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Morphological differences in human zygotes and embryos cultured in different media

Raquel Di Falco Cossiello[2], *Alexandros Aggelis*[2], *Daniel Faúndes*[2] *and Carlos A. Petta*[1,3]
Campinas Center of Human Reproduction; and Department of Obstetrics and Gynecology, University of Campinas, Campinas, São Paulo, Brazil

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Summary

Purpose: To compare the effects of four culture media on the quality of human zygotes and embryos. Methods: Prospective study analyzing 2289 human embryos cultivated simultaneously in two different culture media: HTF, the default medium, with either Universal IVF, Global or IVF-30 as the secondary media. The sibling oocytes by each patient were randomly divided between the two culture media following intracytoplasmic sperm injection (ICSI). On day 1 the pronuclear stage of zygotes were evaluated and on day 2 embryos were evaluated according to the number of cells, percentage of fragmentation and number of nuclei. Z-test and odds ratios were used in the statistical analysis. Results: There was a higher percentage (55.2%) of class A1 + A2 zygotes with IVF-30 compared with HTF, Global or Universal IVF media (49.1%, 44.7% and 44.2%, respectively). The percentage of Top embryos was significantly higher with Global (40.2%) compared with HTF (21.3%), IVF-30 (25.0%) or Universal IVF media (11.2%). Conclusions: Global medium produced more Top embryos evaluated on day 2 of development.

Keywords: Culture media, Embryonic development, Pronuclear stage

Introduction

At the advent of *in vitro* fertilization (IVF), a large number of different media formulations and culture systems was proposed for the development of human zygotes and embryos. Culture media ranged from simple balanced saline solutions to more complex media. Simple culture media were used in assisted reproductive technology (ART) until the mid-1990s. These media, consisting basically of saline solutions, glucose, pyruvate, lactate and carbohydrates supplemented with protein, were effective for culture up to the third day of development. However, the introduction of more complex media (Fissore *et al.*, 1989) that contained amino acids presented new alternatives for *in vitro* culture.

The capacity to simulate natural conditions in the laboratory became fundamental, as the culture of embryos in suboptimal or distressing conditions compels the embryo to undergo physiological adaptations. The features presented by embryos thus affected include delayed cell division (Bowman & McLaren, 1970), poorer incorporation of amino acids (Jung *et al.*, 1987), a reduction in oxidative metabolism and an increase in lactate production (Gardner & Leese, 1990; Gott *et al.*, 1990; Leese *et al.*, 1993). Such factors may lead to consequences that include low pregnancy rates, significantly greater fetal loss (Lane & Gardner, 2007) and low birth weight (Pool, 2005).

Several mechanisms are involved in the development process from oocyte retrieval until embryo transfer to the uterus, with modifications that occur at each stage; however, the embryologist is able to observe and analyze only a few of these steps, one of these being embryo development.

In IVF laboratories, the parameters that are used generally to evaluate embryo quality and consequently the effectiveness of the culture media are based on analysis of the morphologic criteria between days 1 and 5 following fertilization.

For analyses on day 1, embryo pronuclear (PN) classification and morphology represent important variables for assessing different culture media. Evaluation of the pronucleated zygote is based on analysis

[1]All correspondence to: Carlos A. Petta. Campinas Center of Human Reproduction, Rua Eduardo Lane 380, 13073–002 Campinas SP, Brazil. e-mail: cpetta@me.com
[2]Campinas Center of Human Reproduction, Campinas, São Paulo, Brazil.
[3]Department of Obstetrics and Gynecology, University of Campinas, Campinas, São Paulo, Brazil.

of the morphology and position of the pronuclei. Alterations in the appearance or numbers of nuclear precursor bodies (NPBs) (Tesarik & Kopecny, 1989) may cause abnormalities in embryo division and lead to uneven cleavage or fragmentation. Based on these considerations, the morphology of the pronuclei and NPBs has been proposed as a scoring system and a correlation has been suggested between the different patterns observed, embryo development and implantation potential (Montag, 2001).

On day 2 of development, embryos are graded according to blastomere morphology, cleavage stage and fragmentation, and there appears to be a clear relationship between these parameters and implantation and pregnancy rates after IVF (Ziebe *et al.*, 1997). This procedure can also be used to select the best embryos and test the effectiveness of culture media (Ziebe *et al.*, 1997). Moreover the nuclear status of embryo's blastomeres on this day of development is an important evaluation that can predict the implantation rate. Several studies have shown that multinucleated blastomeres are associated with a decrease of implantation chance (Van Blerkom 1990; Ziebe *et al.*, 1997; Van Royen *et al.*, 2003).

The aim of this study was to assess and compare the effects of four different culture media on human zygote and embryo morphology on day 2 of culture.

Material and methods

This prospective study was conducted at the Human Reproduction Center, Campinas, Brazil, in which 2289 embryos were assessed between September 2006 and September 2008 in 319 ICSI cycles. Approximately 80% of the cases in our clinic are ICSI, so we excluded IVF cases to avoid bias regarding the two techniques. All first cycle of each patient was included. ICSI cycles from which spermatozoa were recovered using testicular or epididymal aspiration were excluded from this study. The study was approved by the Institutional Review Board of the University of Campinas (UNICAMP). All patients were informed of laboratory procedures to which they would be submitted and signed an informed consent form prior to the initiation of treatment.

Ovulation induction and oocyte recovery

Ovarian stimulation was initiated following pituitary blockade by leuprolide acetate (Lupron, TAP Pharmaceutical, Abbott Laboratories, Chicago, IL, USA), 0.15 ml/day, beginning on the 21st or 23rd day of the menstrual cycle and maintained until the beginning of the following cycle. On the third day of bleeding, leuprolide was reduced to 0.05 ml/day and ovarian stimulation was initiated with recombinant follicle-stimulating hormone (rFSH) (Gonal F, Serono, São Paulo, SP, Brazil) at a dose of 150–225 IU/day. Follicular development was monitored by transvaginal ultrasonography and the dose of medication was adjusted according to ovarian response.

When at least two follicles $\geq$18 mm in diameter were observed, a single dose of recombinant human chorionic gonadotropin (r-hCG, 250 μg, Ovidrel, Serono, São Paulo, SP, Brazil) was given. Oocytes were aspirated 34–36 h after injection of r-hCG. The oocytes were separated from the follicular fluid under a stereoscope and washed in a modified HTF medium with HEPES (Irvine Scientific, Santa Ana, CA, USA), supplemented with 10% synthetic serum substitute (SSS – Irvine Scientific). All the oocytes were placed in HTF medium (Irvine Scientific) supplemented with 10% SSS and incubated at 37 °C in 5% CO_2 in air.

The cumulus oophorus cells were removed by exposing them to hyaluronidase (H-4272, Sigma, Saint Louis, MI, USA) at a concentration of 80 IU/ml in modified HTF medium with HEPES, supplemented with 10% SSS, for 30 s. The cells were removed with the use of fine hand-drawn glass pipettes. After denudation, the oocytes were washed with HTF culture medium supplemented with 10% SSS and incubated until ICSI was performed (Palermo *et al.*, 1992).

Sperm collection

Semen samples were collected by masturbation immediately after oocyte pickup, evaluated in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and then processed through a discontinuous density gradient (Isolate Lower/Isolate Upper, Irvine Scientific) in accordance with the manufacturer's protocol.

In vitro fertilization and embryo culture

All sibling oocytes were cultured in two different media: human tubal fluid (HTF [Irvine Scientific, Santa Ana, CA, USA]), which was used as the default medium for all the patients, and one of the secondary media (Universal IVF medium [Medicult, Denmark]; LGGG Global [LifeGlobal, Guelph, ON, Canada]; or IVF-30 [VitroLife, CO, USA]). The secondary medium used in each case was determined by local availability.

Following ICSI, the oocytes were separated into the different media. Injected oocytes were transferred alternately to HTF medium or to one of the secondary media until all presumed zygotes were divided between two types of media. The first zygote was randomized following a computer-generated list for one of the media and the following zygotes were distributed alternated, one for each media

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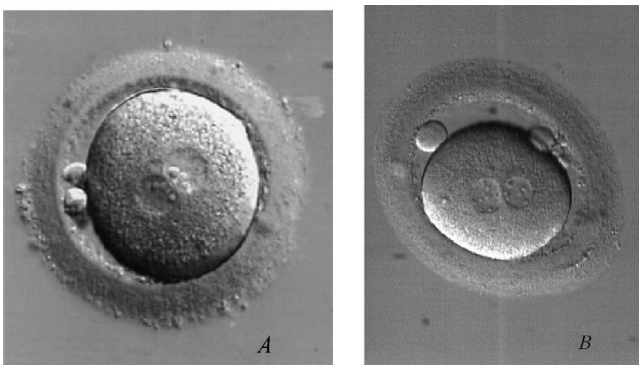

Figure 1 Zygotes morphologies 18–20 h after ICSI. (*A*) Zygote with juxtaposed and centralized pronuclei with aligned nuclear polar bodies (A1). (*B*) Zygote with juxtaposed and centralized pronuclei with scattered nuclear polar bodies (A2).

subsequently. There was no specific period of time that a secondary media was used, they were alternated every month. Embryo culture conditions were 37 °C with 5% CO_2 in air to maintain pH within an optimal range (7.20–7.40) according to the manufacturers' instructions.

Assessment of zygotes

An inverted microscope equipped with Hoffman modulation optics was used to check for the presence of pronuclei and the position of NPBs 18–20 h after ICSI. Based on the classification established by Gianaroli *et al.* (2003), the zygotes were found to be juxtaposed with large centralized pronuclei and aligned NPBs, classified as 'A1', or juxtaposed with centralized pronuclei and large, scattered NPBs, classified as 'A2' (Fig. 1). After fertilization was verified and pronuclei classified, the zygotes were transferred to fresh drops of culture media.

Embryo quality

The individually cultured embryos were evaluated 44–46 h after ICSI on the basis of the number of blastomeres, percentage of fragmentation and presence of multinucleated blastomeres. Embryos were considered to be of top quality (Top) when they had four regular blastomeres, fragmentation in less than 20% of the volume of the embryo and no multinucleated blastomeres (Guerif *et al.*, 2007).

Embryo transfer

The embryo transfer had been carried through at the second day of embryo development. Embryo selection for transfer was based on assessment of morphology of the number of blastomeres, percentage of fragmentation and presence of multinucleated blastomeres (Guerif *et al.*, 2007). All embryo transfers were performed under transabdominal ultrasound guidance with a Frydman catheter (Laboratoire CCD).

Statistical analysis

Quantitative data were assessed for normality and homoscedasticity using the Kolmogorov-Smirnov and F-tests, respectively. The number of mature oocytes (metaphase II; MII) was compared using analysis of variance (ANOVA) followed by Tukey's test for means. The number of blastomeres of the embryos on day 2 was evaluated using the Kruskal–Wallis test followed by a group by group comparison using the Mann–Whitney test. Frequencies of zygotes with A1 and A2 pronuclear morphology and embryos with top quality morphology in each group were compared using the Z-test for two proportions. Parametric data were presented as means ± standard deviations (SD) and non-parametric data were presented as medians and ranges. The Bonferroni correction was used to determine statistically significant differences and significance was defined at $p < 0.008$.

This study was performed with total independence from medium manufacturers.

Result

There was no difference in the age of the woman between the cycles in which the embryos were cultured in different media (HTF 33.6 ± 4.6, Global 32.7 ± 4.4, IVF-30 33.3 ± 4.6, Universal IVF 34.6 ± 4.6), neither in the number of mature oocytes (MII) between cycles (HTF 12 (1–32), Global 11 (2–17), IVF-30 12 (2–32), Universal 16 (3–27)).

A total of 2289 embryos were included in the analysis: 1170 were cultivated in HTF medium; 199 in Global medium; 260 in Universal IVF medium; and 660 in IVF-30 medium. When both the pronuclei and the position of the NPBs were taken into consideration, the percentage of A1 + A2 zygotes was found to be higher with the IVF-30 medium. The frequency of the A1 + A2 pattern was 55.2% in zygotes cultured in IVF-30 medium compared with 49.1%, 44.7% and 44.2% in HTF, Global and Universal IVF media, respectively. The results found with IVF-30 were significantly higher when compared with those of the other media; however, the differences between the Global, HTF and Universal IVF media were not statistically significant (Table 1).

When the number of blastomeres was compared after 44–46 h of culture, the HTF, Global and IVF-30 media produced more 4-cell embryos, while Universal IVF medium produced more 3-cell embryos. In

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Table 1 Comparison of frequencies of pronuclei and NPBs between HTF, Global, IVF-30 and Universal IVF medium

	Other pronuclear pattern than A1 + A2		Pronuclear pattern A1+A2				
Medium	*n*	%	*n*	%		OR (CI 95%)	*p*-value
HTF	595	50.9	575	49.1	1.00	(control)	
Global	110	55.3	89	44.7	0.84	(0.62–1.13)	0.2486
Universal IVF medium	145	55.8	115	44.2	0.82	(0.63–1.08)	0.1514
IVF-30	269	42.5	364	57.5	0.98	(0.68–1.42)	0.9161
Global	110	55.3	89	44.7	1.00	(control)	
Universal IVF medium	145	55.8	115	44.2	1.27	(1.05–1.54)	0.0007
IVF-30	269	42.5	364	57.5	1.52	(1.11–2.09)	0.0098
Universal IVF medium	145	55.8	115	44.2	1.00	(control)	
IVF-30	269	42.5	364	57.5	1.55	(1.16–2.07)	0.0028

embryos cultivated in Global medium, the variation in blastomere numbers on day 2 was less than that found with embryos cultivated in HTF or IVF-30 media, in which there were a significant number of embryos with fewer than four cells.

The presence of Top embryos was evaluated in all the different media between 44 and 46 h after ICSI. The percentage of embryos with four regular blastomeres, fragmentation in less than 20% of the volume of the embryo and no multinucleated blastomeres was significantly higher in Global medium (40.2%) compared with HTF (21.3%), IVF-30 (25.0%) or Universal IVF media (11.2%) (Table 2).

Discussion

In this study, use of the IVF-30 medium resulted in good morphology in a substantial number of zygotes on day 1; however, embryo morphology was found to be better when the embryos were cultivated in Global medium. When the pronuclear and nucleolar parameters were analyzed, Universal IVF medium was found to be associated with fewer zygotes with good (A1 + A2) PN morphology (44.2%) compared with the other culture media, and with fewer top quality day-2 embryos. This finding shows that these parameters should be analyzed together and not individually (James *et al.*, 2006).

The IVF-30 culture medium produced a high number of zygotes with good (A1 + A2) PN morphology (55.2%) but the number of good quality embryos produced with this medium was low. In contrast, Global medium produced the highest percentage (40.4%) of good quality embryos on day 2 although this medium produced fewer good PN on day 1. Universal IVF medium produced the smallest percentage of good quality embryos compared with the other culture media used in this study; nevertheless, there were no differences in the percentage of good PN obtained with this medium compared with the HTF and Global media. With respect to the formation of zygotes with A1 + A2 pronuclear patterns, no difference was found between the HTF medium and the Global and Universal IVF media, and when the production of good quality embryos was compared, results achieved with the HTF medium were found to be similar to those obtained with the IVF-30 medium.

The correlation between zygote and embryo morphology has already been demonstrated (Scott & Smith, 1998; Tesarik & Greco, 1999; Ludwig *et al.*, 2000; Scott *et al.*, 2000); however, this correlation was not linear, suggesting that some degree of predictability is lost when considering either zygote or embryo morphology alone (Rijnders & Jansen, 1998). One study compared the predictive value of different morphological parameters using a score system for human zygotes and preimplantation embryos and showed that zygote and embryo morphology were independent variables predictive of IVF outcome, and that both may be used as criteria to select the best embryos for transfer (De Placido *et al.*, 2002). Another recent study demonstrated that the embryo morphology at second day of development was a better tool than pronuclear analysis (Bar-Yoseph *et al.*, 2011). Picking the top quality embryos is the most

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Table 2 Comparison of the frequency of good quality (Top) embryos between HTF, Global, IVF-30 and Universal IVF medium

	Other than Top embryos		Only Top embryos				
Medium	*n*	%	*n*	%		OR (CI 95%)	*p*-value
HTF	921	78.7	249	21.3	1.00	(control)	
Global	119	59.8	80	40.2	2.49	(1.81–3.41)	<0.0001
Universal IVF Media	231	88.8	29	11.2	0.46	(0.31–0.70)	<0.0001
IVF-30	495	75.0	165	25.0	1.23	(0.98–1.54)	0.0071
Global	119	59.8	80	40.2	1.00	(control)	
Universal IVF Media	231	88.8	29	11.2	0.19	(0.12–0.30)	<0.0001
IVF-30	495	75.0	165	25.0	0.50	(0.36–0.69)	<0.0001
Universal IVF Media	231	88.8	29	11.2	1.00	(control)	
IVF-30	495	75.0	165	25.0	2.66	(1.74–4.06)	<0.0001

important selection criteria to evaluate to transfer, but when the same pattern of quality embryos was present, the pronuclear evaluation was an important criterion to decide the best embryo to transfer.

Nowadays, most IVF laboratories select the best embryo according to embryo morphology parameters on the day of transfer. However, there are additional criteria that may be used to select the best embryo such as evaluation of the pronuclear morphology, NPB size and distribution, cytoplasmic halo and early cleavage (Rienzi *et al.*, 2005; Scott *et al.*, 2007). It is important to determine the pronuclear and NPB morphology of zygotes on day 1 and embryo quality on day 2 of development, as this situation represents a non-invasive instrument for performing a quick, precise and reproducible analysis that does not interfere with the daily clinical work of the IVF laboratory (Gianaroli *et al.*, 2003).

The development of good quality embryos was shown to be related to the pronuclear pattern and was better in the case of zygotes with centralized, juxtaposed pronuclei (pattern A, 96%) compared with the other patterns. Moreover, embryo development was similar in the four patterns of zygotes, whereas chromosomal condition varied depending on nuclear morphology (Gianaroli *et al.*, 2003).

Overall, conflicting results exist and there is currently no consensus on: (1) the optimum day(s) for evaluation; (2) the optimum set of variables that should be used in a predictive model; and (3) model performance benchmarks for future comparison. Answers to these questions are necessary so that quantitative comparisons can be made about the relative efficacies of the different evaluation protocols (Racowsky *et al.*, 2009).

Differences found in the composition of the culture media may explain the variation in Top embryo formation. Global medium was the only monoculture medium used in this study. The formulation used in Global medium is capable of supporting embryo development from pronuclear formation to blastocyst stage. The addition of amino acids is the principal difference between Global medium and the other simple media used in the present study. This feature may explain the higher formation of top quality embryos found with this medium. Previous studies have confirmed that the addition of amino acids to culture medium enhances embryo development, while when mouse zygotes were exposed to medium without amino acids their subsequent potential for development was impaired (Gardner & Lane 1996; Lane & Gardner, 2007).

The addition of ethylenediaminetetraacetic acid (EDTA) is another characteristic that differentiates some media from others used in the present study. The beneficial effects of EDTA are confined to the early cleavage stage embryo (Gardner & Lane, 1996). Of all the media used in this study, only IVF-30 contained EDTA in its composition and this factor may explain the difference in the location and distribution of the pronuclei and NPBs. It has already been shown that human zygote pronuclei are formed in different locations in the oocyte, both migrating towards the centre through time. During this movement, cytoplasmic rotation and cytoskeleton structures are probably involved, possibly co-ordinated by calcium levels. The presence of EDTA in the culture medium may affect the efficiency of the migration of the pronuclei and result in different pronuclear patterns in the zygotes.

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In the present study, Global medium produced more top quality embryos (40.4%) with four regular blastomeres, fragmentation of less than 20% of the embryo and no multinucleated blastomeres. This finding was followed by IVF-30 (25.0%), HTF (21.2%) and Universal IVF (11.1%) media according to the morphological analysis of the embryo on the second day of development.

Several studies that compared different commercial culture media for IVF have been published in the literature. Xella *et al.* (2010) showed that IMS1 (Medicult) culture medium appears to improve the performance of embryonic growth and development and increase pregnancy rates when compared with Universal IVF medium (Medicult). One study compared four media and found better embryo quality with Global medium rather than with cleavage medium, HTF or G1.2 (Aoki *et al.*, 2005). However, no difference was found in day-2 embryo score or pregnancy, implantation or abortion rates between the P-1 and IVF-50 media (Mauri *et al.*, 2001) or in the implantation rates achieved with the different media (Zollner *et al.*, 2004).

This comparison between different media for the culture of sibling oocytes has proven to constitute a valuable tool for the assessment of embryo quality. Traditional studies that compared culture media have evaluated different patients; however, in the present study the same patient received transferred embryos that had been cultivated in different media. Nevertheless, the assessment of implantation and pregnancy rates is problematic due to a high frequency of 'mixed' transfers, resulting in a reduced number of cases in which the embryos transferred were grown exclusively in one type of medium.

Zygote score is a good predictor of embryo quality, but should be used in combination with the evaluation of embryo morphology on the day of transfer. A1 + A2 zygote morphology is associated with chromosomally normal embryos; however, not all chromosomally normal embryos are morphologically normal as well. Top embryos cannot have blastomeres with more than one nucleus or a high percentage of fragmentation, and these characteristics are not directly dependent on pronuclear morphology (Gianaroli *et al.*, 2007).

Another important piece of information is that day 2 or day 3 evaluations alone are sufficient for morphological selection of cleavage stage embryos, which show that our analysis of day 2 are a beneficial tool to choose the best embryo (Racowsky *et al.*, 2009).

One of the principal strengths of the present study is that it is entirely independent, having received no financial support from any of the manufacturers of the different types of media. The experience described here of a single clinic in which two types of culture medium were used concurrently in each cycle may help other services decide which media should be used and also stimulate them to implement this strategy to prevent the unlikely but feasible occurrence that an alteration in one of the media could compromise the whole treatment. Nevertheless, not being able to establish a correlation between the different culture media and pregnancy rate constitutes a limitation to this study. This limitation, however, was unavoidable, as each patient received embryos from different culture media.

In conclusion, the present data show significant differences between the four culture media with respect to pronuclear, NPB and embryo morphology. The use of IVF-30 medium resulted in a higher number of zygotes with centralized pronuclei and juxtaposed or scattered nucleoli, while Global culture medium resulted in the formation of more top quality embryos on day 2 of development. In addition, cleavage rates were lower with the HTF, Universal IVF and IVF-30 media compared with Global medium. More studies with larger sample sizes are needed to permit evaluation of embryo development further to include blastocyst culture, implantation rates in an eSET and pregnancy rates following the culture of sibling embryos in different media.

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