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Original paper

Critical reappraisal of embryo quality as a predictive parameter for pregnancy outcome: a pilot study

R. Campo[1], M. M. Binda, G. Van Kerkhoven, V. Frederickx, A. Serneels, P. Roziers, A. S. Lopes, Sy. Gordts, P. Puttemans, S. Gordts

Leuven Institute for Fertility and Embryology (LIFE), Leuven, Tiensevest 168, 3000 Leuven, Belgium.

Correspondence at: Tel.: +32 (0)16 270 190; fax: + 32 (0)16 270 197; e-mail: Rudi.Campo@lifeleuven.be

Abstract

Aim of the study: Pilot study to analyse the efficacy and embryo morphology using a new human embryo culture medium (GM501) versus the conventional used medium (ISM1).
Methods: Over a four-month period, all patients at the Leuven Institute of Fertility and Embryology (LIFE) were randomly allocated to have their embryos cultured in either the standard sequential culture medium ISM1 (control) or in a new universal medium (GM501) (study group). Primary outcome parameters were clinical pregnancy and live birth rate. The secondary outcome parameter was the correlation of embryo fragmentation rate with pregnancy outcome.
Results: We did not observe any differences between the ISM1 control group and GM501 study group with regard to fertilization, pregnancy, implantation rates, ongoing pregnancy, and babies born. The number of embryos with a minimal fragmentation rate (less than 30%) was significantly higher in the GM501 study group.
Conclusion: Although a significant higher embryo fragmentation rate was seen in *In vitro* culture of embryos in GM501, pregnancy outcome results were comparable to those of embryos cultured in ISM1. According to our results the value of embryo morphological criteria as a parameter for pregnancy outcome should be examined and discussed again.

Key words: Culture medium, fertilization rate, fragmentation, human embryos, IVF, ongoing pregnancy, pregnancy.

Introduction

In vitro, embryos are exposed to stress that can compromise their physiology, gene expression, and development (Gardner *et al*., 2005). In particular, the culture medium is an important determinant of successful *in vitro* interactions between gametes and subsequent embryo development (Gardner *et al*., 1997). Most *in vitro* fertilization (IVF) centers use commercially available culture media, but the formulations of these media are seldom disclosed. In general, manufacturers follow either the "back to nature" or the "let the embryo choose" philosophies (Summers *et al*., 2003). "Back to nature" philosophy attempts to mimic the different biochemical environments that gametes and embryo encounter during the natural reproductive process and provide different media in a sequential order (two-step protocol). The "let the embryo choose" philosophy has led to a family of media in which all of the substances necessary to early embryological development are provided, and there is no need for a media change (one-step protocol).

There is no scientific evidence showing any advantage to one philosophy over the other. Biggers *et al*. (2002) demonstrated the efficacy of a universal medium for human blastocyst development. In a prospective randomized study, no differences were observed for culture and development of human blastocysts, implantation, or pregnancy rates when either a universal or a sequential medium (Macklon *et al*., 2002).

Gynemed GM501 is an optimized variant of KSOM^{AA} medium; it is a novel medium and no comparative data about its use in embryo culture is available. Therefore, we performed a prospective

randomized comparison between this medium and the standard sequential medium ISM1 in terms of pregnancy outcome. Our secondary aim was to investigate the predictive value of embryo fragmentation on pregnancy outcome.

Methods

Population

In a 4-month period, the embryos of all of the patients entering the Leuven Institute of Fertility and Embryology (LIFE) IVF/intracytoplasmic sperm injection (ICSI) program were randomly allocated to either the GM501 culture medium study group or the ISM1 culture medium control group. Blastocyst culture, preimplantation genetic diagnosis (PGD) patients, testicular sperm extraction (TESE), and egg donation procedures were excluded from the study.

Randomization was blinded by providing envelopes filled with an equal amount of adhesive labels printed with either "GM501" or "ISM1." In accordance with Belgian embryo protection law (Gordts *et al*., 2005), the embryo transfer procedures differ depending upon the age of the patient. Therefore, we performed a separate randomization for the < 36 y age group, and the ≥ 36 y age group; the envelopes were contained in different boxes. When the IVF lab forms were prepared, the age of the female patient was determined and an envelope was removed from the appropriate box. The adhesive label taken from the envelope was attached to the lab form, and this determined the culture medium used for all of the embryos from that particular patient.

Culture media

ISM1 (MediCult, Jyllinge, Denmark) was used as the control medium, and Gynemed GM501 (Gynemed GmbH & Co. KG, Lensahn, Germany) was used as the study medium. Both media were supplemented with penicillin and streptomycin. Media were pre-equilibrated for at least 4 h in 5% CO_2 at 37°C prior to use.

Ovarian stimulation and oocyte collection

Patients were treated with a long or short gonadotrophin-releasing hormone agonist (GnRHa) protocol using buserelin (nasal spray, 9 × 100 μg/day; Suprefact, Hoechst, Frankfurt, Germany) starting in the mid-luteal phase of the cycle preceding the IVF attempt for a period of at least 2 wk in the long protocol, or starting on the first day of menstruation following the intake of a contraceptive pill for 2-3 wk in the short protocol.

Hormonal stimulation was performed with urinary gonadotrophins (Menopur; Ferring, Limhamn, Sweden) or recombinant follicle-stimulating hormone (FSH, Puregon; Organon, Oss, The Netherlands). When follicles reached a diameter of 18 mm, 10,000 IU of human chorionic gonadotropin (hCG, Pregnyl; Organon) was administrated and ultrasound-guided oocyte aspiration was performed 35 h later.

Oocyte collection was performed through ultrasound-guided transvaginal aspiration of the follicles under sedation and local anaesthesia. Oocytes were collected in an Earle's Balanced Salt Solution (EBSS) with phenol red (Lonza, Verviers, Belgium) and penicillin (50 U/ml) and streptomycin (50 μg/ml). After collection, oocytes were rinsed with Universal IVF medium (MediCult) and placed in an *in vitro* fertilization four-well plate (Nunclon Surface; Nunc, Roskilde, Denmark). A maximum of four oocytes were placed per well.

Sperm preparation

The sperm sample was obtained through masturbation in a sterile container (the day of oocyte collection: day 0), and immediately placed on a warm plate at 37°C. Sperm concentration and motility were determined before the procedure. Sperm morphology was determined according to Kruger strict morphology criteria. For sperm preparation, a swim-up technique was used: approximately 0.5 ml of the sample was placed at the bottom of 5 ml polystyrene round-bottom tubes (Falcon; Becton Dickinson Labware Europe, Meylan, France) containing pre-equilibrated Universal IVF medium with 5 mM glucose, penicillin, and streptomycin (MediCult; the medium did not contain phenol red). The tubes were incubated for 45 min in a humidified atmosphere of 5% CO_2 at 37°C. Afterwards, supernatants were collected and placed in 15 ml polystyrene conical tubes, which were centrifuged for 10 min at 0.5 × g. The pellet was resuspended in 1 ml of pre-equilibrated Universal IVF culture medium and the sperm concentration and motility were recounted.

Fertilization

Approximately 4 h after oocyte collection, insemination was performed (day 0) by standard IVF or ICSI procedures. For conventional IVF, a maximum of four oocytes/well in a four-well plate with Universal IVF medium were inseminated with one million capacitated spermatozoa and returned to the incubator for 1 h. For ICSI, oocytes were denuded enzymatically with 40 IU/ml hyaluronidase (SynVitro; Hyadase, MediCult), and mechanically

with a glass Pasteur pipette. Afterwards, they were rinsed with Universal IVF medium (MediCult).

After IVF or ICSI, the inseminated oocytes were placed in microdrops under light mineral oil (Irvine Scientific, Santa Ana, California) consisting of pre-equilibrated media: either the control medium (ISM1) or GM501 medium depending on the randomization protocol. Because fertilization techniques can affect pregnancy outcomes, it was important to have the same percentages in both groups. Therefore, either 100% ICSI or IVF was performed, and in some patients 50% IVF–50% ICSI was performed (mixed cycle). Fertilization was checked the morning after IVF or ICSI (day 1) under an inverse microscope at 200× magnification. An oocyte with two pronuclei (2PN) present was considered to be fertilized.

Embryo transfer

Conventional embryo transfer was performed on day 2 or 3. For the purposes of our study, a predefined organizational scheme for conventional transfers was implemented. The day of puncture determined the day of transfer and the length of the *in vitro* embryo culture. Patients were not able to choose between day 2 or day 3 transfer. On the day of the transfer, the embryo morphology was scored and the number of embryos to be transferred was determined in accordance with Belgian legislation (Gordts *et al*., 2005).

For the transfer, pre-equilibrated Universal transfer medium (UTM) with phenol red (MediCult) was used. The transfer was performed with a Semtrac soft catheter (#2000 Semtrac C; Gynétics Medical Products N.V., Hamont-Achel, Belgium), double catheter (#4219 Emtrac Set; Gynétics), or Soft-Trans Embryo Catheter (K-Soft 5100; Cook, Brisbane, Australia).

Transfer catheter policy is standardised in the local working protocol in such a way that an equal distribution of the used catheters is provided for both groups. Furthermore all catheters have received the same in house embryo toxicity tests before use

In all cycles, the luteal phase was supported with 3 × 200 mg of micronized natural progesterone (Utrogestan; Besins International, Brussels, Belgium), administered vaginally.

Follow-up

Following embryo transfer, blood samples were taken for analysis of serum estradiol, progesterone, and hCG concentrations on day 12. A cycle was considered to be a conception cycle when hCG values of > 10 mIU/ml were obtained on day 12 following embryo transfer and any day thereafter, indicating the beginning of implantation. A clinical pregnancy was defined as a conception cycle if there was at least one fetal sac with a positive heartbeat at ultrasound.

Output parameters

The main outcome parameters were fertilization and implantation rates, pregnancy outcome, and babies born rates. Fertilization rate was defined as the number of 2PN/number of oocytes (%); pregnancy rate as the number of patients who were hCG+/number of transfers (%); implantation rate as the number of gestational sacs/number of embryos transferred (%); clinical pregnancy rate as the number of patients who were hCG+ with at least one gestational sac/number of transfers (%); pregnancy loss rate as the number of pregnancy losses/number of patients who were hCG+ (%); ongoing pregnancy rate as the number of patients who were hCG+ with at least one gestational sac with fetal heart rate (FHR) after 12 wk/number of patients who were hCG+ or as the number of patients who were hCG+ with at least one gestational sac with FHR after 12 wk /number of transfers (%); and babies born as the number of babies born/number of transfers (%). For the analysis, the total biochemical pregnancies, miscarriage, and ectopic pregnancies were grouped under "pregnancy losses."

Embryo morphological scoring system

For embryo scoring, we took into account the number of blastomeres, the cell similarity, and the fragmentation rate. The fragmentation rate was classified into three groups: no fragmentation, less than 30% fragmentation, and more than 30% fragmentation. Embryos were scored by an experienced embryologist on the day of transfer.

Multinucleated (MN) embryos where discharged and allocated to the group of no transferable embryo's (no fertilization, degeneration and MN).

Statistics

Data were analyzed using the GraphPad Instat program, version 3.06 for Windows. Fisher's exact test was performed. $P < 0.05$ was considered statistically significant.

Results

Patient and cycle characteristics are summarized in Table 1. A total of 172 patients were randomized: 87 for ISM1 and 85 for GM501. Five patients (four

Table 1. — Patient and cycle characteristics.

Parameter	ISM1	GM501
Age (mean ± SD)	34.7 ± 5.0	34.5 ± 5.1
No. of cycles	87	85
No. of oocytes	652	595
No. of oocytes/ cycle (mean ± SD)	7.5 ± 4.7	7.0 ± 3.9
100% IVF (%)	24.1	21.4
100% ICSI (%)	61.7	61.9
Mixed IVF/ICSI (%)	13.3	16.6
No. of fresh embryos transferred	124	130
No. of embryos cryopreserved	177	181
No. of transfers	83	84
No. of embryos/transfer (mean ± SD)	1.5 ± 0.8	1.7 ± 0.8

in the ISM1 group and one in the GM501 group) were excluded from the study because no transfer was performed; therefore, 83 and 84 transfers were performed for ISM1 and GM501 groups, respectively. There were no significant differences ($P > 0.05$) with regard to age, number of oocytes per cycle, number of embryos per transfer, and technique used for fertilization between the study group and the control group.

Because no significant differences were found between the two age groups for any of the outcomes, we presented the information from both groups together. There were no differences between the two groups with regard to any of the outcomes of this study, i.e. fertilization, implantation and clinical pregnancy rates, pregnancy losses, ongoing pregnancies, and live birth rate (Table 2). When the pregnancy outcomes were analyzed depending on the day of the transfer, day 2 or 3, no significant differences between GM501 and the control medium were observed (Table 3).

On the day of transfer, the best-looking embryos were chosen and the fragmentation rate was recorded (Table 4). No differences in the number and symmetry of the blastomeres were found between the embryos transferred in the two groups. However, fewer nonfragmented embryos (25.4% vs. 49.2%, $p < 0.0001$) and a higher percentage of minimally fragmented embryos, i.e., < 30% fragmentation rate, (70% vs. 47.6%, $p = 0.0003$) were available for transfer when GM501 was used.

Discussion

Optimal IVF and embryo culture conditions depend upon several parameters, including temperature, stable environment, humidity, oxygen and carbon dioxide concentrations, medium pH, and composition of

Table 2. — Fertilization rate, pregnancy outcomes, and delivery rate.

	ISM1	GM501
No. of two pronuclei (2PN) Fertilization rate	446 68.4% (446/652)	433 72.8% (433/595)
No. of hCG+ patients Pregnancy rate	27 32.5% (27/83)	23 27.4% (23/84)
No. of gestational sacs Implantation rate	22 17.8% (22/124)	26 20% (22/130)
No. of patients with at least one gestational sac with FHR Clinical pregnancy rate	22 26.5% (22/83)	19 22.6% (19/84)
Number of pregnancy losses Pregnancy loss rate	8 29.6% (8/27)	5 21.7% (5/23)
No. of ongoing pregnancies Ongoing pregnancy rate per hCG+ Ongoing pregnancy rate/transfer	19 70.4% (19/27) 22.9% (19/83)	18 78.3% (18/23) 21.4% (18/84)
No. of children born	23 27.7% (23/83)	23 27.4% (23/84)

Table 3. — Pregnancy, pregnancy loss, and ongoing pregnancy rates of embryos transferred on days 2 or 3.

	Day 2		Day 3	
	ISM1	**GM501**	**ISM1**	**GM501**
Age	33.6 ± 4.7	34.4 ± 5.2	35.3 ± 4.8	34.3 ± 4.8
No. of transfers	53	56	30	28
No. of patients hCG+ Pregnancy rate	19 35.8% (19/53)	17 30.4% (17/56)	8 26.7% (8/30)	6 21.4% (6/28)
No. of pregnancy losses Pregnancy loss rate	7 36.8% (7/19)	4 23.5% (4/17)	1 12.5% (1/8)	1 16.7% (1/6)
No. of ongoing pregnancies Ongoing pregnancy rate/transfer	12 22.6% (12/53)	13 23.2% (13/56)	7 23.3% (7/30)	5 17.9% (6/28)

the culture media. The ideal culture medium must be designed to mimic the natural environment of the embryo; thus, its composition is a balanced blend of physiological components.

Following the "back to nature" philosophy, media were developed based on the different environments in the fallopian tubes and uterus. To do this, these fluids were collected from these regions and their composition was analyzed. However, there are at least two confounding factors. First, there is fluid exchange between the fallopian tubes and the uterus that is not taken into consideration. As a consequence, the composition of the embryo's microenvironment cannot be clearly defined and most probably varies dynamically. Second, the collection of these fluids and the stabilization of the components until analysis are technically problematic.

The "let the embryo choose" principle is based on the development of "computer-optimized media," which uses a mathematical model to optimize both the combination and concentration of various components. For example, KSOM medium is a variant of Simplex Optimization Medium (SOM), in which potassium chloride is added (Biggers, 1998). Later, essential and nonessential amino acids were added to KSOM to produce KSOM^{AA}, which was successfully used in a one-step protocol to culture human zygotes to the blastocyst stage (Biggers *et al*., 2002). Gynemed GM501 is an optimized variant of KSOM^{AA} medium; it is a novel medium, and thus no comparative data about its use in embryo culture was available.

In this study, we found that good results were obtained using GM501 medium. Equal pregnancy rate, pregnancy losses, clinical pregnancies, ongoing pregnancy, and babies born rates were observed when GM 501 medium was used in comparison with ISM1. The pregnancy outcome was also evaluated depending on the day of transfer (day 2 or 3), and no differences were found between the media.

Embryo selection based on morphological criteria provides a good correlation with embryo viability and pregnancy rates (Scott *et al*., 2008). Gordts *et al*. (2005) emphasized the importance of embryo selection under the restrictions of the Belgian legislation on embryo protection. These authors found that despite an increase in single embryo transfers (from 14% to 49%), consistently good pregnancy rates could be reached (36% vs. 37% for multiple and single embryo transfers, respectively). Thus, Belgian legislation on embryo protection, with its restrictive policy regarding embryo transfer, has not led to lower pregnancy rates. The conclusion that can be drawn from this is that the widely used morphological and biological criteria are sufficient to provide a prognosis for the developmental potential of embryos.

The two parameters most critical to good-quality embryos are cleavage speed and fragmentation level (Cummins *et al*., 1986; Claman *et al*., 1987). That is, an embryo with the best prognosis would have four blastomeres the second day of development and eight cells in the morning of the third day, and not more than 20% of the embryo would be fragmented.

Table 4. — Fragmentation rate of transferred embryos.

Fragmentation rate	**ISM1**	**GM501**
0% Fragmentation (% embryos)	61 (49.2%)	33 (25.4%)*
< 30% Fragmentation (% embryos)	59 (47.6%)	91 (70%)*
> 30% Fragmentation (% embryos)	4 (3.2%)	6 (4.6%)
Total	124	130

*$p < 0.05$ vs. control group (Fisher's exact test).

Cytoplasmatic pitting can also have a negative influence on implantation outcome (Ebner *et al*., 2005). In our trial, better embryo morphology was observed with ISM1, because a higher percentage of non- or low-fragmented embryos were observed in this medium. A possible explanation for this may be differences in the media composition. In addition, ISM1 medium contains phenol red and GM501 does not; it was demonstrated previously that phenol red has estrogenic activity (Ortmann *et al*., 1990; Berthois *et al*., 1986). Whether phenol red might improve embryos morphology must be investigated in future studies.

Although there is general agreement that a positive relationship exists between embryo quality and pregnancy rate, in our study, the pregnancy rate was the same for both media, although more fragmented embryos were transferred using GM501. These results are consistent with those of Aoki *et al*. (2005), who also demonstrated that regardless of the superior embryo morphology achieved with one of the media that they tested, there were no significant differences in implantation rates. There are at least two explanations that could account for the observation that equal pregnancy outcomes were achieved regardless of the differences in fragmentation rates at least up to 30%. First, the number of cells is more important than the fragmentation rate (Giorgetti *et al*., 1995; Ebner *et al*., 2003). Second, embryo fragmentation could be only a momentary state, because spontaneous lyses and resorption has been well documented (Hardarson *et al*., 2001; Van Blerkom *et al*., 2001).

There is some debate as to whether, in addition to light microscopy, other non-invasive methods for judging embryo vitality should be used. Recently, several noninvasive techniques have been discussed, such as measurement of the usage or production of different metabolites, the respiratory activity of the embryo, and the kinetic events during the first cell division steps (Brison *et al*., 2004; Lopes *et al*., 2007). Recently, proteomics and metabolomics have also been used to assess embryo quality and developmental potential (Nagy *et al*., 2008). The use of image analysis systems (time-lapse recordings) to assess embryo developmental timing has also clearly demonstrated differences in the developmental potential of fast- and slow-cleaving embryos with the same cell number and morphological quality (Ramsing *et al*., 2007; Lemmen *et al*., 2008). Several of previous studies demonstrated that levels of glucose (Sallam *et al*., 2006) and oxygen consumption (Lopes *et al*., 2007), amino acid turnover (Brison *et al*., 2004), protein expression (Dominguez *et al*., 2008), metabolomic profiles (Nagy *et al*., 2008), and kinetics (Lemmen *et al*., 2008) of human embryos are significantly correlated with clinical pregnancy and live birth. Moreover, other non-invasive methods for judging the sperm should be used. Gianaroli *et al*. analysed the pattern of birefringence in the sperm during the ICSI to selectively inject acrosome-reacted and acrosome-nonreacted spermatozoa and they demonstarted that, althought there was no effect on the fertilizing rate and embryo development, the implantation rate was higher in oocytes injected with reacted spermatozoa in comparison with those injected with nonreacted spermatozoa (Gianaroli *et al*., 2010). It will be some time before these new technologies can be used as routine procedures; nevertheless, our data and that of previous studies indicate that embryo should be judged less by their morphology than by their content.

Conclusion

In vitro culture of embryos in Gynemed GM501 media yielded pregnancy outcome results comparable to those of embryos cultured in ISM1, in spite of the higher embryo fragmentation rate in GM501. Because embryo culture in GM501 is a one-step procedure, and the medium has a long shelf life of 6 months, this product could represent a more practical and convenient medium for embryo culture in IVF laboratories. Scientific evidence is provided that the implementation of non invasive alternative methods to judge the embryo quality seems to contribute to a more accurate selection of the best embryo.

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