Prospective comparison of two commercially available culture media under the provisions of the German embryo protection law

Alman Embriyo koruma yasasının öngörüsü ışığında iki ticari kültür medyumunun prospektif olarak karşılaştırılması

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Abstract

Objective: Under the provisions of the German embryo protection law allowing a maximum of only three embryos in culture, blastocyst transfers are underrepresented and for obvious reasons usage of sequential media is not required.

Matiral and Methods: This prospective study was set up to evaluate whether a global medium (157 patients) could provide comparable data or even outmatch results achieved with a medium specifically designed for early cleavage stages (116 patients).

Results: Though rates of fertilization, cleavage and pregnancy did not differ between both media ($P>0.05$), interestingly, rate of pregnancy losses was significantly lower in patients with global medium ($P<0.01$). This led to a significantly higher clinical pregnancy rate ($P<0.05$).

Conclusions: Since patient cohorts were comparable in demographic data and all technical details were kept constant it could be hypothesized that culture conditions and/or culture media could have caused this dilemma.

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Key words: Culture conditions, culture media, day 3 transfer, global medium, pregnancy loss

Özet

Amaç: Maksimum sadece 3 embriyo kültürüne izin verilen Alman embriyo koruma yasasının koşulları kapsamında blastokist transferleri yapılır ve bunun açık sebebi ardışık kültür ortamının kullanımın gerekli olmamasıdır.

Gereç ve Yöntemler: Kıyaslanabilir verilerin elde edildiği genel ortam (157 hasta) ile erken klevaj evresi için spesifik olarak tasarlanan ortamdan (116 hasta) elde edilen sonuçları arasındaki ilişkinin değerlendirildiği prosfektif bir çalışma oluşturuldu.

Bulgular: Fertilizasyon, klivaj ve gebelik (kültür koşulları) oranları bütün ortamlarda/gruplarda farklılık göstermemesine rağmen ($P>0.05$), ilginçtir ki; gebelik kayıp oranları genel ortamdaki hastalarda anlamlı derecede düşük ($P<0.01$), klinik gebelik oranı anlamlı derecede yüksek olduğu ($P<0.05$) saptandı.

Sonuç: Diğer tüm şartların eşitlendiği bu durumda, kültür koşullarının ve/veya kültür medyumlarının bu duruma yol açtığı speküle edilebilir. (J Turkish-German Gynecol Assoc 2009; 10: 10-3)

Anahtar kelimeler: Kültür koşulları, kültür ortamı, genel ortam, gebelik kaybı

Introduction

It is important to differentiate between the ability of a particular culture medium to support embryo growth in vitro and the potential of the same medium to give rise to a viable embryo/blastocyst (1). In the past (2) this discrepancy was likely to be associated with the usage of rather simple culture media. As a matter of fact, introduction of more complex tissue culture media helped to further improve day 5 survival but still pregnancy rates were lower compared to day 2 or 3 results of the same IVF program (3).

In-house preparation of a complex embryo culture medium may provide for adequate results (4); however, it may be a time-consuming and expensive task, considering the fact that every batch has to pass quality control, not to mention staff and laboratory time. Thus, the use of pretested, commercially available, embryo culture media gained acceptance in IVF laboratories, keeping inter-batch variation to a minimum (5).

Currently, several types of media are available in the market representing different strategies. Some authors (6, 7) propagate to "let the embryo choose" which medium ingredients to consume or to metabolize. This philosophy culminated in a media family called KSOM in which the appropriate concentrations of constituents are determined by bioassay (8).

The improved understanding of both the physiological changes in oviduct and uterus and the different metabolic needs of cleavage and blastocyst stage embryos led to the development of so-called sequential embryo culture media (9) following the "back to nature" principle.

Since the reason for a sequential approach is to assist prolongation of in vitro culture up to day 5, its routine application in German laboratories may be reduced due to the fact that the national embryo protection law limits the number of embryos

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in culture to three. As a consequence blastocyst transfers are underrepresented and usage of sequential media is not necessarily required.
Thus, this prospective study was set up to evaluate whether a global medium (GM501, Gynemed, Lensahn, Germany) could provide for comparable data or even outmatch results achieved with a medium specifically designed for early cleavage stages (Universal IVF Medium, MediCult, Copenhagen, Denmark) under the provisions of the German embryo protection law.

Materials and Methods

IRB approval was granted since both media tested are commercially available and all companies involved provided certificates of analysis.
In order to achieve an adequate sample and to minimize any patient related bias it was decided to prospectively culture the embryos of all consecutive cycles within one quarter of a year in one medium (MediCult, control group). In a second period (another three months) all embryos were grown in the second medium (Gynemed, study group).
Thus, a total of 273 couples could be recorded within half a year. None of the women (36.4±4.9 years) had more than one treatment per quarter. Within the IVF group (37%) the main indication was found to be tubal blockage (58%). In the ICSI cohort (63%), however, the vast majority suffered from male factor infertility (94%).
For controlled ovarian hyperstimulation (COH) two approaches were applied, either a long or an antagonist one. The former protocol was characterized by the downregulation of the pituitary using GnRH-agonists followed by usage of an individually adjusted dose of gonadotrophins. In the latter regime, stimulation was started on day 2 of the cycle. In addition, a GnRH-antagonist was administered after 5-6 days of stimulation, depending on the presence of a 12-13 mm follicle in the ultrasound scan. As 27 patients did not respond to COH, only 246 patients had their follicle punctured transvaginally after ovulation had been induced.
ICSI and IVF were applied according to internal standardized laboratory techniques. At 16-20 hours after injection or conventional IVF, fertilization was assessed and zygotes were controlled for the presence of two pronuclei. In detail, pronuclear pattern (10) and presence of a halo (11) were documented. Based on these characteristics two zygotes were selected for further in-vitro culture whereas the remaining 2Pn-zygotes were cryostored for later usage according to German law if consent was given.
Preselected zygotes were incubated together in 50 μl drops of medium covered by sterile filtered mineral oil. In the first three-month period of the present prospective study, Universal IVF Medium (MediCult, Copenhagen, Denmark) was the medium of choice and in the second one it was GM501 Culture (Gynemed, Lensahn, Germany). On days 2 and 3, embryos were checked for the number, size, and nuclear status of blastomeres as well as for the presence of fragments.
According to German law, all embryos considered for further culture were transferred into the uterus using special transfer catheter (Labotect, Göttingen, Germany) provided that the embyos were found to be viable on day 3. Beta-hCG was measured 17 days after ovum pick up. A pregnancy was termed clinical as soon as at least one gestational sac could be seen on ultrasound.
Statistical analysis was performed using both t-test and chi-square test. Statistical significance was considered to be reached with a P-value of less than 0.05.

Results

The two study periods did not differ in relevant patient and treatment parameters (as indicated in table 1) such as female age, number of previous cycles, indication, and stimulation protocol, to name just a few. Though significantly more patients failed to respond to controlled ovarian hyperstimulation ($p<0.05$) in the study group as compared to the control group (table 1), no bias was introduced since statistical analysis was much rather based on oocyte collections than on COHs.
A total of 1884 cumulus-oocyte complexes could be collected. Overall, fertilization rate (2Pn) in conventional IVF (66.4%) was comparable to that with ICSI (68.5%); however, these values were not significantly different between study and control group (table 2). The same holds for the rates of 3Pn in both IVF (2.5% vs. 3.2%) and ICSI (0.8% vs. 0.5%).
Cleavage rate and embryo morphology on days 2 and 3 were not different between study and control cohort ($P>0.05$). Thus, as indicated in table 2, the two groups did not differ in the rates of positive β-hCG and pregnancy ($p>0.05$). However, a significantly lower rate of missed abortions in the study group ($p<0.01$) led to a higher clinical pregnancy rate ($p<0.05$).

Discussion

The hypothesis that a two-step protocol is absolutely necessary for an adequate preimplantation development has been based on the work done by Gardner and Lane (12, 13). However, experimental support for this virtual dogma is scarce according to Summers and Biggers (14). Recently, numerous publications emphasized that one global medium can successfully support growth of human and mammalian zygotes to blastocyst stage (7, 15,-17).
Currently, an intense debate on the optimal approach has been ongoing (18, 19) and advocates of both strategies choose their arguments carefully.
Those using sequential media rely on the work of Gardner and co-workers (20) who observed that uterus and oviduct present different environments. This and the finding that several carbohydrates, amino acids, and chelators stimulate or stop development of first cleavages and/or inner cell mass and trophectoderm led them to suggest usage of two distinguishable media for prolonged culture. However, it should be emphasized that placement of an embryo in a new chemical environment could expose it to considerable stress (16). In addition, it is likely that fluids of both uterus and Fallopian tube will mix somewhat, so that the difference is probably less distinctive than expected.
However, for given reasons the rate of patients with blastocyst culture is rather low in countries in which the number of embryos in culture is limited and selection for transfer has to be

Table 1. Comparison of patient related and stimulation data between study and control group

	study group	control group	p-value
Number of patients	157	116	
Number of oocyte collections	136 (86.6)	110 (94.8)	0.025
Number of successful collections	134 (98.5)	110 (100)	0.20
Mean female age	36.7±4.7	35.8±4.3	0.11
Number of ICSIs	82 (60.3)	70 (63.6)	0.59
Long protocols	86/136 (63.2)	86/116 (74.1)	0.07
Number of oocytes	7.0±3.8	6.9±3.7	0.98

In the study group GM501 (Gynemed, Lensahn Germany) was used and in the control group Universal IVF Medium (MediCult, Copenhagen, Denmark). Values in parentheses are percentages

Table 2. Fertilization and pregnancy outcome for both study and control group

	study group	control group	p-value
Fertilization IVF	266/396 (67.2)	175/248 (70.6)	0.37
Fertilization ICSI	411/612 (67.2)	375/536 (70.0)	0.31
number of transfers	128/134 (95.5)	109/110 (99.1)	0.10
Positive β-hCG	56 (43.8)	42 (38.5)	0.41
Pregnancy rate	54 (42.2)	38 (34.9)	0.25
Missed abortions	3 (5.6)	11 (29.0)	0.002
Clinical pregnancy rate	51 (39.8)	27 (24.8)	0.014
Implantation rate	68/283 (24.0)	48/211 (22.8)	0.74
Multiple pregnancy rate	12/56 (21.4)	6/42 (14.3)	0.37

In the study group GM501 (Gynemed, Lensahn Germany) was used and in the control group Universal IVF Medium (MediCult, Copenhagen, Denmark). Values in parentheses are percentages

done at either oocyte stage (Italy) or pronuclear stage (Germany, Switzerland). In these countries, embryo transfer is mostly performed at early cleavage stages on days 2 or 3. Taking this into consideration, practical and financial aspects encourage embryologists to use a more simple approach.

Thus, in the present study a prospective comparison was performed analyzing the utilization of a global medium (which would allow culture up to blastocyst stage) and a universal medium specifically designed for early cleavage stages.

At first glance, both media sufficiently support embryo growth up to day 3 when the embryo transfer was planned. In addition, it turned out that occurrence of pregnancy was not increased using either the universal or the global medium. Interestingly, the latter did better focussing on clinical pregnancy rate which is due to an increase in pregnancy losses in the control group.

Since patients of both groups did neither differ in demographic data nor in stimulation details, it may be assumed that any difference in the rate of missed abortions could be related to culture conditions. This is further supported by the fact that no personal or technical bias was introduced because all cycles were performed by the same embryologists and clinicians. Not to forget that the technical equipment, particularly the incubators, did not change throughout the study periods.

The hypothesis that culture conditions and certain culture media media could be associated with embryo appearance (21, 22) and implantation behaviour (23-25) is not new.

Ebner et al. (25) found a possible relationship between the occurrence of pitted blastomeres and a higher rate of vanishing implantations. These authors speculated that the observed phenomenon could be the effect of some physiological stress that happened to the embryo by an unfavourable culture environment. Their hypothesis was based on the finding that patients whose embryos were cultured in groups showed a higher rate of abortion compared to patients with individual culture which led them (25) to suggest a daily change of medium. In addition, others (23, 24) also suggested a detrimental effect of a certain media composition on spontaneous abortion rate.

Applied to the present data set (group culture, small volumes) this would mean that obviously the global medium could better compensate for a potential disturbance of the physiological environment than the control medium.

This brings one to the main differences between the two media used. One major aspects is that GM501 is a simplex optimized medium enriched with K+ and amino acids. At least the latter components are not present in the control medium. Since amino acids are known to have a pivotal role in media acting as

regulators of embryo metabolism, osmolytes or buffers of internal pH this could be one possible explanation for the observed divergence in implantation outcome.

In addition, the study medium stands out due to its long shelf life (6 months), whereas Universal IVF Medium should be used within 8 weeks from shipment out of Denmark. Introduction of a more stable form of glutamine (Na-alanylglutamine) and use of gentamycine instead of penicilin/streptomycine additionally reduce decay of media components in GM501.

To conclude, it can be stated that under the provisions of the German embryo protection law both media tested gave good results in terms of fertilization, cleavage, and pregnancy. It has to be emphasized that the control group had an abortion rate within empirical ranges and the striking difference was a rather low loss of pregnancies in the study group. Since this phenomenon could be attributable to culture conditions a daily medium change is recommended in order to avoid any impact on the embryos.

References

1. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Hum Reprod Update 1997; 3: 367-82.
2. Bolton VN, Wren ME, Parsons JH. Pregnancies after in vitro fertilization and transfer of human blastocysts. Fertil Steril 1991; 55: 830-2.
3. Noda Y, Goto Y, Umaoka Y et al. Culture of human embryos in alpha modification of Eagle´s medium under low oxygen tension and low illumination. Fertil Steril 1994; 62: 1022-7.
4. Macklon NS, Pieters MHEC, Hassan MA et al. A prospective randomized comparison of sequential versus monoculture systems for in-vitro human blastocyst development. Hum Reprod 2002; 17: 2700-5.
5 Mauri AL, Petersen CG, Baruffi RL et al. A prospective randomized comparison of two commercial media for ICSI and embryo culture. J Assist Reprod Genetics 2001; 18: 378-81.
6. Ho Y, Wigglesworth E, Eppig JJ et al. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. Mol Reprod Dev 1995; 41: 232-8.
7. Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM^{AA} medium: is a two-step protocol necessary. Reprod Biomed Online 2002; 5: 133-40.
8. Lawitts LA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. J Reprod Fertil 1991; 91: 543-56.
9. Gardner DK, Lane M. Culture of viable human blastocysts in defined sequential serum-free media. Hum Reprod 1998; 13 (Suppl 3): 148-59.
10. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. Hum Reprod 1999; 14: 1318-23.
11. Ebner T, Moser M, Sommergruber M et al. Presence of a cytoplasmic halo at zygote stage but not type und extension of the same has a significant influence on preimplantation development und implantation behavior. Human Reproduction 2003; 18: 2406-12.
12. Gardner DK, Lane M. Development of viable mammalian embryos in vitro: evolution of sequential media. In: Cibelli J et al. (eds) Principles of cloning. Academic Press, New York, 2002; 187-213.
13. Gardner DK, Lane M. Towards a single embryo transfer. Reprod Biomed Online 2003; 6: 470-81.
14. Summers MC, Biggers JD. Chemically-defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update 2003; 9: 557-82.
15. Biggers JD, McGinnis LK, Lawitts JA. One-step versus two-step culture of mouse preimplantation embryos: is there a difference? Hum Reprod 2005; 20: 3376-84.
16. Rinaudo P, Schultz RM. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. Reproduction 2004; 128: 301-11.
17. Perin PM, Maluf M, Nicolosi Foltran Jauario DA et al. Comparison of the efficacy of two commercially available media for culturing one-cell embryos in the in vitro fertilization mouse model. Fertility and Sterility 2008, in press.
18. Gardner DK, Lane M. One-step versus two-step culture of mouse preimplantation embryos. Hum Reprod 2006; 21: 1935-6.
19. Biggers JD, McGinnis L, Summers MC. Reply: One step versus two-step culture of mouse preimplantation embryos. Hum Reprod 2006; 21: 1936-9.
20. Gardner DK, Lane M, Calderon I et al. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. Fertil Steril 1996; 65: 349-53.
21. Veeck LL. An Atlas of Human Gametes and Conceptuses. Parthenon Publishing, New York, London, 1999; 64.
22. Morgia F, Torti M, Montigiani M et al. Use of a medium buffered with N-hydroxyethylpiperazine-N-ethanesulfonate (HEPES) in intracytoplasmic sperm injection procedures is detrimental to the outcome of in vitro fertilization. Fertil Steril 2006; 85: 1415-9.
23. Olar TT, Potts AS. Effects of medium composition on murine and human blastocyst formation and hatching rate. J Assist Reprod Genetics 1993; 10: 192-6.
24. Sundström P, Wramsby H. Evaluation of variations in pregnancy rates, implantation rates and early abortion rates within an in-vitro fertilization programme. Hum Reprod 1989; 4: 443-5.
25. Ebner T, Tews G, Sommergruber M et al. Cytoplasmic pitting has a negative influence on implantation outcome. J Assist Reprod Genetics 2005; 22: 239-44.