follows; 1: many cells forming a cohesive epithelium; 2: few cells forming a loose epithelium; and 3: very few cells. 'Top-good quality' blastocyst was defined as advanced blastocyst (type 3 full blastocyst and type 4 expanded blastocyst) with an inner cell mass scored 1 or 2 and multicellular trophectoderm (scored 1 or 2).

Embryo transfer was performed on Day 5; embryos from morula to hatching-blastocyst stage were eligible for embryo replacement. Maximum two embryos were transferred per patient. Those that did not reach the blastocyst stage on day 5 and were not transferred were cultured one more day till day 6 and derived blastocysts were vitrified. In patients at high risk for severe ovarian hyperstimulation syndrome, viable embryos were vitrified at blastocyst stage.

The blastulation rate was the primary outcome and was defined as the number of blastocysts (expansion scored 1 to 4)/ number of zygotes. Secondary outcomes were (i) the cumulative clinical pregnancy rate, defined as the number of clinical pregnancies (with positive cardiac pulsations on ultrasound at week 5–6 after oocyte retrieval) resulting from one aspirated cycle including the cycle when fresh embryos were transferred and subsequent vitrified cycles; (ii) the cumulative implantation rate, defined as the number of gestational sacs (with fetal cardiac activity) observed, divided by the total number of embryos transferred in fresh and vitrified cycles.

Data analysis was performed using Statistics Package for Social Sciences version 18.0 (PASW Statistics 18.0, Chicago, Illinois). Continuous variables were tested for normal distribution using the Kolmogorov–Smirnov test and subsequently analyzed using either the Student's *t*-test (normal data distribution) or the Mann–Whitney *U* test (skewed data) to compare two means (normal data distribution) or medians (skewed data distribution) where appropriate. Categorical variables were analyzed using the Chi-square test or Fisher's exact test. A logistic regression model was used to adjust for baseline variables found to differ between the study groups ($p<0.10$) or known to be influencing the outcome. Results are expressed as mean±standard deviation (SD) or median (range) and Odds Ratio (OR) with 95 % confidence interval (95%CI). A p value< 0.05 was considered statistically significant.

On the basis of the study centers' experience, 40 % blastulation rate from fresh inseminated oocytes was considered the reference value. The study was designed to highlight a 20 % relative increase in this value (corresponding to 48 % blastulation rate). A minimum of 600 zygotes per group were required (type 1 and type 2 errors set at 5 % and 20, respectively) and, estimating a mean number of 8 zygotes per IVF cycle, it was calculated that the number of patients to be enrolled was about 80 per group.

Results

A total of 206 patients with at least 6 zygotes were retrospectively evaluated and an ad-hoc data set was created with their records. Ninety-nine and 107 patients belonged to the RanG and DefG, respectively. On day 3 of embryo culture, 19 (19 %) and 18 (17 %) women in the RanG and in the DefG, respectively ($p=0.72$), underwent embryo transfer and therefore were excluded from the data set. Subsequent evaluation was performed on 80 patients in RanG and 89 patients in DefG.

Table 1 gives detailed information on patients' characteristics of the two groups analysed. Baseline characteristics and indications to in vitro fertilization were comparable. Characteristics of the IVF-ICSI cycles are summarized in Table 2. The medical and laboratory staff, the laboratory and the surgical theatre were the same in the two periods, as were culture media and procedures.

Table 1 Baseline characteristics of women belonging to the two groups

Characteristics	"Random" group $n=80$	"Definite" group $n=89$	p
Age (years)	35.7±3.8	35.9±3.7	0.60
BMI (kg/m^2)	21.9±2.6	22.6±3.7	0.16
Duration of infertility (months)	41±34	39±23	0.65
Day 3 serum FSH (IU/ml)	7.0±2.2	6.9±1.8	0.75
Serum AMH (ng/mL)	2.4±1.8	2.5±1.7	0.71
AFC	13±5	13±5	1.00
Previous deliveries	8 (10 %)	13 (15 %)	0.37
Women at the first IVF-ICSI cycle	43 (54 %)	42 (47 %)	0.44
Main indication to IVF-ICSI			0.54
Male factor	27 (34 %)	22 (25 %)	
Female factor	22 (28 %)	27 (30 %)	
Mixed factor	7 (9 %)	6 (7 %)	
Unknown	24 (30 %)	33 (37 %)	
Main female indication to IVF-ICSI			0.21
Endometriosis	12 (15 %)	19 (21 %)	
Tubal factor	6 (8 %)	8 (9 %)	
PCOS / anovulatory	11 (14 %)	6 (7 %)	

BMI body mass index; *AFC* antral follicle count; *PCOS* polycystic ovarian syndrome

Table 2 IVF-ICSI cycle characteristics

Characteristics	"Random" group n=80	"Definite" group n=89	p
Stimulation protocol			0.133
Long protocol	49 (61 %)	46 (52 %)	
GnRH antagonist	24 (30 %)	39 (44 %)	
Others	7 (9 %)	4 (4 %)	
Total dose of administered FSH (IU)	2,399±949	2,297±1,082	0.52
Duration of stimulation (day)	10.1±1.7	10.6±1.9	0.07
Number of oocyte retrieved	11.9±3.9	12.7±3.8	0.18
Number of inseminated oocytes	9.1±2.0	9.2±1.9	0.74
Technique used			0.87
IVF	16 (20 %)	17 (19 %)	
ICSI	54 (68 %)	58 (65 %)	
Both	10 (12 %)	14 (16 %)	
Fertilization rate (%)	83±12	84±12	0.54
Cancelled embryo transfer			1.00
OHSS (embryo freezing)	5 (6 %)	5 (6 %)	
No viable embryos	0 (0 %)	1 (1 %)	
Blastocysts transferred (FC)			0.28
1	39 (52 %)	36 (43 %)	
2	36 (48 %)[a]	47 (57 %)[b]	

Data is reported as mean±SD or number (%), as appropriate

FC fresh cycle

[a] Includes 1 patient who transferred 2 morulae

[b] Includes 7 patients who transferred 2 morulae

The number of zygotes in the RanG was 594; they were co-cultured with a mean number of 3.3±0.5 zygotes per drop. In the DefG, 649 zygotes were co-cultured with a mean number of 2.9±0.9 zygotes per drop (p<0.001). The blastulation rate both on Day 5 and on Day 6 of culture and the percentage of top quality blastocysts were very similar between groups, as reported in Table 3. In the DefG, a statistically significant higher percentage of blastocysts on Day 5 was observed from type 1 zygotes compared to type 2.

The crude odds ratio (cOR) for total blastulation on D5+D6 of zygotes belonging to RanG compared to those belonging to DefG was 0.96 (95%CI: 0.77–1.205) (p=0.73). The adjusted OR (aOR) obtained using a logistic regression model that included age, parity, number of retrieved oocytes, length of stimulation, total dose of administered FSH, insemination technique, presence of male factor (less than 15 million spermatozoa per ml) was 0.87 (95%CI: 0.57–1.32) (p=0.500). Similar ORs were observed when blastulation on Day 5 and blastulation on Day 6 were considered separately (data not shown).

In the RanG and DefG groups, respectively 32 and 31 % of zygotes developed into top quality blastocysts on Day 5 or Day 6. The corresponding cOR and aOR were 0.97 (95%CI: 0.76–1.23) (p=0.789) and 0.91 (95%CI: 0.58–1.43), respectively (p=0.688).

Table 4 reports the results of the secondary outcomes. In the fresh cycle, 5 patients per group did not perform embryo transfer for ovarian hyperstimulation syndrome (OHSS) and cryopreserved all of the available blastocysts (Table 2). In the remaining patients, the clinical pregnancy rate per fresh cycle was 47 % and 32 % in the RanG and DefG, respectively. The cOR and aOR for clinical pregnancy rate in the fresh cycle were 1.85 (95%CI 0.97–3.52) (p=0.062) and 2.17 (95%CI: 1.07–4.41) (p=0.032). Thirty-one and 35 patients underwent a warming cycle with vitrified blastocysts obtaining 11 (36 %) and 12 (34 %) clinical pregnancies in the RanG and DefG, respectively (p=0.909). The cumulative clinical pregnancy rate per patient was 58 % and 44 %, respectively, with a cOR=1.74 (95%CI: 0.94–3.19) (p=0.077) and an aOR=2.10 (95%CI: 1.08–4.07) (p=0.028).

The cumulative implantation rate was 34 % (52/153) and 25 % (45/183) in RanG and DefG, respectively. The corresponding cOR was 1.58 (95%CI: 0.98–2.54) (p=0.059), while the aOR was 1.78 (95%CI: 1.06–2.97); (p=0.028).

Data were analyzed separately for the two involved units and results are reported in Supplemental Table 1.

Discussion

In 2012, the two Italian laboratories involved in the study implemented the embryo group culture based in general on

Table 3 Blastulation rate

Characteristics	"Random" group, n=80	"Definite" group, n=89		
		Zygotes type 1	Zygotes type 2	Total Zygotes
Number of included zygotes	594	231	418	649
Number of blastocysts				
Day 5	212 (36 %)	97 (42 %)[a]	133 (32 %)[a]	230 (35 %)
Day 6	39 (7 %)	9 (4 %)	29 (7 %)	38 (6 %)
Total	251 (42 %)	106 (46 %)	162 (39 %)	268 (41 %)
Number of top quality blastocysts	190 (32 %)	79 (34 %)	124 (30 %)	203 (31 %)

[a] Values with same superscript are significantly different, p=0.01

Table 4 Secondary outcomes

Variable	"Random" group $n=80$	"Definite" group $n=89$	Odds ratio (95%CI); p-value	Adjusted[a] odds ratio (95%CI); p-value
CPR, fresh cycle[b]	35/75 (47 %)	27/84 (32 %)	1.85 (0.97–3.52)	2.17 (1.07–4.41)*
CPR, vitrification cycle	11/31 (36 %)	12/35 (34 %)	1.06 (0.38–2.9)	1.44 (0.39–5.23)
CPR, cumulative	46/80 (58 %)	39/89 (44 %)	1.74 (0.94–3.19)	2.10 (1.08–4.07)*
IR, fresh cycle	41/111 (37 %)	34/130 (26 %)	1.65 (0.96–2.86)	1.81 (0.99–3.32)
IR, vitrification cycle	11/42 (26 %)	11/53 (21 %)	1.36 (0.52–3.52)	1.94 (0.63–5.96)
IR, cumulative	52/153 (34 %)	45/183 (25 %)	1.58 (0.98–2.54)	1.78 (1.06–2.97)*

CPR clinical pregnancy rate per patient; *IR* implantation rate

*$p<0.05$

[a] For: female age, number of oocytes, days of stimulation, total dose of FSH, FIVET/ICSI, parity, male factor

[b] Patients cryopreserving all embryos are excluded

the strategy of group culture by Ebner et al. [10]. This strategy implied grouping at zygote stage, at a density of three-five zygote per drop with grouped embryos left undisturbed in culture for two periods of 48 h (day 1–3 and day 3–5). Studies addressing the comparison between individual and group embryos vary greatly in regard to incubation volume used, density of grouped embryos, different periods of grouping and different embryo stages. These differences may explain the strong controversies related to the results obtained [6–9, 15]. While some authors failed to observe any differences in IVF outcomes in patients having their embryos either cultivated individually or in groups for a limited period [7], others demonstrated a significant increase in blastocyst developmental rate when embryos were grouped in Day 3 based on the quality [9].

We have chosen to base our implementation program of grouping strategy based mostly on the procedures of Ebner et al. considering (i) the randomized nature of the study and (ii) the similarity of their procedures with those already employed in our laboratories for drop volume, strategy for embryo culture and evaluation and embryo transfer [10]. In the study by Ebner et al., the recruitment was limited to patients with at least nine fertilized oocytes while we have chosen to recruit patients with at least six fertilized oocytes [10]. This may explain the increased rates for some outcomes (i.e. blastocyst formation, clinical pregnancy) reported by Ebner et al. as compared to those of our study.

The idea to group zygotes based on pronuclear morphology was aimed to potentially further ameliorate the efficacy of the grouping strategy. Indeed, assessing pronuclear morphology have proven to be useful for the prediction of embryo developmental potential. A better pronuclear morphology on day 1 is known to be associated with blastocyst develoment [16, 17] and, as a matter of fact, our results confirmed that the blastulation rate was significantly higher for type I zygotes grouped than for type 2 zygotes.

However, when the blastulation rate was compared between random grouping and grouping based on pronuclear morphology, unfortunately we could not detect a better blastulation rate for type II zygotes when they were cultured together with type I. Results were identical between the two groups thus tending to exclude the possibility that modifications of the local environment induced by accumulation of good embryos and theoretically reducing the negative impact of detrimental factors could provide a further additive effect on the blastulation rate to the potential effect already related to the grouping strategy. In other words, we cannot conclude that better embryos (type I zygotes) have a positive effect on the poorer (type II zygotes) embryos.

Conversely and surprisingly, clinical pregnancy rate and implantation rate of fresh cycles resulted significantly higher for random grouping compared to grouping based on pronuclear morphology. Explanations for these findings is not easy to unravel. Indeed, despite greater understanding of the biology of the embryo, the dynamic and complex nature of *in vivo* conditions makes it difficult to completely elucidate factors involved in determining developmental competence [5].

Thus, we have envisaged two possible explanations for our results. Firstly, the strategy based on the pronuclear pattern forced us to separate type 1 from type 2 zygotes and therefore to proceed with a reduced mean number of zygotes for drop compared to the random strategy. We can speculate that the density of the zygotes rather than their quality seems to represent a critical factor on developmental potential. It should be considered in this regard that, while a normal pronuclear pattern can well predict embryo developmental arrest [16], it is not so good as an independent marker of implantation potential [18]. In other words, different factors might be more critical to the outcome in terms of pregnancy than pronuclear morphology and we might limit these factors by reducing the number of grouped embryos. Secondly, it can be hypothesized that the procedure to separate zygotes based on morphology took a longer time than the mere grouping of zygotes without any selection: time outside the incubator is well known to be a critical detrimental factor to embryo development. This explanation is however not very plausible as this procedure is routinely performed by expert embryologists in both

laboratories and we cannot recommend to avoid a careful fertilization check as it represents an essential step during IVF.

This study has some limits. Firstly, this is an observational time-course and not a prospective randomized study. Unbalanced presence of confounding factors due to the retrospective nature of the study may explain the differences, therefore results should be considered with precautions.

The non-simultaneous time period of treatment application should not have been influenced in some way by changes in the personnel or in the laboratory conditions. However, we cannot exclude that our results may be confounded by differences in some patient factors. On the other hand, a similar blastulation rate but a reduction in CPR for fresh cycles were indeed observed even separately by the two IVF laboratories involved and this supports the validity of the results obtained. Secondly, the statistical power to detect differences for the secondary outcomes might have been considered not adequate since the sample size has been calculated on the primary outcome. However, results obtained from the secondary outcomes are very straightforward. A strength of the present study in this regard is represented by the consistency of the results obtained even after controlling for several factors including age, number of oocytes retrieved, male factor by multivariate analysis.

Conclusions

In conclusion, our findings indicate that random grouping of zygotes versus based on morphology may benefit clinical pregnancy and implantation rates in fresh cycles. It is clear that additional work and in particular randomised clinical trials need to be performed to confirm these findings and clarify whether an in depth analysis of every single aspect of embryo grouping including embryo density, embryo quality according to the stage and incubation periods would allow a further amelioration of the clinical outcomes.

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