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ASSISTED REPRODUCTION TECHNOLOGIES

Influence of group embryo culture strategies on the blastocyst development and pregnancy outcome

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Abstract
Purpose To compare two different embryo culture methods and to determine whether grouping embryos based on quality following Day 3 improved outcomes.
Methods Two group embryo culture methods were compared in this study. All zygotes were individually cultured from Day 1 to Day 3. On Day 3, embryos were then cultured in group of 2–5 embryos per droplet until Day 5 or 6. The two group culture methods are: **A**, embryos were randomly grouped regardless of embryo quality; **B**, good and poor quality embryos were separately grouped. Blastocyst development rate, blastocyst utilization rate, implantation rate and pregnancy rate were detected.
Results The group culture of Day 3 embryos, in which good or poor quality embryos were separately grouped, significantly promoted blastocyst development (61.2 %, 289/472) and blastocyst utilization rate (55.9 %, 264/472) in comparison with those embryos that were randomly grouped for culture regardless of embryo quality (44 %, 177/402 and 41.5 %, 167/402). There was no significant difference in the implantation rate and pregnancy rate between two group culture methods.
Conclusions Grouping of embryos after Day 3 based on embryo quality may benefit blastocyst formation. This may be due to secretion of beneficial factors by good embryos, or removal of detrimental factors from poor embryos. No impacts on pregnancy or implantation outcomes were observed.

Keywords Blastocyst development · Group culture · Individual culture

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Capsule Our study demonstrates that Day 3 embryos with good and poor quality may be separately grouped for culture to blastocyst stage. Some unknown factors derived from poor quality embryos (such as arrested embryos) may inhibit the surrounding embryo development. The strategy of the combination of individual culture to Day 3 and good and poor quality of embryos be separately grouped for culture to Day 5 or 6 is recommended for clinical use.

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Introduction

Blastocyst culture and transfer have emerged as important components in human IVF. While the culture of embryos to blastocyst stage for subsequent transfer yields high pregnancy rates and minimizes the risk of multiple gestations, the availability of blastocysts is limited in many IVF clinics because of the high rates of attrition in in-vitro embryo culture [1–3]. Sub-optimal culture conditions can compromise many aspects of embryo development [4]. Therefore, the challenge for many scientists has been to optimize culture conditions so that more human embryos can develop into viable blastocysts.

In recent years, blastocyst development has been improved by a number of ways, such as co-culture of embryos with autologous or heterologous adult somatic cells, or established cell lines [5, 6], use of defined, serum-free, sequential culture media [7–10], use of reduced volumes of culture medium [11, 12], and culture of embryos in group [13–16].

It is hypothesized that one reason for benefit of group embryo culture is embryo modification of their microenvironment due to secretion and/or depletion of various factors in the media [17–21]. However, some other factors derived from poor quality embryos may have negative influences on the development of surrounding embryo. Different quality of companion embryos may differentially secrete or utilize

substances into/from the culture medium. Therefore, there may be a benefit of grouping embryos separately for culture according to their quality. It has been reported that in vitro development of solitary cat embryos is improved by culture with excellent-quality heterospecific companions [22, 23].

Recently, Rebollar-Lazaro and Matson (2010) reported that culturing embryos in groups from Day 1 to Day 3 had no impact on pregnancy and implantation rates compared to those embryos cultured individually [24]. This is similar to a previous report that indicated no benefit of group culture of human embryos prior to Day 2 or Day 3 transfer [25]. In addition, in some cases, the group culture of embryos to blastocyst stage is not always practical because not all patients are blastocyst culture candidates. Therefore, the ability to individually track embryos in these non-blastocyst transfer cases is useful for selection purposes.

The objective of this study was to compare two different embryo culture methods and to determine whether grouping embryos based on quality following Day 3 improved outcomes.

Materials and methods

Patients and stimulation

The study was approved by the local Ethics Review Committee. Data were obtained from 78 patients undergoing IVF (37 cases) and ICSI (41 cases) at the Conceptia Clinic at Moncton, New Brunswick, Canada. They were referred to our clinic with a history of more than 1 year infertility. Patients had the following primary etiologies for their infertility: Male factor, tubal factor, unexplained, endometriosis, anovulation and the polycystic ovary syndrome. Ovarian stimulation was performed with a GnRH analog (Suprefact, Sanofi-Aventis) down regulation utilizing our long or microflare protocols with recombinant FSH (Gonal F, EMD Serono or Puregon, Merck) and LH (Luveris, EMD Serono) or human menopausal gonadotrophin (Menopur or Bravelle, Ferring), dose adjusted according to body mass index. During the ovarian stimulation regimen, the patients underwent transvaginal ultrasonographic evaluation of endometrial thickness and measurement of follicular number and size. Ovulation was induced with recombinant HCG (Ovidrel, EMD Serono) when 3 or more follicles were at least 18 mm in their greatest diameter.

Oocyte retrieval and in vitro fertilization

All oocyte retrievals were done by transvaginal aspiration under ultrasound guidance 36–37 h after hCG administration. The medium used for flushing the follicles is G-MOPS (Vitrolife). After retrieval, oocytes were rapidly isolated from follicular fluid, rinsed, and placed in G-IVF medium (Vitrolife). Oocytes were inseminated 3–5 h later either by classical IVF with a mean concentration of 200 000 motile spermatozoa per ml or by ICSI. Spermatozoa for IVF and ICSI were prepared with the swim-up technique and density gradient centrifugation method according to our standard protocols, respectively. For ICSI cases, cumulus cells were first removed by hyaluronidase (Vitrolife) and mechanical treatments. ICSI was performed as described by Palermo et al. [26]. Single motile spermatozoan of the best available morphologic appearance was injected into each mature oocyte. Care was taken to inject as little extra media volume (2 to 3 pL) as possible.

Day 3 embryo classification

The occurrence of fertilization was determined 16–19 h after insemination. Oocytes with two pronuclei were individually placed in 20 μl droplets of G1-V5 medium (Vitrolife) under Ovoil for culture. Incubation was performed at 37 °C, in a humidified atmosphere containing 6 % CO_2, 5 % O_2 and 89 %N_2 for 48 h. On day 2, embryos were examined for cell stage, fragmentation and multinucleation. Embryos were classified on Day 3 into four categories as follows: grade (G) 1: embryos (≥ 8-cells) have even, regular, spherical blastomeres with no or < 5 % fragmentation. G 2: embryos (≥ 6-cells) have regular, spherical blastomeres, with < 15 % fragmentation. G 3: embryos (≥ 4-cells) have uneven shaped blastomeres, with < 40 % fragmentation. G 4: embryos (< 4-cells) have unequal, dark blastomeres, with > 15 % fragmentation or the number of blastomeres is more than 4-cells, with unequal, dark blastomeres and > 40 % fragmentation. Grade1 and Grade 2 were considered as good quality, Grade 3 and 4 were considered as poor quality.

Embryo group culture strategies

On Day 3, patients who had 4 or more good quality of cleavage embryos were invited to have the embryos cultured to blastocyst stage for transfer. Embryos were then cultured in group of 2–5 embryos per droplet (50 μl G2-V5) until Day 5 or 6. The group culture strategy is: **A**, embryos were randomly grouped regardless of embryo quality; **B**, good and poor quality embryos were separately grouped. All embryos with multiple nuclei or from abnormal fertilization were cultured separately or discarded. Patient cycles with less than 4 good quality embryos or with no poor quality embryos on Day 3 were eliminated in this study.

Blastocyst classification and transfer

On the morning of Day 5, blastocyst formation was determined and each blastocyst was graded by using the system of Gardner and Schoolcraft [27]. Briefly, blastocysts were

given an alphanumeric score from 1 to 6, based on their degree of expansion, hatching status, as follows: 1, early blastocyst, the blastocoel being less than half the volume of the embryo; 2, blastocyst, the blastocoel being half or greater than half of the volume of the embryo; 3, full blastocyst, the blastocoel completely fill the embryos; 4, expanded blastocyst, the blastocoel volume is larger than that of the early embryo and the zona is thinner than before; 5, hatching blastocyst, the trophectoderm has started to herniate through the zona; 6, hatched Blastocyst, the blastocyst has completely escaped from the zona.

The development of the inner cell mass (ICM) was graded as follows: A, tightly packed, many cells; B, loosely grouped, several cells; or C, very few cells. The trophectoderm was assessed as follows: A, many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; or C, very few large cells.

By using this scoring system, maximum of two blastocysts were transferred to patient on Day 5. Those that did not reached the blastocyst stage on Day 5 were cultured one more day till Day 6. The criterion for cryopreservation of blastocysts was that blastocysts with a score of ≥ 3BB at Days 5 or 6 according to Gardner et al. [28]. Blastocysts displaying ICM and/or trophectoderm with very few cells (score as C) were considered as unusable embryos and discarded.

Transfer was performed by using G2-V5 medium, Wallace catheter and ultrasonographic guidance. The chemical pregnancies were defined as a positive pregnancy test result on luteal day 12 with a rising titer confirmed by a second hCG measurement. The clinical pregnancies were diagnosed by ultrasonographic evidence of embryonic heart activity. Statistical analysis was done by using chi-square test.

Results

General observations

A total of 1243 oocytes were collected from 78 patients. 874 of them were fertilized by IVF or ICSI. The fertilization rate was 70.3 %. The percentages of good and poor quality cleavage embryos on Day 3 were, respectively, 76.7 % (670/874) and 23.3 % (204/874). Eighteen cases were eliminated because they had no poor quality embryos on Day 3. There were poor quality embryos on Day 3 in every case. The high percentage of good quality cleavage embryos on Day 3 might be due to a quality culture system in this study.

Comparison of blastocyst development and pregnancy between two methods

Outcome of blastocyst development and pregnancy in two groups were summarized in Table 1. The blastocyst rate in group B (61.2 %) (Good and poor quality embryos were separately grouped) were significantly higher than that in group A (44 %) (Embryos were randomly grouped regardless of embryo quality). There was no statistical difference between two groups in the implantation rate and clinical pregnancy rate.

Effect of group culture methods on blastocyst utilization rates

Blastocyst utilization rates from two different group culture strategies were compared in Table 2. The blastocyst utilization rate was calculated as the number of blastocysts suitable for freezing and transfer/total number of 2 PN embryos cultured to Day 5 and 6. The rates of blastocyst utilization and cryopreservation in group B (55.9 % and 39.2 %, respectively) were significantly higher than those in group A (41.5 % and 25.1 %, respectively). The average number of blastocysts cryopreserved per patient in group B (4.5) was also significantly greater compared with that in group A (2.7).

Quality of blastocysts transferred on Day 5 in both group culture methods

Patients were assigned to one of three situations according to the quality of their blastocysts transferred on Day 5 in both group culture methods. Maximum of two blastocysts were transferred per patient. Patients in situation 1 had two top-scoring blastocysts for transfer (≥ 3AA). Patients in situation 2 had one top-scoring blastocyst (≥ 3AA) with or

Table 1 Outcome of blastocyst development and pregnancy

Group culture method	Patient	Average age	Total oocytes	2PN embryos	Good quality Day 3 embryos	Blastocysts	Embryo implantation	Clinical pregnancy
A	37	34 ± 5.2	563	402 (71.4 %)	304 (75.6 %)	177 (44 %) [a]	27 (40.9 %)	26 (70 %)
B	41	33 ± 4.7	680	472 (69.4 %)	366 (77.5 %)	289 (61.2 %) [b]	29 (36.7 %)	27 (66 %)

In group B, the blastocyst rates for good or poor quality of day 3 embryos were, respectively, 72.4 % (265/366) and 23 % (24/106), the average was 61.2 %

[a,b] Values with different superscript letters within the same column are significant different ($p<0.05$)

Table 2 Effect of group culture methods on blastocyst utilization rates

Group culture method	Patient	2PN embryos	Cryopreserved blastocysts	Transferred blastocysts	Usable blastocysts	Total blastocysts	Average cryopreserved blastocysts
A	37	402	101 (25.1 %) [a]	66	167 (41.5 %) [a]	177 (44 %) [a]	2.7± 0.2 [a]
B	41	472	185 (39.2 %) [b]	79	264 (55.9 %) [b]	289 (61.2 %) [a]	4.5± 0.7 [b]

[a,b] Values with different superscript letters within the same column are significant different ($p<0.05$)

without one blastocyst (< 3AA) for transfer. Patients in situation 3 did not have a top-scoring blastocyst for transfer. There were no significant differences in the top-scoring blastocyst (transferred) rate and the distribution of top-scoring blastocysts in three transfer situations between two group culture methods (Table 3). Six patients in situation 2 (3 from method A, 3 from method B) were selected for doing single embryo transfer.

Discussion

All the procedures taking place in an embryology laboratory are the elements of great importance influencing the efficacy of human assisted reproductive technology. Proper embryo culture strategy is one of the major factors of a successful IVF program. Recently group culture of embryos to blastocyst stage has been beneficially applied in mice [29, 30], sheep [31], cow [32–34] and human [35, 36].

It has been reported that group culture may promote embryo development vie secretion of embryotrophic factors [14, 37, 38], and embryos cultured in group developed better than those cultured individually [39–41]. However, opponents of the approach argue that embryos in group may either deplete the media of substrates or negatively affect nearby embryos via the transmission of other secreted factors. The blastocyst development rate in mouse embryos is promoted by an increase of embryo density in culture drops [20, 39], which suggests that specific factors are secreted from the embryos to influence embryo growth in an autocrine or paracrine manner. However, some other factors derived from poor quality embryos may have negative influences on the surrounding embryo development. It has been reported that the accumulation of toxic substances such as ammonia and oxygen-derived free radicals in the culture medium [42–44] may lead to the late developmental anomalies.

Assessing pronuclear morphology at Day 1 [45, 46] and detecting the presence of multinucleated blastomeres at Day 2 [47, 48] have proven to be useful for the prediction of embryo development potential. In addition, the group culture of embryos to blastocyst stage in humans is not always possible due to the small number of available oocytes at retrieval or the poor quality of embryos on Day 3. Therefore, individual culture and tracking of embryos from Day 1 to Day 3 is preferable because it is able to correlate the embryo morphologic features or cleavage speed evaluated at Day 1 or 2 with the evaluation at Day 3, and with the benefits for selection of embryos to be transferred at Day 3 when there are not enough good quality of Day 3 embryos available for blastocyst culture.

The theoretical reasons and practical evidences of the benefit of group culture to blastocyst stage in the literature as mentioned earlier had prompted the present study to investigate the combination of single culture to Day 3 and group culture to Day 5 or 6 in an attempt to obtain the best of both culture strategies.

It is without question that embryos can modify their surrounding environment by creating localized zones of secreted and depleted factors. Whether these modifications of the local environment are beneficial or detrimental is not clear. The hypothesis generated in this study is that in addition to the positive effects, human embryo development in group culture may also be harmfully affected by negative factors that derived from poor quality of embryos. In this study, the culture of Day 3 embryos, in which good and poor quality embryos were separately grouped, significantly promoted both blastocyst development and blastocyst utilization rate in comparison with those embryos that were randomly grouped regardless of embryo quality. Even

Table 3 Quality of blastocysts transferred and distribution of top-scoring blastocysts in three transfer situations

Group culture method	Patient	Distribution of top-scoring blastocysts in three transfer situations			Total transferred blastocysts	Top-scoring blastocysts (≥ 3AA) transferred
		1, Two blasts ≥ 3AA	2, One ≥ 3AA ± one < 3AA	3, One or two blasts <3AA		
A	37	20 (54 %)	11 (30 %)	6 (16 %)	66	51 (77 %)
B	41	26 (63 %)	11 (27 %)	4 (10 %)	79	64 (81 %)

though there was no significant difference in the implantation rate and the pregnancy rate between two group culture methods, with the strategy that good and poor quality embryos be separately grouped for culture, the IVF program and patient may be benefited by improving the overall embryo quality, by increasing the number of good blastocysts cryopreserved and by increasing cumulative pregnancy rates with frozen-thawed blastocyst transfer. The reason why there were no significant differences in the implantation and pregnancy rates might be that both group culture methods had similar distribution of top-scoring blastocyst rates in all their three transfer situations.

Our results also raise the question of whether quality of the entire cohort is intrinsic due to the shared origins of the embryos, or if it is merely a result of group culture in vitro. The further study is needed to find out the differences in embryo phenotypes or embryo derived factors between good and poor quality embryos [49], and their effects on embryo development. If the benefit of group culture is the paracrine effect of embryonic factors which are secreted from good quality embryos, identification and addition of these factors to culture media may permit individual embryo culture to perform similarly to the group embryo culture.

Various factors that affect blastocyst formation and quality could not be controlled in one study. This study focused on the group embryo culture methods. However, in efforts to sweep generalizations, many other factors need to be evaluated that potentially impact outcomes. These include fertilization procedures (IVF or ICSI), stimulation protocols, number of oocytes, causes of infertility, embryo density, embryo spacing and type of culture media.

In conclusion, our findings indicate that grouping of embryos after Day 3 based on embryo quality may benefit blastocyst formation. This may be due to secretion of beneficial factors by good embryos, or removal of detrimental factors from poor embryos. The strategy of the combination of individual culture to Day 3 and good and poor quality of embryos be separately grouped for culture to Day 5 or 6 is recommended for clinical use.

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