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Optimization and Simplification of Culture Conditions in Human in Vitro Fertilization (IVF) and Preembryo Replacement by Serum-Free Media

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The results of 220 consecutive IVF treatments are presented, comparing the use of culture media supplemented with either patient serum (Group 1; n = 110), or Medi-Cult SSR 2 synthetic serum replacement with pyruvate, and human serum albumin (HSA) (GEA BioTech, Hvidovre, Denmark) (Group 2; n = 110). In both groups the Medi-Cult Hybritest was used for routine quality testing. A significantly ($P < 0.05$) increased rate of deliveries/ongoing pregnancies was observed with the Group 2 medium. However, no significant differences in fertilization rate, cleavage rate, or implantation rate were observed. It is concluded that the serum-free culture medium described and the testing for absence of cytotoxicity in a sensitive bioassay (Hybritest) have yielded culture conditions capable of sustaining the development in vitro of human preembryos without impairing the fertilization process or the implantation rate, ultimately resulting in a significantly increased rate of deliveries/ongoing pregnancies and an apparently decreased abortion rate. The potential harmful effects of serum and the need for blood sampling and preparation further increase the advantages of replacing serum with the synthetic serum replacement SSR 2 in an IVF program.

KEY WORDS: culture media; embryonic development; in vitro fertilization (IVF); serum-free culture; serum substitute.

INTRODUCTION

Usually, culture media for human in vitro fertilization and embryo replacement (IVF-ER) have been supplemented with serum from various sources in order to improve culture conditions. Serum is normally also added to media used for the preparation of sperm by the "swim-up" technique. There are, however, several advantages to a standardized synthetic serum replacement: most important, serum may be contaminated with virus or other harmful substances (i.e., HIV, hepatitis, etc.). Furthermore, serum contains an unknown amount of various antibodies, the possible contents of sperm antibodies clearly interfering with the fertilization process. Other substances, including inflammatory mediators released from platelets and white blood cells, are present in an unknown and variable amount (1–3). For example, adenosine diphosphate released from platelets during blood coagulation has been suggested to be the main factor responsible for the embryo-toxic effect of serum (1). Finally, the time-consuming procedure of preparation and heat inactivation of serum can be omitted by using a serum replacement.

Control of the quality of embryo growth is of utmost importance in any IVF program, and the testing of culture media, water, equipment, etc., must therefore be considered mandatory. So far, the one- or two-cell mouse embryo test (4,5) and sperm survival tests (6) have been the only methods available for quality control in IVF programs. However, as we (7,8) and others (9) have pointed out previously, the sensitivity and reproducibility of these tests may be questioned. The cell culture assay newly described (7) seems to provide a simple yet sensitive and reproducible bioassay for quality control and may, therefore, represent an important improvement in clinical IVF.

We here describe our IVF procedure and com-

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pare the results obtained by using culture media supplemented with patient serum, and a specially developed serum-free medium, Medi-Cult EBSS-P-SR2 with SSR 2, pyruvate, and 1% human serum albumin (HSA) (GEA Biotech, Hvidovre, Denmark). The Medi-Cult Hybritest for routine quality testing of culture media, water, and equipment was used in both groups. We have compared fertilization rate, cleavage rate, implantation rate, and pregnancy rate.

MATERIALS AND METHODS

Culture Media

Culture Media Supplemented with Patient Serum (Group 1)

Ham's F10 medium (GEA BioTech, Cat. No. 1015) was used for insemination, cleavage, and preembryo replacement. The following components had been added to the medium: 2.2 g/liter $NaHCO_3$, 50,000 U/liter penicillin, and 50 mg/liter streptomycin. Heat-inactivated patient serum was added and constituted 10% (v/v) of the insemination medium, 15% (v/v) of the cleavage medium, and 65% (v/v) of the medium for preembryo replacement. For the preparation of patient serum blood was drawn through an indwelling venous catheter into glass tubes on the day after ovulation induction with hCG. Serum was separated by immediate centrifugation, filtered through 0.22-μm sterile filters (Millipore Millex GV 0.22, Millipore, Oslo, Norway), and heat inactivated at 56°C for 35 min. Serum was kept at +4°C until addition to the culture media. For the preparation of sperm we used the same Ham's F10 medium as described, supplemented with 10% (v/v) heat-inactivated patient serum, and 25 m*M* Hepes. Flushing medium, i.e., for flushing of the needle and aspiration system before puncture and, if necessary, for flushing of the follicles, consisted of Ham's F10 medium supplemented with 1% (v/v) patient serum and 25 m*M* Hepes. The concentration of $NaHCO_3$ in the flushing medium was reduced to 1.1 g/liter.

All culture media (for both Group 1 and Group 2) were freshly made up (GEA BioTech, Denmark) and were tested in the Hybritest before use. Since we have found (10) that culture media are rapidly degraded by exposure to light, special care was taken to avoid this during the production of media and by handling in the IVF laboratory. Culture media were stored for a maximum of 4 weeks at +4°C. The osmolality of both types of media was 280 mOsm and the pH was 7.4.

Serum-Free Culture Media Supplemented with SSR 2, Pyruvate, and HSA (Group 2)

The composition of the serum-free culture medium (EBSS-P-SR2 with HSA, GEA BioTech, Cat. No. 1031) is shown in Table I. The synthetic serum replacement SSR 2 is completely chemically defined and is totally devoid of proteins, lipids, and glycans, except for the presence of small quantities of insulin (0.5 μg/ml). Full control of all metals in solution, i.e., iron, trace elements, and heavy metals, has been achieved by a novel metal ion buffer. All metals necessary for cell growth, including Fe, Zn, Cu, Mn, Ni, Al, Cr, Co, and Se, were presented as stabilized chelated compounds without transferrin or other binding proteins. Pyruvate (0.8 m*M*) was added to the culture medium to ensure the metabolic requirements of pronucleate preembryos and to function as an antioxidant. The HSA (1% = 10 mg/ml) used was of highest quality and purity. All units of plasma used for the preparation of HSA had been tested for absence of anti-HIV (ELISA test) and HBsAG (third-generation test) and found to be negative. HSA also passed a pyrogen test, a safety test, and a sterility test.

The Group 2 medium was used without further supplementations for insemination, cleavage, and preembryo replacement. For the preparation of sperm and for flushing, the culture medium was supplemented with 25 m*M* Hepes. For flushing the concentration of $NaHCO_3$ was lowered to 1.1 g/liter, and HSA was reduced to 0.1%, i.e., 1 mg/ml.

Table I. Composition of EBSS Supplemented with SSR 2, Pyruvate, and HSA

$CaCl_2$ (anhyd.)	0.19 g/liter
KCl	0.38 g/liter
$MgSO_4$ (anhyd.)	0.0929 g/liter
NaCl	6.46 g/liter
$NaH_2PO_4 \cdot H_2O$	0.133 g/liter
D-Glucose	0.95 g/liter
Phenol red	0.0095 g/liter
Medi-Cult SSR 2, component A	1 ml/liter
Medi-Cult SSR 2, component B	1 ml/liter
Na-pyruvate (0.8 m*M*)	0.097 g/liter
$NaHCO_3$	2.2 g/liter
Pencillin	50,000 U/liter
Streptomycin	50 mg/liter
HSA	10 g/liter

Hybritest

The Hybritest has been described in detail previously (7,8). Briefly, the main principle of this cell culture assay is that rapidly growing and anchorage-independent hybridoma cells (1E6) are cultured under defined protein-free conditions, thereby greatly increasing the sensitivity to toxic substances due to the absence of binding proteins. The cytotoxicity testing of various products used in IVF is performed by mixing the compound or incubating the equipment to be tested in control medium (RPMI-SR3, GEA BioTech, Cat. No. 1008) before adding the indicator hybridoma cells. Test and control cultures are incubated at 37°C in 5% CO_2 in air for 4 days, whereafter cell counting of the test and control cultures is carried out. A cytotoxicity index (percentage inhibition of cell growth) is calculated using the following formula:

$$(N_2 - N_1)/N_2 \times 100,$$

where N_1 is the final cell number in test cultures, and N_2 the final cell number in control cultures. A cytotoxicity level <10% is not regarded as significant and is interpreted as a negative test. At ≥10% inhibition of cell growth, the test is considered positive.

Patients

Two hundred twenty consecutive treatment cycles leading to oocyte retrieval (OR) were included in this study. In 110 patients the culture medium (Ham's F10) was supplemented with patient serum (Group 1), and in the next 110 patients culture medium (EBSS) supplemented with SSR 2, pyruvate, and HSA was used (Group 2). A description of the patients enrolled is presented in Table II.

Table II. Patient Characteristics: Group 1, Serum-Supplemented Culture Medium; Group 2, Serum-Free Culture Medium

	Group 1	Group 2
Age (years)[a]	32.2 (3.3)	31.6 (3.1)
Indication for IVF-ER (*n*)		
Tubal infertility	101	100
Endometriosis	3	2
Unexplained infertility	6	8
Stimulation regimen (*n*)		
CC-hFSH-hMG	51	53
hFSH-hMG	59	57

[a] Mean (SD).

IVF Procedure

Controlled ovarian hyperstimulation was achieved by one of the following regimens: (i) 100 mg clomiphene citrate (Pergotime, Serono, Stockholm, Sweden) from cycle day 2 to cycle day 6, with administration, on cycle days 4 and 5, of 225 and 150 IU, respectively, pure follicle-stimulating hormone (hFSH; Fertinorm, Serono, Stockholm, Sweden) and injection, from cycle day 6 onward, of 150 IU human menopausal gonadotropin (hMG; Humegon, N.V. Organon, Oss, Netherlands); and (ii) 150 IU hFSH + 150 IU hMG on cycle days 3 and 4, with injection, from cycle day 5 onward, of 150 IU hMG.

Follicular development was monitored by transvaginal ultrasonography (Brüel & Kjær, Type 8538, Nærum, Denmark) and daily serum estradiol measurements. When the largest follicle(s) reached 18 mm in diameter and serum estradiol levels were appropriate for the number of large follicles present (0.9 nmol/liter per follicle of ~14-mm diameter), ovulation was induced with 9000 IU hCG (Physex, Leo, Copenhagen, Denmark), and oocyte aspiration was performed about 35 hr later by transvaginal ultrasound-guided puncture in local anesthesia (lidocaine chloride, Xylocaine, Astra, Södertälje, Sweden, 10 mg/ml, 20 ml) after premedication with midazolam (Dormicum, F. Hoffman-La Roche, Basle, Switzerland, 5–7.5 mg i.m.). We used a puncture needle with a 1.0-mm inner diameter (Swe-Med Lab, Gothenburg, Sweden, Cat. No. 3990) connected to a suction unit giving a negative pressure of about 100 mm Hg.

All cell cultures were maintained in a CO_2 incubator at 37°C in a humidified atmosphere of 7% CO_2 in air.

Sperm was prepared using the "swim-up" technique. Briefly, sperm was washed and centrifuged twice in culture medium at 300 and 200*g*, respectively. The pellet was resuspended in 2 ml medium and incubated for 120 min at 37°C in the CO_2 incubator to allow for the most motile spermatozoa to migrate into the medium.

All oocytes were preincubated for 6 hr before insemination in four-well culture dishes (Nunc, Roskilde, Denmark, Cat. No. 134673). About 100,000 motile spermatozoa were used for fertilization of the oocytes in 1 ml of insemination medium. Cumulus cells were removed at 16 to 20 hr after insemination by gentle pipetting. Fertilization was recognized by the presence of two or more pronu-

clei. Preembryos were transferred into 1 ml of fresh cleavage medium for further culture and reexamined in about 24 hr for cleavage.

Preembryo replacements were performed with the patients in the lithotomy position using Wallace catheters (Cat. No. 1816, H. G. Wallace Ltd., Colchester, UK). Flushing medium was used to rinse the catheter and the attached 1-ml tuberculin syringe and then to preload the entire replacement device. Transfer medium was aspirated into the catheter, followed by 3–4 cm of air and the preembryos in 10–20 μl of medium. The catheter was gently placed with the tip in the uterine fundus and the preembryos were expelled. One to four preembryos were replaced.

No luteal support was given. Blood samples were drawn 10, 20, and 34 days after ER for the measurement of β-hCG. By positive and increasing values of β-hCG, pregnancy was confirmed by a transvaginal ultrasound examination of 6–7 weeks after the last menstrual period.

Statistical Analyses

For statistical analyses, Student's *t* test and chi-square tests were used where appropriate. *P* values <0.05 were considered significant.

RESULTS

Between the two groups studied there was no significant differences concerning age, indication for IVF-ER, or stimulation regimen used (Table II). Also, the number of oocytes recovered was similar in the two experimental groups. On average, 7.9 oocytes were retrieved in Group 1, and 8.6 oocytes in Group 2. There was no significant difference in the fertilization rate (Table III). Nor was the stage of preembryo development at the time of replacement different (Table IV). The number of preembryos replaced showed no differences between the two experimental groups, i.e., 3.0 (Group 1) and 3.1 (Group 2) per patient, respectively. Likewise, the number of ERs was identical in the two groups. The implantation rate, i.e., the proportion of preembryos actually implanting as demonstrated by ultrasound examination, relative to the number replaced, was slightly higher in Group 2 (14.4%, vs 11.0% in Group 1) (Table V). The total clinical pregnancy rate was 29% per ER in Group 1 and 34% per ER in Group 2; a difference not reaching statistical significance. The relative number of deliveries or normal ongoing pregnancies, however, was significantly higher in Group 2 (28% per ER, compared to 16% in Group 1) ($P < 0.05$) (Table VI). In Group 1, two twin pregnancies and one set of triplets occurred, whereas in Group 2 five sets of twins and two sets of triplets were established. Four twin pregnancies and one set of triplets have been delivered. The other multiple pregnancies are normal ongoing (>18 weeks).

Table III. Fertilization Rate Following Insemination and Culture of Oocytes: Group 1, Serum-Supplemented Culture Medium; Group 2, Serum-Free Culture Medium

	No. of oocytes		Fertilization rate (%)
	Inseminated	Fertilized	
Group 1	867	498	57.4
Group 2	949	563	59.3

Table IV. Stages of Cleaved Preembryos 40 to 44 hr Following Insemination: Group 1, Serum-Supplemented Culture Medium; Group 2, Serum-Free Culture Medium

	No. of embryos	% embryos at each cleavage stage		
		2 cells	3–4 cells	≥5–6 cells
Group 1	480	18.9	66.5	14.6
Group 2	547	16.3	64.9	18.8

DISCUSSION

The present study shows that culture conditions in human IVF-ER can be optimized and simplified by using the serum-free culture medium described, i.e., a basal medium (EBSS) supplemented with a chemically defined, synthetic serum replacement (SSR 2), pyruvate, and 1% HSA. Generally, in order to achieve beneficial and reproducible results in IVF treatment, we believe that it is of particular importance that each batch of culture medium and its single components is routinely tested for cyto-

Table V. Implantation Rate Following Preembryo Replacement: Group 1, Serum-Supplemented Culture Medium; Group 2, Serum-Free Culture Medium

	Preembryos		Implantation rate (%)
	Replaced (*n*)	Implanting (*n*)	
Group 1	310	34	11.0
Group 2	326	47	14.4

Table VI. Outcome of Pregnancies Following Oocyte Retrieval (OR) and Preembryo Replacement (ER): Group 1, Serum-Supplemented Culture Medium; Group 2, Serum-Free Culture-Medium

	Group 1	Group 2	*P* (by chi-square test)
No. of ORs	110	110	
No. of ERs	105	105	
Total clinical pregnancies (*n*)	30	36	
Per OR (%)	27	33	NS
Per ER (%)	29	34	NS
Clinical abortions (*n*)	9	6	
Rate (%)	30	17	NS
Ectopic pregnancies (*n*)	4	1	
Rate (%)	13	3	NS
Deliveries/ongoing pregnancies[a] (*n*)	17	29	
Per OR (%)	15	26	<0.05
Per ER (%)	16	28	<0.05

[a] Normally ongoing pregnancies at >20 weeks after the last menstrual period.

toxicity in a sensitive bioassay (i.e., Hybritest). Furthermore, it is important that culture media are freshly made up using a high-quality sterile water, as we have shown previously (7,8). It is also of consequence that culture media are not stored for more than 4 weeks at +4°C and that exposure to light is avoided, since the media otherwise are rapidly degraded (10). When using HSA it is important to employ HSA of the highest quality and purity. Thus, in our opinion it is indispensable for a high success rate in clinical IVF that the general quality requirements outlined are followed, Then, a rate of deliveries/ongoing pregnancies per OR or per ER of 26–28% can be achieved (Table VI), provided that the other steps in the IVF treatment are performed thoroughly. Furthermore, the abortion rate tends to decrease when using serum-free culture conditions, and this approach does not seem to impair the fertilization process or implantation rate.

A point of particular interest to be deduced from this study is that serum apparently is not necessary in human IVF, preembryo culture and replacement, and for the capacitation of sperm. This has also been shown by Menezo and co-workers (11). Their culture medium (B_3 medium) was also supplemented with HSA. Otherwise, the B_3 medium must be considered an ordinary basal medium, i.e., a salt solution with glucose, amino acids, and vitamins, and supplemented with lipids and cholesterol, besides HSA. Apparently favorable clinical results can be obtained with the B_3 medium (11,12). This culture medium, however, does not contain any metal trace elements or metal stabilizing chelators. Thus, metals necessary for cell growth and differentiation are not adequately presented. Particularly, there is strong evidence that the presence of transferrin or other Fe-presenting substances is essential for oocyte maturation in preparation for subsequent fertilization and cleavage (13). The reproducibility of the B_3 medium in an IVF program must therefore be questioned. Furthermore, a possible negative effect on embryo growth of purines and pyrimidines contained in the B_3 medium cannot be ruled out. It has been reported that purine derivates maintain oocyte meiotic arrest (14) and have a detrimental effect on the development of two-cell embryos in mice (1).

Recently, the use of another serum substitute (UltroSer G) in IVF has been reported (15). This study concluded that UltroSer G sustained successfully the development in vitro of mouse and human preembryos. However, this serum substitute, at the concentration used, significantly impaired the fertilization process (15). According to the manufacturer's information. UltroSer G contains multiple serum fractions (i.e., mineral trace elements, hormones, binding proteins, and vitamins), supplemented with highly purified growth factors and attachment factors, and a trypsin inhibiting factor. Opposed to SSR 2, UltroSer G is not chemically defined and its purity must be questioned. Furthermore, UltroSer G does not optimize preembryo growth and may also contain inhibiting factors since hybridoma cells and lymphocytes cannot be cultured with this serum substitute, whereas both hybridoma cells (7) and lymphocytes (16) can be cultured easily with SSR.

SSR 2 must, on the other hand, be regarded a universally applicable synthetic serum replacement for practically all types of cells, although the possible need for special growth factors or attachment factors for various cell types has to be examined. The universality of SSR 2 has been made possible by a novel metal ion buffer, chelators, and a synthetic replacement for transferrin, thereby presenting all metals necessary for growth and differentiation to the cells.

Interestingly, Jinno *et al.* recently reported (17) that the addition of EDTA, a well-known chelating substance, significantly enhanced the development of mouse preembryos derived from oocytes matured in vitro, both to the two-cell stage and to blastocysts. This substantiates, at least indirectly, the use of SSR 2-containing culture media for IVF,

since it shows the value of chelating agents in this type of product.

Pyruvate was added to the culture medium since this substance seems to be needed to support the development of pronucleate preembryos as glucose cannot be utilized until cleavage has begun (18). Furthermore, pyruvate has antioxidative effects which are advantageous both for preembryo growth and during the preparation of sperm.

The addition of HSA (1%) to the serum-free culture medium must be viewed in the light of its general function as an "environmental buffer" (19–21). Thus, HSA has the ability to bind various substances, both necessary and toxic ones. By adding HSA to a serum-free medium, lipids and other nutrient molecules may be made available to cells. Particularly, the HSA–lipid complex may be advantageous for the in vitro culture of human preembryos (20,21). Furthermore, the supplementation of HSA to the culture medium is important for the fertilizing capacity of the spermatozoa. HSA may be involved in sperm capacitation and has been shown to induce the acrosome reaction, and also appears to maintain sperm viability (22). Additionally, because the occurrence of interfering substances and batch variability is prevented by using the serum-free medium described, it may prove to be of value for the standardization of the human sperm zona-free hamster oocyte penetration test (SPA) (23). In this way, the SPA may yield a more precise prediction of the fertilizing capacity of spermatozoa in an IVF program.

The use of bovine serum albumin (BSA) has also been advocated in human IVF (11,24). However, one has to consider that BSA is a slaughterhouse-derived material and, as such, is therefore in principle hampered by impurities. There are considerable variations in quality between different batches of BSA, and a toxic effect on cell cultures cannot be ruled out (20, unpublished results). For these reasons, the use of BSA cannot be recommended in human IVF treatment.

In conclusion, supplementation of SSR 2, pyruvate, and HSA to a basal medium (EBSS) and testing for the absence of cytotoxicity in a sensitive bioassay (Hybritest) have yielded a culture medium capable of sustaining the development in vitro of human preembryos without impairing the fertilization process or the implantation rate, ultimately resulting in a significantly increased rate of deliveries/ongoing pregnancies and an apparently decreased abortion rate. The potential harmful effects of serum in an IVF program and the need for blood sampling and preparation make the advantages of replacing serum with the synthetic serum replacement SSR 2 obvious.

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