Improved blastocyst formation with reduced culture volume: comparison of three different culture conditions on 1128 sibling human zygotes

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Abstract

Purpose The aim of the present randomized, comparative study was to evaluate the effect of reduced culture volumes on sibling human embryo development.

Methods Firstly, sibling injected oocytes obtained from 88 out of 165 consenting couples undergoing infertility treatment were cultured either in large (35 μ l) or in small drops (15 μ l) of culture medium. Secondly, sibling injected oocytes from 77 couples were cultured either in large (35 μ l) or in mini drops (7 μ l). Embryo quality on day-2 and day-3 and blastocyst formation rate on day-5 were evaluated.

Results No statistically significant difference in terms of embryo quality was detected comparing embryos cultured either in large (35 μ l) or small (15 μ l) drops until blastocyst stage. Similarly, no difference appeared between large (35 μ l) or mini (7 μ l) drops until day-3, however a significantly higher blastocyst formation rate was observed in mini (7 μ l) drops on day-5.

Conclusions Reduced culture volume seems not to influence early embryo development but a reduction of medium appears to positively affect blastocyst development. This supports the hypothesis that the pre-implantation embryo produces autocrine factors which exert a positive effect on embryo development when culture is performed in a reduced volume.

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

Introduction

Embryo culture is a critical aspect in IVF and plays a key role in achieving good clinical results. An adequate in vitro culture system should reproduce as much as possible the natural environment of human embryos, however, important differences exist.

Studies concerning embryo physiology and metabolism have highlighted the importance of autocrine and paracrine growth factors affecting embryo development in vivo [1–10]. However, recent data have shown that preimplantation embryos promote their own development in vitro also in absence of paracrine factors secreted by the female reproductive tract [4, 6, 7, 11, 12]. It seems that embryos generate an autocrine loop of growth stimulation, producing their own growth factors and receptors [1, 4, 6, 7, 13]. Thus, in vitro culture is of great importance for an optimal embryo development, in particular, culture volume plays a key role since large culture volumes may dilute these beneficial (autocrine) factors impairing growth. As a consequence, the reduction of incubation volume has been considered a strategy to improve embryo culture since small incubation volumes would prevent dilution, enhancing the autocrine loop [14]. Contrarily, in small volumes, embryos are more likely to be exposed to embryotoxic metabolic products (eg. ammonium) which progressively accumulate during embryo culture and which can cause developmental delay or arrest [15–17]. Hence, it is important to find an optimal medium volume to balance the right culture volume balancing the beneficial effect of autocrine factors and the negative effect of embryotoxic substances.

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Capsule Reducing the amount of culture media does not influence early embryo development but can positively affect blastocyst formation.

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Murine and bovine data have suggested that also embryo grouping improves IVF cultures since paracrine interaction among embryos may complement those of reproductive tract origin [1, 2, 4, 6–8, 10, 18, 19]. However, in the literature, data concerning a positive effect of group culture on human embryo development are conflicting [6–8, 10, 14, 20, 21]. Furthermore, culturing embryos in group is not a suitable method for who routinely monitors the development of individual embryos from early stages of fertilization [6, 7, 22, 23].

This led us to perform a study concerning individual embryo culture and their ideal culture environment. Specifically, the study aims to evaluate the effect of a reduced culture volume on embryo development and blastocyst formation of individually cultured human embryos.

Materials and methods

In our routine, embryos are cultured individually in 35 μ l drops covered with mineral oil. In this study, embryo development of 1128 sibling human zygotes was evaluated in three different volumes of culture drops: Large Drops of 35 μ l (laboratory standard), Small Drops of 15 μ l and Mini Drops of 7 μ l. The assessed outcome measures were embryo quality on day-2 and day-3 and blastocyst formation rate on day-5.

Patients

The study group included a total of 165 couples after written consents undergoing an assisted reproductive program. Selected patients have six or more mature oocytes available for sperm injection. Female patient characteristics were the following: age ≤ 37 (mean age $\pm$ SE 36.0 ± 4.5), basal FSH ≤ 10 IU/l (mean value 6.93 ± 2.54), body mass index (BMI = weight (kg)/height (m)²) ≤ 27 (mean value 20.92 ± 4.46), menstrual cycle range 24–35 days (intra-individual variability ± 3 days) and normal karyotype of both subjects. Male partner semen characteristics are: sperm count $\geq 15 \times 10^6$ /ml with sperm mobility of A+B ≥ 50 % according to the World Health Organization criteria [24] and normal morphology ≥ 4 % according to Kruger's strict criteria [25]. Specific disorders such as PCOS, pelvic endometriosis, metabolic and quality autoimmune syndromes and couples in which semen derives from a cryopreserved or surgical sample were excluded from the study in order to prevent a technique-derived bias. Ovarian stimulation was conducted in all patients as described elsewhere [26] using a GnRH agonist (buserelin acetate 0.2 mg twice daily; Suprefact; Aventis Pharma, Milan, Italy) started on day 21 of the menstrual cycle, recombinant FSH (Puregon 100 IU; Organon, Rome, Italy) and HCG (Gonasi; Amsa, Rome, Italy).

Phases

The study consisted of two phases. During the first part of the study, the development of embryos obtained from 88 ICSI cycles (from October to December 2010) and cultured either in large or small drops was observed (Part I: 35 μ l vs. 15 μ l). For each of these 88 patients, injected oocytes were randomly and individually allocated in a one-to-one ratio into two different dishes containing 35 μ l and 15 μ l respectively. In the second part, the development of embryos obtained from 77 ICSI cycles (from May to July 2011) and cultured either in large or in mini drops was examined (Part I: 35 μ l vs. 7 μ l). Injected oocytes of these 77 patients were randomly and individually cultured in two different dishes containing 35 μ l and 7 μ l respectively. Randomization was performed by a computer generated randomized one to one ratio, according to a balance block system immediately after oocyte injection.

Microdrop preparation

Either large, small or mini drops were prepared on 35 $\times$ 10 mm polystyrene dishes (Becton Dickinson Labware, NJ, USA) under the following condition: absence of airflow and work-surface temperature $\sim 23^\circ$ (RT). In details, large drops (35 μ l) were prepared pipetting the amount of 25 μ l of media directly onto the dish, overlaid by 3 ml oil (Oil for Tissue Culture, SAGE, USA) and then, refilled with 10 μ l of the same media. Small drops (15 μ l) were prepared pipetting the amount of 8 μ l of media directly onto the dish, overlaid by 3 ml oil and then, refilled with 7 μ l of the same media. Mini Drops (7 μ l) were prepared pipetting the amount of 4 μ l of media directly onto the dish, overlaid by 3 ml oil and then refilled with 3 μ l of the same media. Dishes were prepared once at a time.

Oocyte and embryo culturing preparation

After retrieval, Cumulus Corona Complex COCCs were incubated in fertilization medium (Quinn's Advantage Protein Plus Fertilization Medium, SAGE) until denudation. For all oocytes, denudation was obtained by brief incubation for 10 s in hepes-buffered medium (Quinn's Advantage® Medium with Hepes, Sage, USA) containing 20 IU/ml of hyaluronidase fraction VIII (Hyaluronidase 80 U/ml in HEPES-HTF, Sage, USA). Subsequently, oocytes were gently aspirated in and out of a plastic pipette (Flexipet, 170 and 140 μ m i.d., COOK, Australia) to allow the complete removal of cumulus and corona cells. Only oocytes with first polar body extruded (metaphase II) were selected for sperm injection. Finally, injected oocytes were moved to cleavage medium (Quinn's Advantage Protein Plus Cleavage Medium, SAGE). No medium change-over was performed during embryo culture. Only embryos cultured to blastocyst stage were moved for sequential culture in a dish containing drops of the same size

of fresh blastocyst medium (Quinn's Advantage Blastocyst Medium, SAGE) on day-3.

Sperm samples evaluation and preparation

Sperm samples were collected by masturbation after 3–5 days of sexual abstinence and examined by microscopy at 40× magnification after liquefaction. Sperm concentration was assessed using a Makler counting chamber, motility was assessed according to World Health Organization criteria [24] and morphology according to Kruger's strict criteria [25]. All sperm samples were prepared by swim-up technique immediately after oocyte retrieval. The whole semen sample was washed by centrifugation at 400g for 10 min in HEPES-buffered medium supplemented with human serum albumin (Quinn's® Sperm Washing Medium, Sage, USA). Then, 0.3 ml of medium (Quinn's Advantage® Fertilization HTF Universal Medium, Sage, USA) was gently layered over the resuspended pellet and the sample was incubated in an atmosphere of 37 °C and 6 % CO₂ for 30 min. Finally, the upper layer containing motile sperm was removed and placed in a new tube and maintained in an atmosphere of 37 °C and 6 % CO₂ until ICSI was performed.

Microinjection

Intracytoplasmic sperm injection (ICSI) was performed at 38 h post hCG administration at 400 magnification under an inverted microscope equipped with Hoffman Modulation contrast (Nikon Diaphot 300, Tokyo, Japan). The microtools used for sperm injection are commercially available (Origio Humagen pipets, Charlottesville, VA). Microinjection dishes (cat. No. 1006, Falcon; Becton-Dickinson Labware) consisted in 10 µl microdroplets of HEPES-buffered medium (Quinn's Advantage® Medium with Hepes, Sage, USA) supplemented with 5 % human serum albumin (Human Serum Albumin, Sage, USA) and one 10 µl microdroplet of 7 % polyvinylpyrrolidone solution (PVP 7 % Solution, Sage, USA). In order to prevent evaporation, mineral oil was layered on the microdroplets of the injection dish. Sperm injection was performed as described elsewhere [27].

Assessment of fertilization, zygote and embryo quality

The same inverted microscope used for sperm injection was used for assessment of fertilization and further development. Fertilization was assessed 16–18 h after injection using the scoring system developed by Tesarik and Greco [28]. Embryo morphology was recorded on day-2 and 3 using the scoring system reported by Rienzi et al. [27]. Briefly, for each embryo, the number and size of the blastomeres were observed, as well as the percentage of anucleate fragments. Cleaved embryos with 20 % anucleated fragments and with equal-sized

blastomeres were considered type A. When the percentage of anucleate fragments was between 20 and 50 % the embryos were considered type B. Finally, when 50 % anucleated fragments were present, embryos were considered type C. On day-5, the percentage of blastocyst formation was determined: a blastocyst was recorded when even a small cavity was visible and each blastocyst was assigned a score by using the system of Gardner and Schoolcraft [29]. Embryo transfers were performed on day-2, day-3 and day-5. The choice of the embryo/s to be transferred was carried out on a morphological basis, independently on the volume in which it was cultured. Often, embryos cultured in different drop volumes were transferred simultaneously in the same patient, for this reason, data concerning pregnancy rates are not conclusive and they have not been analysed.

Ethics

Embryo cultures in volumes between 5 and 50 µl are standard methods routinely used in the present laboratory work as already reported in the manuscript by Ebner and colleagues in 2010 [14]. For this reason, the Institutional Ethics Committee of the European Hospital has decided to approve the present study proposal. This study is aimed to evaluate the effect of reduced culture volumes on sibling human embryos development. It emerges that data were collected as part of routine diagnosis and treatment without affecting in any way the good practice of the fertility center.

Statistics

Statistical analysis was performed using Fisher's exact test at the level of $P \leq 0.05$.

Results

During the first part of the study, sibling injected oocytes from 88 consenting patients were randomly and individually cultured either in 35 µl or 15 µl. In this group (Group I) a total of 574 sibling zygotes were obtained (308 cultured in 35 µl and 266 in 15 µl) and embryo development was examined and recorded on day-2, day-3 and day-5. In the second part of the study, sibling injected oocytes from 77 consenting patients were randomly and individually cultured either in 35 µl or 7 µl. In this group (Group II) a total of 554 sibling zygotes were obtained (294 cultured in 35 µl and 260 in 7 µl) and embryo development was recorded until day-5.

No statistically significant differences were observed between the two groups with regard to the mean age of female patients (35.8 ± 3.9 and 36.2 ± 5.0 respectively for Group I and II).

In Group I, a total number of 282/308 (91.6 %) type A+B embryos were observed in 35 μ l -subgroup and 238/266 (89.5 %) in 15 μ l -subgroup (NS) on day-2. On day-3, type A+B embryos were 208/264 (78.8 %) in 35 μ l -subgroup and 172/217 (79.3 %) in 15 μ l -subgroup (NS). Thirteen patients had embryo transfer on day-5 with a rate of blastocyst formation of 45.3 % (24/53) and 47.7 % (21/44) in 35 μ l -subgroup and in 15 μ l -subgroup respectively (NS) (Table 1).

In Group II, a total number of 264/294 (89.8 %) type A+B embryos were obtained in 35 μ l -subgroup and 236/260 (90.8 %) in 7 μ l -subgroup (NS) on day-2. On day-3, type A+B embryos were 218/264 (82.6 %) in 35 μ l -subgroup and 189/235 (80.4 %) in 7 μ l -subgroup (NS). Finally, 19 patients had embryo transfer on day-5 with 50.5 % (50/99) in 35 μ l -subgroup and 70 % (56/80) in 7 μ l -subgroup of blastocyst formation rate (Fisher's exact test $P \leq 0.05$) (Table 2).

Discussion

Different studies concerning embryo physiology and metabolism, together with the analysis of tubal and uterine fluids, have highlighted the importance of growth factors in embryo development [3, 10].

In animal models, small culture volume as well embryo grouping have been proposed as two different strategies to improve embryo developmental potential [2, 4, 5, 7, 8, 10, 18, 19, 30] since autocrine and paracrine factors of embryonic origin are likely to mimic in vivo conditions [1, 4, 5, 7, 9, 13]. In a recent review, Reed and coauthors [7] describe the impact of group versus individual cultures on embryo development. In group cultures, a beneficial effect on embryo quality, due to embryotrophic factors secreted from neighboring embryos, was supposed. Anyway, it is still not possible to isolate or identify such secreted factors. However, when performing group cultures, an accumulation of detrimental metabolic factors may impair embryo development. On the contrary, in individual cultures the issue of a potential depletion of

Table 2 Embryo quality and blastocyst formation with regard to culture volume (LD vs. MD)

	Excellent/good embryos Day-2	Excellent/good embryos Day-3	Blastocyst Day-5
LD (35 μ l)	264/294 (89.8 %)	218/264 (82.6 %)	50/99 (50.5 %) ^a
MD (7 μ l)	236/260 (90.8 %)	189/235 (80.4 %)	56/80 (70.0 %) ^a
	NS	NS	$P < 0.05$

Values in parentheses are percentages. The table reports the number of excellent/good embryos on day-2 and day-3 and the number of blastocysts on day-5 with regard to the volume of media in which embryos were cultured, Large Drops (LD) and Mini Drops (MD), respectively

^a Fisher's exact test $P < 0.005$

nourishing substrate factors is avoided as well as the negative impact of toxic factors secreted by sibling embryos. In addition, individual cultures allow to identify and evaluate each embryo which is very useful and practice in the daily routine [7]. However, in humans, studies comparing individual and group culture show controversial results [6, 7, 10, 14, 20, 21, 31] and few data concerning the effect of a reduced culture volume on embryo development are available.

Our study involves the observation of sibling human embryos from the zygote to the blastocyst stage avoiding to compare embryos generated from different simulation protocols [20, 21] and it is the first study centered on individual embryo culture and on the effect of reduced culture volumes (7 μ l, 15 μ l, 35 μ l) on human embryo development. Our results show that no statistically significant difference in terms of embryo morphology exists comparing embryos cultured either in large (35 μ l) or small (15 μ l) drops until blastocyst stage. The same was observed for embryos cultured either in large or mini drops until day-3, but a significant higher blastocyst formation rate was observed in mini drops (7 μ l) with respect to large drops (35 μ l) on day-5 (50.5 % vs. 70 % respectively). This finding is in agreement with a previous study conducted by Rijnders and Jansen [21] who performed a study on group and individual human embryo cultured in large (160 μ l) or small volumes (5 μ l). It involved the observation of embryos from day-3 of development until blastocyst stage and the authors reported that single culture in a small volume showed the highest blastocyst formation rate. However, in this case, the difference was not significant. Also study conducted in mice by Lane and Gardner [2] showed no significant difference in blastocyst formation between single embryos cultured in three different volumes, 320 μ l, 20 μ l, 5 μ l. While a study performed by Melin and colleagues [32] even showed that reducing culture volume to 5 μ l from 20 μ l did not “rescue” mouse embryo development, but resulted in development further compromised (blastocyst developmental rate were 50 % and 86,6 % respectively). However, all these studies adopted 5 μ l as the “reduced volume” and it is possible

Table 1 Embryo quality and blastocyst formation with regard to culture volume (LD vs. SD)

	Excellent/good embryos Day-2	Excellent/good embryos Day-3	Blastocyst Day-5
LD (35 μ l)	282/308 (91.6 %)	208/264 (78.8 %)	24/53 (45.3 %)
SD (15 μ l)	238/266 (89.5 %)	172/217 (79.3 %)	21/44 (47.7 %)
	NS	NS	NS

Values in parentheses are percentages. The table reports the number of excellent/good embryos on day-2 and day-3 and the number of blastocysts on day-5 with regard to the volume of media in which embryos were cultured, Large Drops (LD) and Small Drops (SD), respectively

that this amount did not respect the ideal balance between the negative effect of toxic metabolic molecules and the positive effect of beneficial autocrine factors.

Also for this reason we decided to test 7 μ l instead of 5 μ l as “reduced volume” in our study since even just 2 more microliters may be important to prevent evaporation and, as a consequence, change in osmolarity, which can significantly impact embryo development [33]. In addition, we believe that using small culture volumes can be critical if some environmental conditions are not respected during dish preparation. Airflows and the use of heated stages should be avoided since they can increase media evaporation, in particular when dealing with small volumes and dishes should be prepared once at a time with attention to quickly overlaying mineral oil on top of the media microdroplets. Probably, these precautions have minimized the negative effects deriving from the use of reduced volumes (evaporation and osmolarity variation) allowing beneficial factors deriving from the embryo itself (autocrine factors) positively influence embryo development.

Finally, a further consideration should be made. Since randomization was performed immediately after injection, the culture volume could have impacted fertilization rate, selecting out the less developmentally competent oocytes, which in turn, may have resulted in the different blastocyst formation rate observed between the groups 35 μ l vs. 7 μ l.

In conclusion, our study confirm that media volume plays a key role in individual embryo culture, where paracrine factors are absent and the embryos relies just on autocrine factors. In fact, results demonstrate that a reduced volume, balancing the beneficial effect of autocrine factors and the negative effect of embryotoxic substances and evaporation, improves embryo development in vitro.

Since embryos for transfers were selected on a morphological basis, independently to the drop size, data about pregnancy and implantation are not conclusive. In order to obtain these clinical data, a prospective study is in progress, were all patients enrolled are entirely allocated in the two different volume drops groups (35 μ l vs. 7 μ l).

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Author contributions M.G.M. was involved in study conception and design, data interpretation, acquisition of data, critical revision of the article and final approval. G.F. was involved in drafting manuscript and final approval. V.C. played a central role in all laboratory procedures and was involved in final approval. A.M.L. was involved in all laboratory procedures and in final approval. A.C. was involved in all laboratory procedures and in final approval. F.S. was involved in all laboratory procedures and in final approval. E.G. was involved in patient recruitment and preparation and critical revision of the article.

Conflict of interest The corresponding author discloses any potential conflict of interests for any of the submitting authors, in reference to the submitted material.

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