Reproductive Sciences
https://doi.org/10.1007/s43032-024-01480-4

EMBRYOLOGY: ORIGINAL ARTICLE

Impact of Group vs Individual Embryo Culture Strategies on Blastocyst and Clinical Outcomes

Miguel Herreros[1] · Laura Martí[1] · Nerea Díaz[1] · María Carmen Tió[1] · Adoración Rodríguez-Arnedo[1] · Jaime Guerrero[1] · José A. Ortiz[2] · Andrea Bernabeu[3] · Rafael Bernabeu[3] · Jorge Ten[1]

Received: 14 April 2023 / Accepted: 7 February 2024

Abstract

Embryo culture is one of the most important steps in an assisted reproduction laboratory. Embryos can be cultured individually, one embryo per media drop, or in groups, culturing several embryos in the same media drop. Due to the controversy generated on this subject, we wondered which embryo culture method would have the best results in terms of quality and blastocyst formation rate. We designed a prospective randomized study comparing two different embryo culture strategies: group and individual embryo culture. The data were obtained from 830 embryos from 103 egg donation treatments. The zygotes were randomized into two groups: individual culture (group 1) or group culture (group 2). The embryos were cultured in 35-µl drops until day 5 when they were classified morphologically. We observed a significant increase in the blastocyst formation rate and in the usable embryo rate in individual culture on day 5 compared to group culture. However, good embryo quality (A/B blastocysts), implantation, and pregnancy rates were similar regardless of the type of embryo-culture. As a conclusion, individual culture may increase blastocyst formation rate and may benefit embryo quality on day 5. Our results support previous reports suggesting that individual culture could improve embryo development.

Keywords Embryo culture · Individual culture · Group culture · Human IVF · Blastocyst · Embryo quality

Introduction

From the first homemade culture media, where glucose, water, and egg white were used, to today's culture media that contain up to 80 different ingredients, the culture medium improvement has made possible to increase the embryo performance, increasing its quality [1, 2]. This has consolidated the strategy of single blastocyst stage embryo transfer increasing the pregnancy rate and decreasing multiple gestation risks [3]. It is necessary to point out that embryo culture depends on different variables such as gamete preparation techniques, the medium, the culture dishes used, and the culture strategy itself [4].

✉ Miguel Herreros
mherreros@institutobernabeu.com

Laura Martí
lmarti@institutobernabeu.com

Nerea Díaz
ndiaz@institutobernabeu.com

María Carmen Tió
mtio@institutobernabeu.com

Adoración Rodríguez-Arnedo
drodriguez@institutobernabeu.com

Jaime Guerrero
jguerrero@institutobernabeu.com

José A. Ortiz
jaortiz@institutobernabeu.com

Andrea Bernabeu
abernabeu@institutobernabeu.com

Rafael Bernabeu
rbernabeu@institutobernabeu.com

Jorge Ten
jten@institutobernabeu.com

[1] Reproductive Embriology, Instituto Bernabeu, Alicante, Spain

[2] Molecular Laboratory, Instituto Bernabeu Biotech, Alicante, Spain

[3] Reproductive Medicine, Instituto Bernabeu, Alicante, Spain

Published online: 20 February 2024

In recent years, different strategies to improve the embryo culture have been tested, among them are the following: sequential or continuous [5–7], individual or group culture [8], and more recently, mixed culture in microwells with time-lapse system [9]. Another important factor is the culture medium volume used for each embryo, also known as embryo density. This is calculated by dividing the medium volume by the number of cultured embryos. An optimal embryo density could benefit the embryo development [9]. Specifically, the best option would be to grow four embryos per 50 µl of culture medium [10], although each laboratory must adapt the number of embryos cultured in each drop of medium according to their protocols and results. Culture conditions and type of mineral oil employed could also have an influence in this strategy.

The debate on whether to use individual or group embryo culture is still open. With individual culture, we are able to follow each embryo individually, but there is no communication between them by paracrine signaling. Embryos can communicate with each other through various signaling factors such as proteins, lipids, saccharides, microRNAs, and other molecules [11]. With group culture, embryos could benefit from these signals and improve embryo quality [12]. On the other hand, it has been shown that the accumulation of waste products, such as ammonium [13] and free radicals derived from poorer quality embryos, could negatively affect embryos, which may not reach the blastocyst stage or become poorer quality embryos [14–16].

In this context, the main objective of this study was to compare two methods of embryo culture: single culture and group culture, to determine in our laboratory setting which of them have a higher number of blastocysts and better embryo quality at day 5 of embryo development.

Materials and Methods

Study Population

This prospective randomized study was performed between November 2020 and December 2021. Data were obtained from a total of 103 egg donation treatments. A total of 1081 fresh oocytes were donated, from which 995 metaphases II (MII) were microinjected, obtaining 830 zygotes. Randomization was carried out by fertilized oocyte, dividing cohorts from the same donor into individual and group cultures. Ninety-seven embryo transfers were performed at Instituto Bernabeu. Inclusion criteria for the oocyte reception cycles were as follows: normal uterine cavity, use of fresh oocytes, normal sperm samples according to the World Health Organization [17], both fresh or cryopreserved, and continuous culture until day 5 of development. Only cycles with a minimum of five fertilized oocytes were considered for the study. Preimplantation genetic testing (PGT) cycles were not included.

Oocyte Donors and Ovarian Stimulation

Our strict donor selection protocol was followed, which considers the following characteristics: being between 18 and 32 years of age, BMI between 18 and 25, minimum height of 1.55 m, and having good physical and emotional health (pass a psychological test). In the case of piercings or tattoos, they must have been made prior to 6 months of the donation. Of course, avoid being pregnant and, according to the Spanish law, having fewer than six descendants in Spain, counting your own children, and children born from previous donations.

All donors employed a short protocol starting in follicular phase with an initial dose of 100–300 UI/day of FSHr (Bemfola®, Gedeon Richter, Madrid, Spain) according to the antral follicle count (AFC) and the body mass index (BMI). In addition, a dose of 200 mg/day of micronized natural progesterone was administered orally (Utrogestan®, SEID S.A., Barcelona; Spain) from the first day of stimulation until the day before the trigger to prevent premature LH surge [18]. Ovulation was induced with a GnRH agonist (triptorelin (Decapeptyl®, Ipsen Pharma, Spain), 0.2 mg) when at least three follicles had a diameter ≥ 18 mm. After 36 h, ultrasound-guided transvaginal follicle aspiration was performed.

Endometrial Preparation

The vast majority of patients received hormone replacement therapy for endometrial preparation, and all those showing ovarian function were downregulated with a depot gonadotrophin-releasing hormone agonist (Ginecrin® depot 3.75 mg, administered intramuscularly as a single dose) (Abbott Laboratories, Madrid, Spain) given in the midluteal phase (approximately day 21) of the previous cycle [19]. On day 1 of subsequent menstruation (or at any point in recipients without ovarian function), estrogen treatment was started using either daily oral oestradiol valerate (Progynova®) (Schering Spain, Madrid, Spain) or transdermal oestradiol every 2 days (Progynova® transdermal patch) (Schering Spain, Madrid, Spain). The dosage was 4 mg (or 100 µg of transdermal oestradiol) during the first 7 days and 6 mg (or 150 µg of transdermal oestradiol) from day 8 onwards. In the fresh embryo transfer group, starting in the evening of oocyte retrieval, 400 mg twice daily of micronized progesterone pessaries (Cyclogest®, Gedeon Richter Ibérica, S.A., Barcelona, Spain) were added. In the FET group, a similar dose of micronized progesterone was started 5 days before the day of embryo transfer. Hormone replacement therapy was maintained until the end of the

12th gestational week, as described in a previous publication from our group [20].

One patient underwent a modified natural cycle preparation, with monitoring until criteria for HCG-triggered ovulation were met (leading follicle > 17 mm). A mandatory endometrial thickness of 7 mm was required for embryo transfer. After ovulation, 400 mg daily of Utrogestan® progesterone pessaries were used until the pregnancy test and confirmation of a gestational sac [21].

In Vitro Fertilization, Randomization, Embryo Culture, and Embryo Transfer

After recovery of the oocyte-corona-cumulus complexes, they were rapidly isolated from the follicular fluid, washed with Global Total LP w/ HEPES (LifeGlobal; Guilford, CT, USA), and placed in Global Total LP for fertilization medium (LifeGlobal; Guilford, CT, USA). After 1 h, the cumulus cells were removed mechanically using hyaluronidase (FertiPro; Beemem, Belgium). The oocytes were placed back into Global Total LP for fertilization.

Semen samples were collected or thawed on the day of oocyte retrieval. Sperm for intracytoplasmic sperm injection (ICSI) was prepared by density gradient centrifugation using isolate concentrate (FUJIFILM Irvine Scientific; The Hague, The Netherlands) and sperm washing (FUJIFILM Irvine Scientific; The Hague, The Netherlands) at two different concentrations (80% and 40%).

Mature oocytes (MII) were placed on 10-µl microdrops of Global Total LP w/ HEPES under LiteOil (LifeGlobal; Guilford, CT, USA) mineral oil, where they were microinjected. After ICSI, the oocytes were placed in a culture dish with 50-µl drops of Global Total for fertilization medium covered with LiteOil mineral oil.

After 16–18 h post ICSI, fertilization was assessed (presence of two pronuclei and two polar bodies). The fertilized oocytes were then randomized and divided into two groups:

Group 1: individual culture (399 embryos) in which only one zygote was included per drop of culture medium.
Group 2: group culture (431 embryos) in which 2–5 zygotes were included per drop of culture medium, varying according to the total number of fertilized oocytes.

The culture dishes were previously prepared by placing 35-µl drops of Global Total LP medium, covered with LiteOil mineral oil on which the zygotes were deposited according to the group assigned after randomization. We did not adjust the volume of the culture medium based on the number of zygotes, since it is the volume that we use in our laboratory protocol.

On day 3 of embryo development (67–69 h after microinjection), we scored the embryo quality according to the number of blastomeres, fragmentation, and multinucleation. The quality was evaluated according to the Spanish Association of Reproductive Biology (ASEBIR) criteria [22].

Day 5 blastocysts (114–118 h after ICSI) were evaluated according to the Gardner and Schoolcraft's criteria [23]. We simplified the Gardner classification into four categories: type A for AA, BA, and CA embryos; type B for AB, BB, and CB embryos; type C for AC, BC, and CC embryos; and type D for AD, BD, and CD embryos, and all embryos whose inner cell mass was classified as type D. On the other hand, the expansion range was considered as early blastocyst type 2, expanding blastocyst type 3, expanded blastocyst type 4, hatching blastocyst type 5, and hatched blastocyst type 6. At this moment, we calculated the blastocyst formation rate and the percentage of useful embryos. Embryos deemed as usable embryos were those acceptable for transfer and/or cryopreservation using Gardner's criteria [24].

The best quality embryo in the cohort was transferred to the recipient uterus using a flexible catheter (Rocket Medical; Washington, England) under ultrasound control. The rest of embryos were vitrified or discarded according to embryo quality.

Culture Conditions

Embryo culture was carried out in Planer BT37 (CooperSurgical; Trumbull, CT, USA) or MINC Cook (Cook Medical; Bloomington, IN, USA) benchtop incubators. The culture conditions were 6% O_2, 7% CO_2, and 37 °C with humidity.

Outcome Measures

Blastocyst formation rate (%) was the primary variable in this study. It was defined as [number of blastocysts on day 5/number of fertilized oocytes] × 100 [25]. The secondary variables studied were embryo quality according to Gardner [23] and useful embryos [transferred or frozen embryos on day 5/number of fertilized oocytes] × 100. Pregnancy and implantation rates after fresh transfer of a single blastocyst were also evaluated.

Transferred or frozen embryos were those with high quality (types A and B). Some slow developing embryos and good morphology (type C) were also used.

Embryo quality was evaluated by two senior embryologists to reduce variability between operators. Internal and external quality controls for embryo assessment are performed periodically to ensure updating and similarity between embryologists.

Statistical Analysis

Our sample size calculation was based on data from a previous pilot study. Accepting an alpha risk of 0.05 and a

beta risk of 0.20 in a two-sided contrast (statistical power of 80%), a sample size of 712 embryos (356 in each study group: individual vs group) is required to detect a minimum difference of 10% in the blastocyst formation rate between the two groups. Estimating a drop-out loss rate of 15%, a sample size of 830 is required (415 per group). The randomization table was carried out prior to the study start using a computer-generated list for a 1:1 ratio. Randomization was carried out using the !RNDSEQ macro of SPSS Statistics [26], so that both groups had the same probability of being assigned at each point in time.

The descriptive statistical methods used in this study depend on the type of the variable analyzed. In the case of qualitative variables, the following descriptive statistics will be obtained: frequency and percentage. Pearson's chi-squared and Fisher's exact test were used to analyze the association between variables. Continuous variables were presented as number of cases, mean, and SD. The Shapiro–Wilk tests were used to assess whether the continuous variables were normally distributed. Depending on whether the variable has a normal distribution, the comparison between means was carried out using Student's *t* test or the Wilcoxon rank sum test. Values of $p < 0.05$ will be considered statistically significant.

R Statistical Software version 4.0.3 (The R Foundation) and Statistical Package for the Social Sciences (SPSS) software (version 20.0, SPSS, Inc., Chicago, IL, USA) were used for statistical analysis.

Results

A total of 995 MII oocytes (91.3%) were microinjected. Fertilization rate was 80.03%, obtaining 830 zygotes.

The characteristics of the patients are shown in Table 1. The characteristics collected were as follows: the age of the donors, the recipients and the males, egg phase (follicular or luteal), sperm status (fresh or frozen), donor BMI and recipient BMI, recipient stimulation, and race of donors and recipients. Only sperm status was significantly different between the control group and the study group. This variable was considered as confounding factor and was taken into account in the subsequent analysis.

Table 2 shows the comparison of good quality (A and B), fair quality (C), and poor quality embryos (D) in the three groups between group 1 and group 2. The percentage of good quality, fair quality, and bad quality embryos on day 3 was similar in group 1 compared to group 2 (79.2% vs 80.9%; 8% vs 7.2%; 12.8% vs 11.8%, respectively, $p = 0.81$). However, on day 5, more good quality embryos (55.9% vs 52.4%) were observed in group 1 compared to group 2, but fair quality (7.5% vs 9.7%) and bad quality (36.6% vs 38.1%) were similar in both groups without reaching a significant difference ($p = 0.402$). The blastocyst formation rate is reflected in Table 3. Here, we can observe a statistically significant increase in group 1 compared to group 2 (65.7% vs. 58.7%, respectively, $p = 0.048$). Also, we obtained a statistically significant difference in favor of group 1 compared to group 2 in the percentage of useful embryos (63.9% vs 56.4%, respectively, $p = 0.028$) (Table 3).

Table 1 Patient's and donor's characteristics. Mean (SD); *n*/*N* (%). Wilcoxon rank sum test; Pearson's chi-squared test; Fisher's exact test

Characteristic	Group 1	Group 2	*p*-value
Donor age	25.79 (3.76)	24.36 (4.11)	0.072
Recipient age	41.83 (3.06)	41.91 (4.30)	0.425
Male age	40.54 (6.62)	42.93 (6.81)	0.099
Egg phase			0.988
Follicular	20/48 (41.7%)	23/55 (41.8%)	
Lutea	28/48 (58.3%)	32/55 (58.2%)	
Sperm status			0.015
Fresh	45/48 (93.8%)	42/55 (76.4%)	
Frozen	3/48 (6.3%)	13/55 (23.6%)	
Donor BMI (Kg/m^2)	23.02 (2.39)	23.22 (3.35)	0.717
Recipient BMI (Kg/m^2)	23.26 (4.00)	23.60 (4.42)	0.828
Recipient preparation			0.130
Agonist/antagonist + estrogen + progesterone	37/47 (78.7%)	31/50 (62.0%)	
Estrogen + progesterone	9/47 (19.2%)	15/50 (30.0%)	
Modified natural cycle	1/47 (2.1%)	4/50 (8.0%)	
Donor race			0.717
Caucasian	47/48 (97.9%)	54/55 (98.2%)	
African	0/48 (0.0%)	1/55 (1.8%)	
Hispanic	1/48 (2.1%)	0/55 (0.0%)	
Recipient race			0.719
Caucasian	46/48 (95.8%)	54/55 (98.2%)	
Afro-American	0/48 (0.0%)	1/55 (1.8%)	
African	1/48 (2.1%)	0/55 (0.0%)	
Gypsy	1/48 (2.1%)	0/55 (0.0%)	

Table 2 Quality of embryos in day 3 and day 5. *n*/*N* (%). Pearson's chi-squared test

Quality embryos	Group 1	Group 2	*p*-value
Day 3			0.810
Good (A and B)	316/399 (79.2%)	349/431 (81.0%)	
Fair (C)	32/399 (8.0%)	31/431 (7.2%)	
Bad (D)	51/399 (12.8%)	51/431 (11.8%)	
Day 5			0.402
Good (A and B)	223/399 (55.9%)	225/431 (52.2%)	
Fair (C)	30/399 (7.5%)	42/431 (9.7%)	
Bad (D)	146/399 (36.6%)	164/431 (38.1%)	

Table 3 Blastocysts formation rate and useful embryo rate. *n*/*N* (%). Pearson's chi-squared test

	Group 1	Group 2	*p*-value
Blastocyst formation	262/399 (65.7%)	253/431 (58.7%)	0.048
Useful embryo	255/399 (63.9%)	243/431 (56.4%)	0.028

Table 4 Implantation and pregnancy. *n*/*N* (%). Wilcoxon rank sum test

	Group 1	Group 2	*p*-value
Implantation	25/41 (61%)	35/56 (62.5%)	0.879
Pregnancy	19/41 (46.3%)	30/56 (56.3%)	0.541

Regarding clinical outcomes, 94.1% (97/103) of recipients underwent a fresh transfer of a single blastocyst. In the remaining six patients, elective embryo vitrification was carried out. Implantation (61% vs 62.5%, $p=0.879$) and clinical pregnancy (46.3% vs 56.3%, $p=0.541$) between group 1 and group 2, respectively, did not reach significant differences (Table 4).

Only one of the cycles resulted in no blastocyst development, and this was not excluded from subsequent analysis.

Discussion

Embryo culture is one of the main pillars in an assisted reproduction treatment. Some studies support embryo culture in group [8, 27], showing better results up to blastocyst stage. This may be due to some factors secreted by embryos, such as embryo-derived platelet activating factor (EPAF), to promote embryonic development [28].

Other studies, however, argue that group culture may deplete the resources necessary for embryo growth and, in addition, load this medium with waste components secreted by the embryos. Ammonia and free radicals are the components most likely to negatively influence embryo development [15, 29].

On the other hand, we cannot always group embryos. The clearest example is when embryos are biopsied, and therefore, we must keep them separate and well numbered for their subsequent diagnosis and transfer. A single embryo culture may also be necessary in cases of low number of embryos, for example, in patients with low ovarian reserve/response or low fertilization [30]. In addition, an individual embryo culture can increase the cost per cycle as it uses a larger volume of medium and sometimes even more culture dishes.

This controversy led us to consider which method of embryo culture would give the best results. We have been able to verify that single culture provides a higher blastocyst formation rate and more importantly, a higher rate of useful embryos, i.e., embryos to be cryopreserved or transferred. This leads us to believe that the components released by the embryos into the environment could be more detrimental than beneficial to other embryos. In addition, a single embryo having all the resources for itself in one drop of medium could be beneficial for its growth.

In terms of clinical outcomes, no differences were obtained. However, it would be possible to hypothesize that obtaining more and better quality blastocysts could increase the cumulative pregnancy rate. This will be an aspect to verify in a subsequent analysis. The main strength of this study is its prospective design with randomization at the zygote level and the use of donor oocytes with defined inclusion and exclusion criteria. Although the age of the donors was statistically significant between the two groups, we consider that this has no impact since it is a group of young patients (less than 29 years old) with proven fertility. Paternal age was also statistically significant, but the difference was less than 2 years between both groups and never reaching more than 50 years old where there may be a decline in semen volume, sperm motility, and sperm morphology [31]. The significant differences between fresh and frozen semen may be due to the difference in the number of samples between one group and the other. The final results were obtained and corrected including these values as confounding factors.

In addition, prior to the study, a statistical power calculation was performed to estimate a 10% improvement in the blastocyst formation rate. At the end, this result was not reached, obtaining a difference of almost 6% in the blastocyst formation rate between the individual culture and the group culture. However, this result was enough to find significant differences.

As a study limitation, we could mention that it was carried out with donated oocytes, and therefore, the results have not been verified by age range or other patient's characteristics. On the other hand, no differences in clinical outcomes were obtained. However, this result should be interpreted with caution as this study was not designed for this purpose. Prospective studies designed to establish whether there are clinical differences would be necessary. Another important factor that we must take into account is that we used a continuous medium, so the results with sequential media could be different.

Most of studies comparing individual and group culture are made, taking into consideration different criteria and methodology which means that the results cannot be extrapolated or generalized, for example, studies on animals or humans, day 3 or day 5 culture, use of sequential or continuous media, and different volumes in the drops of medium according to the number of embryos. In our study, we adapted to one of the conventional protocols used in

non-time-lapse incubators. For this reason, we defined to carry out this study on day 5 of culture, using continuous culture medium and without changing the volume of the drops. We believe that this is how we could really see the difference in the consumption of resources of the embryo itself or how the waste components that they can release affect other embryos. Although the incubators and culture media improvement in recent years has been exponential, especially with the introduction of time-lapse systems [32, 33], there are still many laboratories that continue to perform embryo culture in conventional incubators, so studies like this would help to better understand under what conditions embryos develop best. Further research comparing single culture, group culture, and the mixed culture provided by time-lapse systems would be interesting. Finally, the most important thing is that each laboratory should adapt its embryo culture to its own needs and limitations.

Data Availability The data obtained are available to you if necessary.

Code Availability Not applicable.

Declarations

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University Hospital of San Juan de Alicante (25.11.2020; No. 20/045 Tut).

Consent to Participate All study participants signed an informed consent to participate in this study.

Consent for Publication All study participants gave their consent to publish the final data.

Competing Interests The authors declare no competing interests.

References

1. Wiemer KE, Anderson AR, Kyslinger ML, Weikert ML. Embryonic development and pregnancies following sequential culture in human tubal fluid and a modified simplex optimized medium containing amino acids. Reprod Biomed Online. 2002;5(3):323–7. https://doi.org/10.1016/s1472-6483(10)61840-8.
2. Chronopoulou E, Harper JC. IVF culture media: past, present and future. Hum Reprod Update. 2015;21(1):39–55. https://doi.org/10.1093/humupd/dmu040.
3. Karaki RZ, Samarraie SS, Younis NA, Lahloub TM, Ibrahim MH. Blastocyst culture and transfer: a step toward improved in vitro fertilization outcome. Fertil Steril. 2002;77(1):114–8. https://doi.org/10.1016/s0015-0282(01)02939-9.
4. Swain JE. Optimal human embryo culture. Semin Reprod Med. 2015;33(2):103–17. https://doi.org/10.1055/s-0035-1546423.
5. Sepúlveda S, Garcia J, Arriaga E, Diaz J, Noriega-Portella L, Noriega-Hoces L. In vitro development and pregnancy outcomes for human embryos cultured in either a single medium or in a sequential media system. Fertil Steril. 2009;91(5):1765–70. https://doi.org/10.1016/j.fertnstert.2008.02.169.
6. Paternot G, Debrock S, D'Hooghe TM, Spiessens C. Early embryo development in a sequential versus single medium: a randomized study. Reprod Biol Endocrinol. 2010;8:83. https://doi.org/10.1186/1477-7827-8-83.
7. Stimpfel M, Bacer-Kermavner L, Jancar N, Vrtacnik-Bokal E. The influence of the type of embryo culture media on the outcome of IVF/ICSI cycles. Taiwan J Obstet Gynecol. 2020;59(6):848–54. https://doi.org/10.1016/j.tjog.2020.08.001.
8. Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. Reprod Biomed Online. 2010;21(6):762–8. https://doi.org/10.1016/j.rbmo.2010.06.038.
9. Lehner A, Kaszas Z, Murber A, Rigo J Jr, Urbancsek J, Fancsovits P. Embryo density may affect embryo quality during in vitro culture in a microwell group culture dish. Arch Gynecol Obstet. 2017;296(2):345–53. https://doi.org/10.1007/s00404-017-4403-z.
10. Gardner DK, Lane M. Culture of the mammalian preimplantation embryo. In: Gardner DK, Lane M, Watson JA, editors. A laboratory guide to the mammalian embryo. Oxford University Press, Oxford: UK; 2004. p. 41–61.
11. Wydooghe E, Vandaele L, Heras S, De Sutter P, Deforce D, Peelman L, De Schauwer C, Van Soom A. Autocrine embryotropins revisited: how do embryos communicate with each other in vitro when cultured in groups? Biol Rev Camb Philos Soc. 2017;92(1):505–20. https://doi.org/10.1111/brv.12241.
12. Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update. 2003;9(6):557–82. https://doi.org/10.1093/humupd/dmg039.
13. Kleijkers SH, van Montfoort AP, Bekers O, Coonen E, Derhaag JG, Evers JL, Dumoulin JC. Ammonium accumulation in commercially available embryo culture media and protein supplements during storage at 2–8°C and during incubation at 37°C. Hum Reprod. 2016;31(6):1192–9. https://doi.org/10.1093/humrep/dew059.
14. Johnson MH, Nasr-Esfahani MH. Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? BioEssays. 1994;16(1):31–8. https://doi.org/10.1002/bies.950160105.
15. Virant-Klun I, Tomazevic T, Vrtacnik-Bokal E, Vogler A, Krsnik M, Meden-Vrtovec H. Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. Fertil Steril. 2006;85(2):526–8. https://doi.org/10.1016/j.fertnstert.2005.10.018.
16. Costa-Borges N, Bellés M, Meseguer M, Galliano D, Ballesteros A, Calderón G. Blastocyst development in single medium with or without renewal on day 3: a prospective cohort study on sibling donor oocytes in a time-lapse incubator. Fertil Steril. 2016;105(3):707–13. https://doi.org/10.1016/j.fertnstert.2015.11.038.
17. Boitrelle F, Shah R, Saleh R, Henkel R, Kandil H, Chung E, Vogiatzi P, Zini A, Arafa M, Agarwal A. The sixth edition of the WHO manual for human semen analysis: a critical review and SWOT analysis. Life (Basel). 2021;11(12):1368. https://doi.org/10.3390/life11121368.
18. Guerrero J, Castillo JC, Ten J, Ortiz JA, Lledó B, Orozco D, Quereda F, Bernabeu A, Bernabeu R. Random-start ovarian stimulation in an oocyte donation programme: a large, single-centre, experience. Reprod Biomed Online. 2024;48(1):103572. https://doi.org/10.1016/j.rbmo.2023.103572. Epub 4 Oct 2023.
19. El-Toukhy T, Taylor A, Khalaf Y, Al-Darazi K, Rowell P, Seed P, Braude P. Pituitary suppression in ultrasound-monitored frozen embryo replacement cycles. A randomised study. Hum Reprod. 2004;19(4):874–9. https://doi.org/10.1093/humrep/deh183.

20. Llácer J, Díaz N, Serrano-Revuelta E, Álvarez E, Castillo JC, Ortíz JA, Ten J, Bernabeu A, Bernabeu R. Vitrification does not affect birth weight: lessons from the oocyte donation model. Reprod Biomed Online. 2022;45(2):355–63. https://doi.org/10.1016/j.rbmo.2022.02.023.
21 Mackens S, Santos-Ribeiro S, van de Vijver A, Racca A, Van Landuyt L, Tournaye H, Blockeel C. Frozen embryo transfer: a review on the optimal endometrial preparation and timing. Hum Reprod. 2017;32(11):2234–42. https://doi.org/10.1093/humrep/dex285.
22. Cruz M, Gadea B, Garrido N, Pedersen KS, Martínez M, Pérez-Cano I, et al. Criterios ASEBIR de valoración morfológica de oocitos, embriones tempranos y blastocistos humanos. 3ª Edición. 2015. http://www.asebir.com/cuadernos-asebir/criterios-asebirde-valoracion-morfologica-de-oocitosembriones-tempranos-yblastocistoshumanos. Accessed 12 Jan 2023.
23. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. Curr Opin Obstet Gynecol. 1999;11(3):307–11. https://doi.org/10.1097/00001703-199906000-00013.
24 Shakartzi H, Gilroy L, Amaral S, Leondires MP, Paganetti E, Richlin S. Usable blastocyst rate in donor oocyte cycles: performance of frozen sperm with ICSI in an extended culture system. Fertil Steril. 2018;110(4):e199–200. https://doi.org/10.1016/j.fertnstert.2018.07. Gynaecol.
25. Hong YH, Kim HK, Nho EJ, Youm HW, Kim SK, Lee JR, Jee BC, Kim SH. Predictors of blastocyst formation rate in elective day 5 transfer cycle. J Obstet. 2020;40(6):863–8. https://doi.org/10.1080/01443615.2019.1676212.
26. Domenech JM, Granero R. Macro !NSize for SPSS Statistics. Sample size and power: comparisons and estimations for independent proportions, means, correlations, risks and rates [computer program]. V.2012.02.10. Bellaterra: Universitat de Aùtonoma de Barcelona; 2012. http://www.metodo.uab.cat/macros.htm. Accessed 14 Oct 2023
27. Tao T, Robichaud A, Mercier J, Ouellette R. Influence of group embryo culture strategies on the blastocyst development and pregnancy outcome. J Assist Reprod Genet. 2013;30(1):63–8. https://doi.org/10.1007/s10815-012-9892-x.
28. Fujita T, Umeki H, Shimura H, Kugumiya K, Shiga K. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. J Reprod Dev. 2006;52(1):137–42. https://doi.org/10.1262/jrd.16084.
29. Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. Biol Reprod. 1993;48(2):377–85. https://doi.org/10.1095/biolreprod48.2.377.
30. Rijnders PM, Jansen CA. Influence of group culture and culture volume on the formation of human blastocysts: a prospective randomized study. Hum Reprod. 1999;14(9):2333–7. https://doi.org/10.1093/humrep/14.9.2333.
31. Kidd SA, Eskenazi B, Wyrobek AJ. Effects of male age on semen quality and fertility: a review of the literature. Fertil Steril. 2001;75(2):237–48. https://doi.org/10.1016/s0015-0282(00)01679-4.
32. Márquez-Hinojosa S, Noriega-Hoces L, Guzmán L. Time-lapse embryo culture: a better understanding of embryo development and clinical application. JBRA Assist Reprod. 2022;26(3):432–43. https://doi.org/10.5935/1518-0557.20210107.
33. Sciorio R, Meseguer M. Focus on time-lapse analysis: blastocyst collapse and morphometric assessment as new features of embryo viability. Reprod Biomed Online. 2021;43(5):821–32. https://doi.org/10.1016/j.rbmo.2021.08.008.