Reduced blastocyst formation in reduced culture volume

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Received: 13 May 2015 / Accepted: 14 July 2015 / Published online: 21 August 2015
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Abstract

Purpose The aim of this prospective sibling oocyte study was to evaluate whether reduced culture volume improves blastocyst formation.

Methods Twenty-three patients with extended embryo culture until day 5 were selected for the study. After injection, 345 sibling oocytes were individually cultured in either 25 or 7 µl droplets of Origio cleavage medium under oil. On day 3 of development, embryos were transferred to droplets with the corresponding volume of Origio blastocyst culture medium. Fertilization and embryo quality on day 3 and day 5/6 were evaluated. **Results** No statistically significant difference ($p=0.326$) in fertilization rate was observed (81.3 versus 83.0 %). There was no significant difference in terms of the number of excellent and good-quality embryos obtained on day 3 between both groups ($p=0.655$). Embryo culture in 25 µl droplets led to more embryos with a higher cell number when compared to 7 µl culture ($p=0.024$). On day 3, 132 and 131 embryos were considered for further culture until day 5/6. Blastulation rates were significantly higher in the 25 µl group (75.0 versus 61.6 %; $p=0.017$) and significantly more day 5 embryos with excellent and good quality were found in this group (54.5 versus 40.5 %; $p=0.026$). Finally, the utilization rates

expressed per mature oocyte (41.4 versus 29.8 %; $p=0.043$), per fertilized oocyte (50.7 versus 36.6 %; $p=0.023$), and per day 3 embryo undergoing extended culture to day 5/6 (54.5 versus 39.7 %; $p=0.019$) were all significantly higher in the 25 µl group.

Conclusion Reduced culture volume (7 µl) negatively impacts early development by reducing the cell number on day 3 and both blastocyst formation and quality.

Keywords Embryo culture · Media volume · Embryo quality · Blastocyst formation · Blastocyst quality

Introduction

The optimal selection of a culture system is paramount for a successful outcome in IVF clinics. Despite the long history of IVF, the best culture system is yet to be elected and the majority of the labs use different methods to culture their embryos. At present, three major embryo culture systems are used: (i) grouped culture, with three to five human zygotes/embryos cultured together [22, 5, 12, 13], (ii) individual culture, where every embryo is cultured in an individual droplet [22, 10, 12], and (iii) individual culture with medium exchange between embryo droplets, where although the embryos are cultured individually, the presence of small communicating orifices in the walls allow the passage of medium [5]. Besides the culture system, the choice of the culture medium is also important and may be either: (i) single media, in which the zygote/embryo is cultured from day 0/1 until day 5 or (ii) sequential media, composed of both a cleavage and a blastocyst medium, in which a consecutive medium change is performed on day 3 of embryo development. Finally, different culture volumes can be applied for every culture system and culture medium [22, 5, 10]. Any selection of the abovementioned

Capsule Reducing culture volume from 25 to 7 µl significantly reduces blastocyst formation and quality.

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parameters should account for an optimal balance between the need to limit the concentration of toxic metabolic molecules, such as ammonium, and the positive effects of the beneficial autocrine factors on embryo development [21].

The ultimate goal of any IVF laboratory is to identify the best culture system that will lead to increased embryo and blastocyst quality and, most importantly, increased implantation rates and healthy liveborns.

Our current embryo culture system includes individual culture in 25 μ l droplets of sequential media. A recent report by Minasi et al. [10] concluded that a reduction in culture volume from 35 to 7 μ l is associated with a significantly higher blastulation rate on day 5. In order to confirm these results, we designed a prospective sibling study in which injected oocytes were cultured individually in either 25 or 7 μ l droplets until day 5 or 6 after oocyte retrieval.

Materials and methods

Patients

The study comprised 23 cycles (in 23 couples) performed in our center between March and April 2015. Only cycles with ≥ 6 mature oocytes in which ejaculated sperm (fresh or frozen) was used for intracytoplasmic sperm injection (ICSI) were included. Oocyte retrieval was scheduled 36 h after ovulation induction.

Study protocol

Injected sibling oocytes were randomly allocated to either 25 or 7 μ l droplets. The first oocyte was allocated to the group (25 or 7 μ l) based on a computer-generated randomization list.

Preparation of the dishes

All dishes (IVF round dish, $\varnothing$ 60 mm, 353652, Falcon) were prepared at room temperature the day before use in order to allow equilibration. The 25 μ l dishes were prepared as follows: 7 ml Ovoil (Vitrolife, Sweden) was placed into the culture dish; with an automated dispenser (multipetteplus, Eppendorf, VWR), 12 individual droplets were circularly placed under the oil at the bottom of the dish, and 4 washing droplets were placed in the center of the dish. The 7 μ l dishes were prepared using the same procedures previously described for the 25 μ l dishes; however, the droplets were placed under the oil by manual pipetting. In order to allow sufficient embryo rinsing, the four washing droplets also contained 25 μ l. After preparation, culture dishes were equilibrated for 24 ± 4 h in 5 % O_2 and 6 % CO_2 at 37 °C. Embryo culture was performed in cleavage medium (83040010A, Origio, Denmark, pH 7.2)

from day 0 post-injection until day 3. On day 3, the embryos were transferred to blastocyst medium (83060010A, Origio, Denmark, pH 7.3) until day 5 or 6.

Sperm preparation

Nineteen autologous fresh sperm samples were collected by masturbation, and 4 frozen sperm samples (one autologous and three heterologous) were used. All sperm samples were prepared by 90/45 % density gradient centrifugation (Spermint, CookMedical). Prepared sperm samples were kept at room temperature until the moment of injection.

Oocyte retrieval and injection

After oocyte retrieval, oocytes were incubated in fertilization medium (83020060A, Origio, Denmark) until denudation. Denudation [4] and mature oocyte injection [20] were performed as previously described. After injection, the oocytes were allocated to treatments of either 25 or 7 μ l droplets of Origio cleavage medium (at 37 °C in 5 % O_2 and 6 % CO_2), based on the randomization list.

Fertilization and embryo quality

Fertilization was assessed 16–18 h after injection by the presence of two pronuclei (PN). Day 3 embryos were scored on average $66 \text{ h } 23 \text{ min} \pm 1 \text{ h } 33 \text{ min}$ post-injection, and the evaluation was based on the number and symmetry of the blastomeres, percentage of fragmentation, vacuolization, granulation, and multinucleation. Based on all these parameters, an embryo quality score (EQ) was assigned to all normally fertilized embryos based on a predefined algorithm, which divides them in four categories: excellent, good, moderate, or poor. Excellent embryos had at least seven blastomeres that did not differ or only differed marginally in size and had ≤ 10 % fragmentation ($\sim$ Alpha grade 1). Good embryos had at least six blastomeres and/or ≤ 20 % fragmentation ($\sim$ Alpha grade 2) and/or blastomeres which differed only marginally in size. Moderate embryos had either at least four cells and/or 20–50 % fragmentation (with or without a combination of vacuoles or granulation) and/or multinucleation in ≤ 50 % of the blastomeres. For embryos considered to be of poor quality, ≥ 50 % fragmentation and/or multinucleation in > 50 % of the blastomeres (with or without a combination of vacuoles) was observed. Blastocysts (BL) were scored according to the grading system developed by Gardner and Schoolcraft [8]. Based on (i) the expansion stage, (ii) the number of cells joining the compaction or blastulation, and (iii) the appearance of the trophoctoderm (TE) and inner cell mass (ICM), one of the following blastocyst quality scores was given to every day 5 or 6 embryo: excellent, good, moderate, or poor. Excellent blastocysts were at least expanded with TE type A and ICM

type A or B. Good blastocysts were either early blastocysts with many cells and in which all cells were participating in the blastulation or expanded blastocysts with a TE type B and ICM type A or B. Moderate quality was obtained in either early blastocysts with a limited cell number or if not all cells were participating in the blastulation, or, additionally, to expanded blastocysts with a type C TE or ICM. Poor-quality embryos were scored as such if they were arrested, fragmented, degenerated, or lacked a TE or ICM. On day 5, fully compacted embryos, early blastocysts, or fully expanded or hatching blastocysts with a visible trophectoderm (TE) and inner cell mass (ICM) were considered adequate for transfer. Supernumerary blastocysts were cryopreserved on day 5/6 if they reached at least full blastocyst stage with an ICM and TE type A or B. Early blastocyst stages 1 and 2 were further cultured until day 6 and cryopreserved if they developed until a fully expanded blastocyst with an ICM and TE type A or B. A utilization rate was calculated based on all the blastocysts that were transferred or cryopreserved.

Statistics

Univariate statistical analysis was performed using either the Fisher's exact (for categorical variables) or Student *t* tests (for continuous variables). *p* values below 0.05 were considered statistically significantly.

Results

Mean female age was 32.0 ± 5.3 years (range of 24–42 years). Five patients performed ovarian stimulation with a GnRH agonist co-treatment for pituitary downregulation, while 18 were administered a GnRH antagonist. Patients were stimulated on average for 10 ± 1.4 days, and ovulation was induced with exogenous human chorionic gonadotropin (hCG) in all cases except for one, in which triptorelin was used.

In 13 cycles, a fresh blastocyst transfer was performed on day 5, with an average of 1.2 embryos per transfer. Supernumerary embryos were vitrified on day 5 or 6. In 10 cycles, a “freeze all” strategy was scheduled and all blastocysts fulfilling the minimum quality criteria were cryopreserved on day 5 or 6.

The relevant embryological data up to day 3 is presented in Table 1. In the 23 cycles included, a total of 453 cumulus-oocyte complexes (COCs) were obtained after oocyte retrieval, 345 (76.2 %) of which were mature. After injection, 174 oocytes were cultured in 25 μ l droplets and 171 in 7 μ l droplets. Fertilization rate did not differ significantly between both groups (81.3 versus 83.0 %, respectively, $p=0.326$). The percentage of good-quality day 3 embryos (excellent + good) was 81.7 and 78.9 %, respectively ($p=0.655$). When embryos were subdivided according to their cell number on day 3, a

significant difference was found between both groups ($p=0.031$). Specifically, the mean cell number on day 3 was $8.2 (\pm 2.9)$ in the 25 μ l group and $7.4 (\pm 2.5)$ in the 7 μ l group ($p=0.024$).

All excellent, good, and moderate day 3 embryos ($n=263$), except two, were transferred to blastocyst medium (25 or 7 μ l droplets); a total of 132 and 131 embryos respectively were cultured to day 5, and the results are shown in Table 2. Both the blastulation rate and the number of good-quality embryos (excellent + good) were significantly higher in the 25 μ l group ($p=0.017$ and $p=0.026$, respectively). The total number of utilized embryos was 72 in the 25 μ l group and 52 in the 7 μ l group. The utilization rates per mature oocyte ($p=0.034$), per fertilized oocyte ($p=0.023$), and per day 3 embryo going for day 5 culture ($p=0.019$) were all significantly higher in the 25 μ l group.

Discussion

The aim of this prospective sibling oocyte study was to analyze if reduced embryo culture volume indeed led to increased blastocyst formation. Our results revealed that culturing embryos in 25 μ l was associated with a significantly higher cell number on day 3, a higher blastulation rate, and higher number of good-quality blastocysts when compared to those cultured in 7 μ l culture droplets. Overall, the utilization rate per mature oocyte, per fertilized oocyte, and per embryo going for day 5 culture were significantly higher in 25 μ l droplets.

The recent report by Minasi et al. [10] comparing embryo culture in either 35 or 7 μ l droplets reported comparable embryo quality on day 3 (~80 %), but significantly higher blastulation rates on day 5 when embryos were cultured in 7 μ l droplets (50.5 versus 70.0 %, $p<0.05$). It should be noted that the authors did not present data on cell number on day 3 nor on expansion stage and blastocyst quality on day 5. Although our study also showed similar overall embryo quality distributions on day 3, a significant difference in cell number was detected, a difference which was previously otherwise undescribed and which could impact IVF outcome. According to the Alpha grading system, a day 3 embryo should have eight cells at 68 ± 1 h post-insemination, though without distinction between IVF or ICSI [2]. In our study, evaluations were performed on average $66:23 \pm 1:33$ h post-injection and an average cell number of 8.2 (25 μ l) and 7.4 (7 μ l) was observed. This number is in line with the expected cell number on day 3, especially when taking into account that developmental kinetics can differ between culture media. Furthermore, on day 5 of embryo development, our study revealed contrasting results, namely, significantly higher blastulation rates (75.0 % in 25 μ l versus 61.6 % in 7 μ l) and more good-quality blastocysts obtained in 25 μ l culture droplets (54.5 versus 40.5 %).

Table 1 Embryological data up to day 3

	25 μ l	7 μ l	<i>p</i> value
No. of MII (345)	174	171	
Fertilization			0.326
Fertilized (%)	142 (81.3)	142 (83.0)	
1PN (%)	4 (2.3)	3 (1.8)	
3PN (%)	5 (2.9)	3 (1.8)	
Degenerated (%)	3 (1.7)	9 (5.3)	
Not fertilized (%)	20 (11.5)	14 (8.2)	
Day 3 embryo quality			0.655
Excellent + Good (%)	90 (63.3)+26 (18.3)	79 (55.6)+33 (23.2)	
Moderate + Poor (%)	17 (11.9)+9 (6.3)	20 (14.1)+10 (7.0)	
Day 3 cell number			
<6	19	24	0.031
6–7	23	33	
=8	45	52	
>8	43	30	
C1	12	3	
Average cell number ($\pm$ SD)	8.2 $\pm$ 2.9	7.4 $\pm$ 2.5	0.024

A total of 453 cumulus-oocyte complexes (COCs) were obtained after oocyte retrieval, and 345 of which were mature. The number of cycles is 23. The table shows the allocation of the mature oocytes to both groups. Fertilization is given as normally fertilized oocytes, oocytes with one or three pronuclei (PN), degenerated oocytes or not fertilized after injection. Day 3 embryo quality and cell number is only presented for the fertilized oocytes

MI mature oocyte, *C1* compacting

Table 2 Embryological data from day 3 to day 5

	25 μ l	7 μ l	<i>p</i> value
No. of embryos for day 5 culture	132	131	
Day 5 evaluation			
Total blastocyst formation	99/132 (75.0)	80/131 (61.6)	0.017
Arrested	10	22	
Degenerated	9	9	
Fragmented	4	1	
C1/2	10	19	
BL1	12	21	
BL2	21	11	
BL3	18	22	
$\geq$ BL4	48	26	
Day 5 embryo quality			
Excellent + Good (%)	26 (19.7)+46 (34.8)	15 (11.5)+38 (29.0)	0.026
Moderate + Poor (%)	28 (21.2)+32 (24.4)	34 (26.0)+44 (33.6)	
Utilization			
No. of embryos transferred on day 5	9	7	
No. of embryos cryopreserved on day 5	43	30	
No. of embryos cryopreserved on day 6	20	15	
Per no. of mature oocytes (%)	72/174 (41.4)	52/171 (29.8)	0.043
Per no. of fertilized oocytes (%)	72/142 (50.7)	52/142 (36.6)	0.023
Per no. of embryos for day 5 culture (%)	72/132 (54.5)	52/131 (39.7)	0.019

A total of 132 and 131 embryos were cultured to day 5/6. Utilization rate is defined as the total number of embryos transferred and cryopreserved
C1 compacting, *C2* compacted

The cell number already reached a significant difference on day 3, and this significance persisted up to day 5. These data are in contrast with those of Minasi et al. [10], which may be related to the difference in culture media (Origio in the present study versus Sage by Minasi et al.). The two culture media have a different amino acid constitution and concentration and were supplemented with different protein sources. Any absorption or secretion in the smaller culture volume (7 μ l) may lead to a concentration change which is more pronounced as compared to a larger volume (25 μ l). The different concentration of components, such as amino acids with internal pH (pHi) buffering capacity [6], may induce a negative shift in the pHi. Even though the external pH (pHe) in 7 and 25 μ l droplets is the same for both cleavage (7.28 and 7.29) and blastocyst media (7.35 and 7.34) of the Origio media, it is likely that this negative shift in pHi is obtained in the smaller culture volume. It is known that a drift in pHe from the pHi might induce stress to the embryo. Since pHi is regulating a variety of cellular processes, it is possible that this shift is responsible for the early impaired development in the 7 μ l group, at least in the case of Origio culture media [16]. Since the formation of tight junctions at the compaction stage allow for a better control over the pHi, the harmful effect of the 7 μ l Origio culture volume might have been better controlled from day 4 onwards [7]. Moreover, the protein supplement between media also varied: specifically, human serum albumin (HSA) in our Origio culture system and serum protein supplement (SPS) in the Sage media used by Minasi et al. A recent article by Morbeck et al. [11] analyzed the composition of different protein supplements and their influence on blastocyst formation in a murine model. Not only was the composition of HSA and SPS rather different but they also resulted in a different time to reach the eight-cell stage and in a different number of expanded blastocysts. Taking into account the abovementioned differences in amino acid constitution and protein supplements between both media and their influence on embryo culture, one cannot predict which embryo culture medium will lead to an improved development in a reduced culture volume.

Another explanation for the impaired embryo development could be a more pronounced oxygen depletion in the reduced culture volume. As shown in a mathematical model by Byatt-Smith et al. [3], the culture of human embryos in a static microdrop may become marginally hypoxic, especially at lower oxygen levels (5 % O_2).

In addition, the control group in each study was always the standard volume as normally applied in the lab: 25 μ l in our study and 35 μ l in the study of Minasi et al. [10]. To this extent, one could argue that a 35 μ l culture droplet could induce impaired development due to the greater dilution of autocrine factors. However, Minasi et al. [10] also compared the embryo development between 15 and 35 μ l droplets and observed no difference in the developmental capacity between

the two groups (47.7 versus 45.3 %). Therefore, we believe that constitutional differences between the Sage and Origio media may, at least in part, justify the contrasting conclusions in terms of embryo development found in both studies.

Most data on culture systems is available from animal models, but these are not always the best models for human embryo culture. A recent review on mammalian culture systems by Vajta et al. [18] described the effect of different culture systems and their effects on blastocyst formation or progeny. It seems that pig, instead of mouse and cattle, may be considered as a more appropriate animal model for human embryo culture. Regarding the embryo culture studies performed on human embryos, it is difficult to compare our results with older studies using more basic culture media under high oxygen [22, 14]; especially since it is known that the current culture media are highly enriched and that the use of low oxygen concentration is now consensual for all mammalian embryo culture systems [18]. Most data on culture media derives from studies comparing two different media and these results have been well summarized by Mantikou et al. [9]. However, little information is available on studies in which one culture medium is used in different setups (grouped culture or not, different culture volumes). In the study by Ebner et al. [5], grouped culture was compared to individual culture with or without possible medium exchange, and a higher blastulation rate was observed in the grouped culture (55.8 versus 40.8 and 45.2 %, respectively, per normally fertilized zygote). An equally low blastocyst formation rate (~40 %) was observed in the study of Restelli et al. [13] where random versus “definite” (based on the zygote type) grouping was compared. The study of Rebollar-Lazaro and Matson [12] showed a significantly higher rate of usable blastocysts when grouped culture was performed until day 3 (51.3 %) compared to single culture to day 5 (46.5 %). The choice of an embryo culture system, being grouped or individual, also depends on the needs and preferences of each individual lab. In our unit, especially for day 3 transfers, we rely on the division rate on day 1 and day 2 in order to select the best embryo among equal-quality embryos on day 3.

Minor adaptations to the culture system in order to generate higher blastulation rates may seem, at first glance, as a very attractive research field and an adequate surrogate for the overall performance of a system. However, the type of blastocyst that is obtained should be the main focus of our attention as embryologists. Previous studies have shown that blastocyst expansion [19] and TE morphology result in higher clinical pregnancies and live birth rates [1, 17]. Furthermore, the number of blastomeres on day 3 is also an important determinant of blastocyst yield [15]. Therefore, studies on blastulation rate should also take into account these parameters.

In conclusion, the results of the current sibling study, performed in Origio sequential media, do not support the

previously reported hypothesis of an increased blastocyst formation when culture is performed in a reduced volume. Depending on the culture system, culture medium, and conditions, every laboratory should investigate which is the optimal culture volume in order to achieve the best blastocyst qualities in their own setting.

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