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Middle East Fertility Society Journal Vol. 9, No. 2, 2004

Comparison of embryo development in sibling oocytes cultured in two different sequential media

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ABSTRACT

Objective: To compare the efficiency of two different sequential media for the cultivation of sibling embryos until the blastocyst stage.

Design: A prospective analysis was conducted on 113 ART cycles with the indication of severe male factor infertility in Istanbul Memorial Hospital ART and Genetics Unit. Setting: After insemination, oocytes were randomly divided into two groups and cultured with either ISM1/ISM2 or G1.2/G2.2 sequential media until embryo transfer. Embryo development parameters were recorded for every embryo on consecutive days of preimplantation embryo development, growing in each set of culture media.

Results: 16.7±6.4 MII oocytes were retrieved. Out of 1434 MII oocytes injected, 1044 were fertilized (72.8%). There was no statistically significant difference in the rate of fertilization ($p>0.05$). Also, there was no difference in embryo development on the second day of cultivation. On the 3rd day of cultivation, the mean number of blastomeres was significantly higher in embryos cultivated within ISM1 ($p<0.01$). There was no statistically significant difference among the two groups in terms of blastulation rate.

Discussion: Both G1.2-G2.2 and ISM1-ISM2 sequential cultures are equally effective for in-vitro cultivation of embryos until the blastocyst stage. Our results show that both commercial media can be used as valuable and efficient alternatives to each other for sustaining blastocyst development in extended embryo culture programs. ISM1 may be preferred when blastomere biopsy for preimplantation genetic diagnosis is planned as more embryos with 7-8 blastomeres can be available with the use of ISM1.

Key Words: Human embryo development, sibling oocytes, sequential media, severe male infertility

Recent findings as well as the understanding of the metabolic requirements of preimplantation stage embryos led scientists to improve existing laboratory protocols that more closely match the changing physiological requirements of developing embryos (1,2). That is, a fertilized zygote has completely different needs of nutrients and other molecules when compared to a fifth-day blastocyst. Therefore it is generally thought that the use of only one type of embryo culture media throughout the preimplantation development may decrease the efficiency of an overall ART outcome and therefore an extended culture requires the use of sequential culture media.

Although initial studies resulted in poor outcomes, investigators afterwards have reported the increased pregnancy and implantation rates when sequential media combined with blastocyst

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transfer were applied on selected patients (2,3,4). When compared with day 3 embryo transfers, Marek et al. has found increased implantation rates in day 5 transfers (5) and others have shown that day 3 embryo morphology is not a good predictor for blastocyst development (6,7). There are also several reports arguing that blastocyst transfers have no advantage on cleavage stage transfers and there exists a possibility that some of the healthy embryos could be lost due to suboptimal culture conditions (8,9,10). However, it has been generally accepted that under suitable conditions, blastocyst stage transfer using sequential culture are advantageous and can be used effectively to decrease multiple pregnancy rate (11).

Besides several difficulties observed during the establishment of day 5 transfer protocols and policies in a laboratory, one of the main problems is the selection of a proper sequential media that can safely support an embryo from pronuclear through blastocyst stage. For this reason, in our lab we aimed to develop a strategy to minimize the possible negative effects of culture media conditions by using two sequential culture media systems- one being a routine culture media and one being a backup. However, such a backup system requires well-defined embryo culture parameters; one has to be sure that both media can work efficiently and effectively. Therefore, the aim of our study was to compare two different commercially available sequential media in terms of their individual embryo developmental capacity by using sibling oocytes obtained from the same cohort.

MATERIALS AND METHODS

Patient selection

This prospective randomized study was realized in Istanbul Memorial Hospital ART and Reproductive Endocrinology Center between February 2001 and June 2001 and approved by an Institutional Review Board at the Istanbul Memorial Hospital. A total of 113 cycles undergoing ICSI for severe male infertility were included.

Table 1. Overall cycle outcome of 95 cycles included in the comparison study.

Cycles	95
No.of prezygotes scored	1044
Female age	30.0±4.8
Male age	34.7±5.6
Duration of infertility	8.3±4.8
Oocyte retrieved/cycle	18.6±5.8
Fertilization rate %	72.8
Mean no. of emb. Transferred	2.7
Pregnancy (hCG(+) %	52.6
Pregnancy FHR(+) %	46.3
Implantation rate%	24.5

Ovulation Induction and Oocyte Recovery

Pituitary down-regulation was performed by using a gonadotrophin-releasing hormone analogue (Buserelin Suprefact ®, Hoecst AG, Frankfurt, Germany) and follicular development was then stimulated with an injection of follicle stimulating hormone (FSH) (Gonal-F®; Serono, Turkey; Puregon®; Organon, Turkey; Metrodin®; Serono, Turkey), human menopausal gonadotrophin (HMG) (Humegon®; Organon, Turkey) and human chorionic gonadotrophins (HCG; Profasi®, Serono, Turkey or Pregnyl®, Organon, Turkey). The stimulation protocols were performed as described in our previous publication (12). Patients who produced greater or equal to 8 MII oocytes were included in this study.

ICSI and Embryo Culture

Transvaginal-ultrasound-guided oocyte retrieval was performed 36 hours after the injection of 10.000 IU hCG (Profasi, Ares Serono Laboratories Co, Welwyn Gordon City, UK). After oocyte retrieval, oocyte-cumulus complexes were evaluated with an inverted microscope (Olympus inverted microscope; IX70; Japan) and incubated in 100 ul droplets of IVF-20 (Vitrolife; Gothenburg, Sweden) at 37^{o}C with 5 % CO2. Approximately 2 hours after oocyte retrieval, cumulus cells and the corona radiata were removed by a brief exposure to 40 IU/ml hyaluronidase (type VIII, specific activity 320 IU/mg, H 3757®; Sigma Chemical

Co.). Intracytoplasmic sperm injection was then performed with metaphase II stage oocytes.

Immediately after ICSI, injected oocytes were transferred into the corresponding culture media from each sequential media set (G1.2: Vitrolife and ISM1: Medicult) and cultured in these media until day 3 of embryo development. In the morning of day 3, embryos were transferred into G2.2 and ISM2 as outlined in Figure 1. When day 5/6 embryo transfer was planned, culture media was again changed in the afternoon of day 4.

Fertilization was assessed at 16 -18 h after injection. It was determined as normal fertilization when two clearly distinct pronuclei containing precursor nucleolar bodies (PNBs) were observed under an inverted microscope. After the assessment, the zygotes were transferred to fresh droplets of the corresponding medium. The state of embryo cleavage and quality were assessed after a further 24, 48, 72 and 96 hours of in-vitro culture. The embryos were evaluated according to the number of blastomeres, blastomere size equality, presence or absence of granularity and the relative proportion of anucleate fragments by at least two experienced embryologists by 600X magnification on an inverted microscope.

Embryo Transfer and Pregnancy Test

Embryo transfer was performed on the 3rd, 4th 5th or 6th day of embryo culture according to the developmental potential of the transferrable embryos and the supernumerary embryos were cryopreserved. Pregnancy was first evaluated by serum HCG levels assay 12 days after embryo transfer. Clinical pregnancy was diagnosed by the presence of a gestational sac by echographic screening approximately 7 weeks after the embryo replacement procedure.

Statistical Analysis

Results were expressed as mean±SD. Data was analyzed and compared by using χ^2 test. A $p \leq 0.05$ was defined as statistically significant.

Table 2. Overall embryo development for sibling oocytes cultured in ISM or G series.

	ISMTM	G1.2/G2.2	P
Prezygotes scored (n)	524	520	
Group II prezygotes (%)	13.2	9.0	Ns
4-cell embryos on day 2 (%)	59.3	54.8	Ns
Embryos scored on day 3 (n)	460	425	
Embryos <5 cells (%)	18.7	32.7	p<0.01 a
6-8 cells (%)	72.8	59.8	p<0.01 a
Embryos scored on day 4 (n)	405	381	
<10 cells (%)	24.9	23.1	Ns
Compaction/morula (%)	61.0	61.2	Ns
Embryos scored on day 5 (n)	296	275	
Morula/Early cav. (%)	52.7	57.8	Ns
Late cav/blastocyst(%)	23.3	22.2	Ns

(a): Chi-square test; Ns: Not significant

RESULTS

Two different commercially available media were used in this study; G1.2-G2.2 media and ISM1/ISM2. The sibling oocytes from 95 consecutive ICSI cycles were cultured simultaneously in both media. Cycle characteristics are shown in Table 1. Mean female and male ages in these cycles were 30.0 ± 4.8 and 34.7 ± 5.6 respectively. Mean duration of infertility was found to be 8.3 ± 4.8 years. In these cycles, 16.7 ± 6.4 oocytes were retrieved per cycle. ICSI was performed for all cases and 72.8% of the injected oocytes were found to be fertilized. A mean number of 2.7 embryos per cycle were transferred giving an overall clinical pregnancy and implantation rates of 46.3% and 24.5% respectively.

Table 3. Cycle parameters for 18 consecutive cycles

Patients	9
Cycles	18
No.of prezygotes scored	102
Female age	35.9±3.4
Male age	38.9±7.2
Duration of infertility	8.2±5.1
Oocyte retrieved/cycle	10.1±4.5
Fertilization rate	72.5%

Immediately after ICSI, sibling oocytes were transferred into G1.2 and ISM1 media and cultured with these media until the morning of day 3. A total of 524 prezygotes were cultured in ISM1 and

520 prezygotes were cultured in G1.2. The results of the embryo culture are summarized in Table 2. Fertilized zygotes were classified according to the nuclei formation characteristics as well as the distribution of prenucleolar bodies as outlined in our previous study (12). In this manner, for ISM1 culture, we have observed a slightly but not statistically significant increase in group II prezygotes that are termed as a poor morphology group which have a higher chance of showing early developmental arrest as well as a higher probability of having chromosomal abnormality. There was no statistically significant difference when the pronuclear stage arrest rate was compared in two groups ($p>0.05$; data not shown). Day 2 embryo development characteristics were also similar in terms of blastomere numbers. However, we have observed a significant difference in cleavage rates of embryos cultured in both media on day 3 of embryo development. 72.8% of the embryos cultured in ISM1 were found to have 6-8 blastomeres whereas in G1.2 media this rate was shown to be 59.8% ($p<0.01$). This lower cleavage rate was accompanied by a higher percentage of embryos having less than 6 bastomeres in the morning of day 3. Embryo arrest rate was similar in both groups (data not shown).

Table 4. Overall embryo development in consecutive cycles

	ISMTM	G1.2/G2.2	P
Prezygotes scored (n)	49	53	
Group II prezygotes (%)	16.3	9.4	Ns
4-cell embryos on day 2 (%)	30.6	39.6	Ns
Embryos scored on day 3(n)	43	48	
Embryos <5 cells (%)	30.2	41.7	Ns
6-8 cells (%)	65.1	56.3	Ns
Embryos scored on day 4(n)	32	15	
<10 cells(%)	34.4	46.7	Ns
Compaction/morula(%)	43.8	40.0	Ns
Embryos scored on day 5(n)	4	13	
Morula/Early cav. (%)	(3)	46.2	
Late cav/blastocyst (%)	(1)	23.1	

Chi-square test; Ns: Not significant

When 6 or more developing embryos were observed on the 3rd day, further embryo culture was performed for 24, 48 and 72 hours where available. As shown in Table 2, no statistically significant difference was observed in the two groups in terms of extended embryo culture with G2.2 and ISM2 ($p>0.05$).

For nearly all cycles, embryos from both culture groups were selected for transfer therefore the individual pregnancy rates were not determined.

In parallel with the above study on sibling oocytes, these two embryo culture media were also used on consecutive cycles for 9 patients in 18 cycles. When a cycle with a given culture media failed to result in a pregnancy, the next cycle was performed with the other embryo culture media. Cycle parameters and embryo development profile were documented in Table 3 and 4. Although embryo development was similar with the previous results, unfortunately no pregnancy was obtained for these 9 patients with on consecutive trials.

DISCUSSION

In this study, two sequential culture media, G1.2/G2.2 and ISM1/ISM2 were compared in terms of embryo developmental capacity on specific time points starting from fertilization until the day of embryo transfer.

There are very few prospective studies that compare the effectiveness of sequential media on supporting embryos up to the blastocyst stage (3,8,9,10). However, there is only one such study performed on sibling oocytes and in that, Staessen and his colleagues compared the potential of three different commercial media on sustaining embryo development during first cleavage stages (13). Our results on sibling oocytes that are followed and compared until day 5 or day 6 demonstrated that both sequential media are equally as effective in supporting the embryos through extended culture. Therefore, both can be used as a back up media for the other.

However, we have observed a higher numbers of embryos with 6 or more blastomeres, 72 hours after insemination, when cultured in ISM1 medium. Since the oocytes from the same cohort were used and the embryo culture conditions were the same, the difference in mean blastomere numbers on day 3 of embryo development can be

explained as the ability of Medi-Cult's ISM1 to increase the mitotic index of developing embryos.

Which factor(s) might be responsible for this increase is not known as the exact media composition is not available. However, the difference in concentration and/or composition of amino acids and (possibly) growth factors may contribute to this increase by increasing the rate of biochemical metabolism and cleavage of embryos. Amino acids, nucleic acid precursors and maternal growth factors are the most important candidates for the improvement of sequential culture media. However, adding or removing some factors may also select and promote blastocyst formation in abnormal embryos which otherwise should be eliminated via culture selection. Therefore more research is needed in order to clarify the exact role and the possible benefit(s) of adding growth factors and other components into the culture.

On the other hand, the pregnancy outcome when either media is used was not assessed. Instead, a cumulative clinical outcome was given for the analyzed groups in this study since in nearly all cases, embryos developing from both media were selected for transfer.

Besides its advantages on embryo development and pregnancy outcome, sequential culture can be more laborous and require more strictly-controlled laboratory conditions that are not readily available in some laboratories. It has also been argued that there may exist some disadvantages such as the osmotic shock to embryos that may be created while switching from one media to another, deprivation of specific growth factors and autocrines that are produced in the first culture period. Therefore, an alternative strategy of using only one single culture media that can support embryos until the blastocyst stage has been recently discussed (14). Also, there may be cases in which no blastocysts can be observed or most of the embryos can be eliminated during extended culture (15,16). The reasons in part can be attributed to the culture systems since although their composition and quality are being improved everyday, they are far from fulfilling all of the needs for an embryo. Moreover, there may exist batch to batch variations which may negatively affect most of the cycles for a given period. Other factors can be numerous including patient specific factors, factors that arise from suboptimal conditions and factors whose presence or absence in the culture media can create impaired embryo development for a subset of patients.

In some but not all studies, embryo development and quality were found to improve when certain co-culture methods were used (17,18,19,20). In our clinic, we also apply autologous endometrial culture in cycles with poor embryo development and repeated implantation failure cases without a possible genetic cause. In these patients, we have observed an overall rate of decreased cytoplasmic fragmentation as well as increased embryo quality, suggesting that there are in fact some factors which are not functional in a conventional culture media but can be effective in coculture systems (data not shown). These observations further imply that sequential culture media still needs to be improved.

PGD procedures are routinely applied in our clinic with the indication of single gene disorders, advanced maternal age, translocations, recurrent early pregnancy loss, repeated implantation failure and severe male infertility. For this reason availability of embryos with 6 or more cells for blastomere biopsy on the third day of embryo development is of importance. One possible advantage of the increased cleavage rate with ISM1 in this study is that, for the cycles in which a PGD procedure is planned, it may be advisable to use ISM1/ISM2 media since more embryos with 7 or more blastomeres can be available for the embryo biopsy on the day 3 of the embryo development, increasing the efficiency of the PGD outcome.

In conclusion, although the media compared in this study were produced by different commercial companies, our results indicate that both sequential media are equally efficient in sustaining fertilization and embryo development until embryo replacement. Therefore G1.2/G2.2 media and ISM1/ISM2 media can be used as valuable alternatives to each other. However, ISM1/ISM2 media can be used as a source if PGD is planned since more embryos with 7 or more blastomeres on day 3 can be obtained.

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Received on September 21, 2003; revised and accepted on May 17, 2004

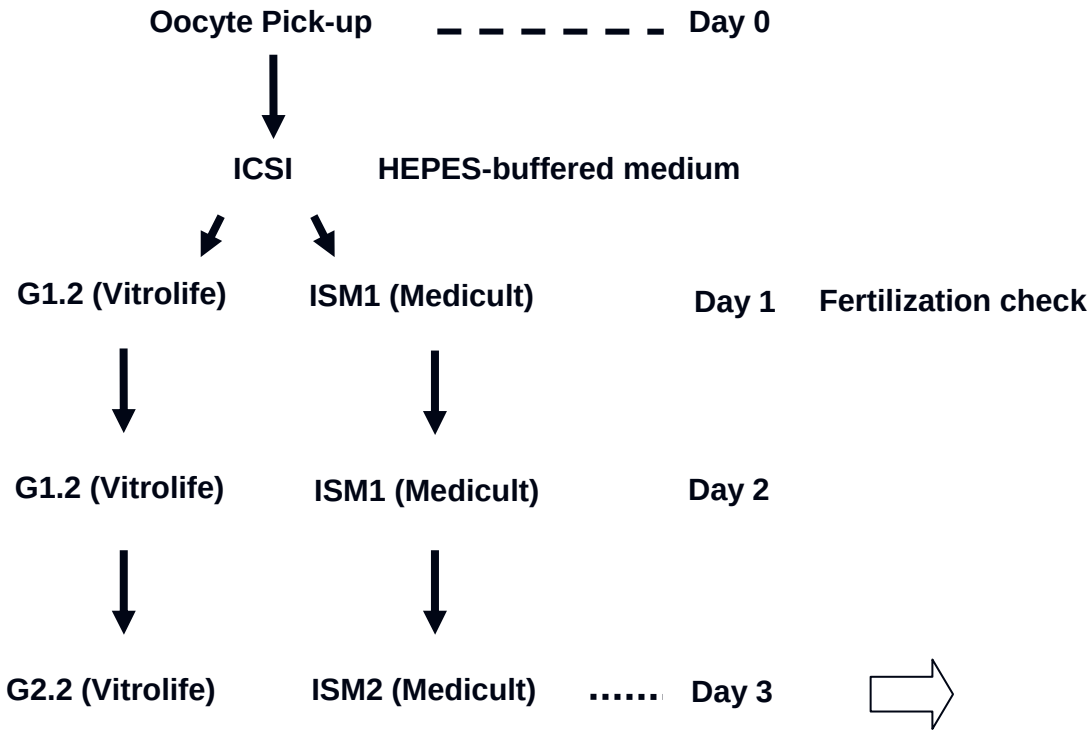

Figure 1