A prospective randomized sibling-oocyte study of two media systems for culturing cleavage-stage embryos—impact on fertilization rate

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Abstract

Purpose Although several media systems have been developed, data from prospective randomised clinical studies are still lacking. In the present study we compared the effects of 2 different media systems on embryo morphology and development at days 2/3 using sibling oocytes.

Methods In this prospective sibling-split trial, 1206 oocytes from 110 women were divided via alternate allocation to fertilization and culture in media system A (G-IVF™ v5 PLUS/ G-1™ v5 PLUS) or for fertilization and culture in media system B (Universal IVF medium/EmbryoAssist™).

Results The use of media system A significantly increased the normal fertilization rate (73.5% versus 67.2%; $p=0.030$) and embryo utilization rate (55.5% versus 42.9%; $p=0.001$), whereas polyploidy and embryo quality were similar in the two groups.

Conclusion The different impacts on fertilization and early embryo development between the two commercially available and commonly used media systems show the importance of evaluation of the efficacy of existing sequential culture media and the need to further improve media for in vitro development of human embryos.

Keywords Sibling oocytes · Culture media · Fertilization rate

Background

The human oocyte is fertilized in the Fallopian tube and is developing during the transport into the uterus, where it is implanted at the blastocyst stage [1–3]. The ampulla region of the Fallopian is the natural environment for fertilization and the first mitotic divisions occurs as the pre-embryo passes along the Fallopian tube to reach the uterus [3]. The in vivo environment is optimal for the developing preimplantation embryo as it moves and develops along this route. Media intended for use in IVF treatment should be optimised in order to reflect the environment in the Fallopian tube and endometrium, as the culture conditions in which the zygote forms and divides plays an important part in dictating its development and subsequent achievement of a successful pregnancy [4]. The change in environment in the different parts of the Fallopian tube has lead to development of different culture media systems.

Development of new culture media is continuously ongoing, but so far none of the media used are as complex as the tubal and endometrial fluid. Simple media has gradually been replaced with more complex culture systems; but the ability of different media to support the fertilization and culture of preimplantation embryos up to cleavage stage remains uncertain [5]. Although the use of commercial media is usually beneficial [6], these media are still under investigation and their most optimal composition remains to be determined.

The aim of the present study was to compare the effects of two different commercial culture media on early embryo

Capsule Two commercially available and commonly used media systems show different impacts on fertilization and early embryo development

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development using a prospective randomized sibling design. The primary outcome measure was normal fertilization rate. The secondary outcome measures were polyploidy rate, embryo utilization rate and embryo quality (EQ) at day 2 and 3.

Material and methods

Design of the study

This trial was designed as a prospective randomized sibling oocyte split study and included all oocytes from women undergoing IVF treatment at the Centre for Reproduction, Uppsala University Hospital, Sweden, between October 2007 and December 2007. The age limits of the patients ranged from 24 to 40 years, with a mean ($\pm$ SD) of 33.9 ± 3.8 years. The oocytes were randomly allocated into one of two groups at the time of ovum pick up. Allocation was not blinded. Oocytes in media system A ($n=622$) were fertilized in fertilization media A (G-IVFTM PLUS, Vitrolife AB, Kungsbacka, Sweden) and further cultured in culture media A (G-1TM PLUS v5, Vitrolife AB, Kungsbacka, Sweden). Oocytes in media system B ($n=584$) were fertilised in fertilization media B (Universal IVF medium, MediCult A/S, Jyllinge, Denmark) and cultured in culture media B (EmbryoAssistTM MediCult A/S, Jyllinge, Denmark). When there were uneven numbers of oocytes, the additional oocyte was allocated to media system A, which was the standard system in the clinic at the time of this trial.

Ovarian stimulation and oocyte retrieval

Down-regulation for ovarian hyperstimulation was achieved by using either a gonadotropin-releasing hormone (GnRH) agonist or antagonist protocol (Suprecur Hoechst, Frankfurt, Germany, Synarel, Syntex Nordica AB, Södertälje, Sweden; Cetrotide, Serono Laboratories, Aubonne, Switzerland or Orgalutran, Organon, Oss, the Netherlands), starting on either cycle day 1 or day 21. Following down-regulation, ovarian stimulation was induced by using either recombinant FSH, (Gonal-F, Serono Laboratories, Aubonne, Switzerland, or Puregon, Organon, Oss, the Netherlands) or urine-derived Menopur[®] (Saint-Prex, Switzerland). The starting dose was dependent on the woman's age and/or previous response to ovarian stimulation. The ovarian response was monitored by means of serum oestradiol assays and vaginal ultrasonographic scans of follicles. Recombinant FSH or Menopur[®] was administered until the leading follicle had a diameter of at least 17 mm. Maturation of the oocyte was triggered by s.c injection of hCG (Pregnyl, Organon, Oss, the Netherlands or Ovitrelle, Serono Laboratories, Aubonne, Switzerland). Oocytes were retrieved by transvaginal

needle aspiration under ultrasonographic guidance 34–36 h after hCG administration. The cumulus oocyte complexes were randomly allocated to fertilization medium A or fertilization medium B.

Semen preparation and fertilization

All semen preparation was carried out in Nidacon sperm preparation media. A two-density gradient (47.5/95%) (PureSperm[®] 100, Nidacon International, Mölndal, Sweden) was used for isolation of motile sperm. Briefly, liquefied semen was gently overlaid the 47.5% layer and centrifuged for 15 min at $300 \times g$. The supernatant was aspirated and 0.5 ml of the pellet was transferred into a new centrifuge tube and washed twice in 5 ml equilibrated room-temperature PureSperm[®] Wash (Nidacon) for 10 min at $500 \times g$. Sperm concentration was determined by pipetting an aliquot of 5 μ l semen into a Makler Counting Chamber (Sefi Medical Instruments, Israel), followed by counting under a phase contrast microscope.

Conventional IVF was performed in 20 μ l fertilization medium A or fertilization medium B under mineral oil and about 15,000 spermatozoa were used. The compositions of the two media used for fertilization is shown in Table 1. According to lab praxis, insemination was done at about 13.00 and fertilization check in the morning between 07.45 and 09:00 (18–20 h after insemination).

Embryo culture and grading

During the study period, the culture conditions were kept identical as regards temperature, and pH. The only difference was the culture media used and protein supplement. To ensure optimal fertilization and culture conditions in the incubator, daily manual temperature readings were performed. The pH of the media was checked once a week and the CO₂ level adjusted for optimal pH range of 7.2–7.4. Using these criteria, the pH for both media was within the optimal range (7.2–7.4) as recommended by the manufacturers.

Following fertilisation after IVF or ICSI, oocytes were evaluated and cultured in groups of 3–5 under oil in 20 μ l droplets of either culture media A or culture media B at 37°C in a dual gas incubator (6% CO₂ and 94% air). Composition of the two media used for culture is shown in Table 2. On the morning of day 2 or day 3 the embryo quality was assessed morphologically on the basis of the number of blastomeres (cells), the fragmentation rate (volume of embryo with anucleate fragments) and multinucleation of blastomeres, as described previously [7, 8]. Each embryo received a score of 0 (top quality), 1 (good quality), 2 (fair quality) and 3 (poor

Table 1 Composition / Information on ingredients of the fertilization and culture media

G-IVF.v5 Plus (Vitrolife)	Universal IVF (Medicult)	GI.v5 Plus (Vitrolife)	EmbryoAssist (Medicult)
Alanyl-Glutamine	Water	Alanine	Water
Calcium chloride dihydrate	Sodium Chloride	Alanyl-glutamine	Sodium Bicarbonate
Citrate	Human Serum Albumin	Asparagine	Human Serum Albumin
EDTA	Glucose	Aspartate	Sodium Chloride
Fructose	Potassium Chloride	Calcium chloride	Amino Acids
Gentamicin	Calcium Chloride	EDTA	Hepes Acid
Glucose	Sodium Phosphate monobasic	Gentamicin	Calcium Lactate
Glycine	Magnesium Sulphate	Glucose	Potassium Sulphate
Human serum albumin	Sodium Pyruvate	Glutamate	Sodium phosphate
L-Alanine	Sodium Bicarbonate	Glycine	Vitamins
L-Asparagine H ₂ O	Penicillin Sodium Salt	Human serum albumin	Glucose
L-Aspartate	Streptomycine Sulfate Salt	Hyaluronan	EDTA
L-Glutamate	SSR [®] (Synthetic Serum Replacement) ^a	Lipoic acid	Sodium Pyruvate
L-Proline		Magnesium sulphate	Magnesium Sulphate
L-Serine		Methionine	Penicillin Sodium Salt
Magnesium sulphate heptahydrate		Potassium chloride	Streptomycine Sulfate Salt
Potassium chloride		Proline	SSR [®] (Synthetic Serum Replacement) ^a
Sodium chloride		Serine	
Sodium dihydrogen orthophosphate 1-hydrate		Sodium bicarbonate	
Sodium hydrogen carbonate		Sodium chloride	
Sodium lactate		Sodium citrate	
Sodium pyruvate		Sodium dihydrogen phosphate	
Taurine		Sodium lactate	
Water for injection (WFI)		Sodium pyruvate	
		Taurine	
		Water for injection (WFI)	

^a Contains Human Insulin recombinant.

quality). Embryos with a score of 0–2 were used for direct embryo transfer either on day 2 or day 3, or frozen for later use by the patient (supernumerary embryos). In this trial embryos were regarded as of ‘good quality’ when they were at the 4-cell stage at ~46 h post insemination (day 2) or at the 8-cell stage, ~ 70 h post insemination (day 3) and had a score of 0–2. Three embryologists graded the embryos and together decided on which embryo had the highest quality. The embryologists assessing embryo grading and selecting embryos for transfer or cryopreservation were not blinded as to which media was used. According to clinic policy one embryo was transferred in women ≤38 years old and two embryos in women >38 years old. In cases when embryo quality was not good, two embryos were transferred regardless of the woman’s age.

Embryo transfer

Embryo transfers were performed using a soft transfer catheter (K-JETS-7019-SIVF, Cook, Brisbane, Australia) under transabdominal ultrasonographic guidance.

Outcome measures

Normal fertilization rate was defined as number of oocytes containing two pronuclei (2PN) divided by total number of mature oocytes (MII). *Polyploid rate* was defined as number of oocytes containing >2PN divided by total number of mature oocytes (MII). Embryo quality (EQ) was defined as number of 4-cell embryos on day 2 and number of 8-cell embryos on day 3 with a grade of 0–2, divided by number of normal fertilized embryos. Embryo

Table 2 Demographic data of patients included in the study is shown

Patients included (<i>n</i>)	110
Female age, mean±SD (years)	33.9±3.8
Infertility diagnosis, <i>n</i> (%)	
Tubal factor	8 (7.3)
Endometriosis	8 (7.3)
Unexplained	30 (27.3)
Anovulation	12 (10.9)
Male factor	37 (33.6)
Other	15 (13.6)
No. of oocytes retrieved per patient, mean±SD	10.9±4.8

utilization rate was defined as the number of embryos transferred and the number of embryos frozen in relation to the total number of embryos available. The *positive hCG rate* was determined as a ratio between the number of positive hCG tests and the total number of embryo transfers. *Clinical pregnancy rate* was defined as the number of cases with evidence of at least one gestational sacs divided by the number of transfers. *Implantation rate* was defined as the number of gestational sac/sacs verified by vaginal ultrasonography divided by the number of embryos transferred. The *Delivery rate* was defined as a ratio between deliveries and embryo transfers.

Statistical analysis

SPSS statistical software (Chicago, IL, USA) was used to calculate statistics. Power analysis showed that to detect an increase of 10% in fertilization rate between the two media systems with a power of 0.8 (alpha-level 0.05), we needed at least 269 oocytes in each group. Comparison of normal fertilisation rates, polyploid rates, embryo quality and embryo utilization rates was performed using the Chi-square test. Positive hCG rates, clinical pregnancy rates, implantation rates and delivery rates were compared by using the Fisher's exact test (two-tailed). A value of $p < 0.05$ was considered statistically significant.

Table 3 Outcome of 1206 sibling oocytes assigned to media system A or system B.

*Statistically significant ($p < 0.05$, Chi-square test)

	Group A	Group B	<i>P</i> -value
Number of oocytes inseminated, <i>n</i>	622	584	0.122
Mature oocytes (MII), <i>n</i> (%)	520/622 (83.6)	469/584 (80.3)	0.137
Normal fertilized oocytes (2PN), <i>n</i> (%)	382/520 (73.5)	315/469 (67.2)	0.030*
Polyploid (>2PN), <i>n</i> (%)	29/520 (5.6)	34/469 (7.3)	0.282
Embryo quality (grade 0–2), <i>n</i> (%)			
with 4-cells at day 2	210/382 (54.9)	163/315 (52.7)	0.396
with 8-cells at day 3	23/382 (6.0)	11/315 (3.5)	0.123
Embryos cryopreserved, <i>n</i> (%)	124/382 (32.5)	111/315 (35.2)	0.439
Embryo utilization, <i>n</i> (%)	212/382 (55.5)	135/315 (42.9)	0.001*

Results

Normal fertilization rate, polyploid rate, embryo quality and embryo utilization rate

In total, 1206 oocytes from 110 women were included in this study. A little over half of them (622; 51.6%) were fertilized and cultured in media system A (group A) and 584 were fertilized and cultured in media system B (group B). Patient demographic data including female age, infertility diagnosis and number of oocytes retrieved are given in Table 2. Regarding the causes of infertility, 7.3% were due to tubal factor, 7.3% due to endometriosis, 27.3% were unexplained, 10.9% and 33.6% were due to anovulation and male factor respectively, and 13.6% due to other factors

This was a sibling-split study and therefore differences in demographic data were eliminated. The rate of normal fertilization and the embryo utilization rate were significantly higher in group A than in group B (73, 5% and 55.5% compared with 67.2% and 42.9% respectively, $p = 0.030$ and 0.001). There were no differences in polyploidy rate, embryo quality and the number of embryos cryopreserved between the groups (Table 3).

Embryo transfer

A total of 108 embryo transfers were performed. Seventeen of these were mixed transfers, were two embryos, one from each group were transferred (data not shown). Three embryologists together agreed on the best quality embryos to be used in immediate transfer. Additional embryos of high quality were frozen. A Significantly higher number of transfers were performed after culture in media system A (75.8%) compared with system B (24.2%; $p < 0.0001$), as shown in Table 4.

Clinical outcomes

The positive hCG rate, the clinical pregnancy rate, the implantation rate and the delivery rate were similar in the two groups (Table 4).

Table 4 Clinical outcome of 91 embryo transfers, comparing the two media systems is shown.*Statistically significant ($p < 0.05$, Fisher's exact test (two-tailed))

	Group A	Group B	P-value
Embryo transfer (<i>n</i>)	69 (75.8)	22 (24.2)	<0.0001*
Positive hCG, <i>n</i> (% per embryo transfer)	34 (49.3)	11 (50.0)	1.00
Clinical pregnancies, <i>n</i> (% per embryo transfer)	32 (46.4)	8 (36.4)	0.467
Implantation rate, <i>n</i> (%)	36/88 (40.9)	9/24 (37.5)	0.818
Deliveries, <i>n</i> (% per embryo transfer)	24 (34.8)	8 (36.4)	1.000

Discussion

In most mammalian species, the intrinsic quality of the oocyte before it is fertilized determines whether a zygote has the potential to progress through pregnancy [4]. Human embryos used for IVF are generally derived from a cohort of oocytes collected after ovarian stimulation. These oocytes differ in quality between women and the only way to study the effect of different media systems is to compare oocytes from individual women. Therefore, this study was designed as a sibling-split study, where oocytes from each woman were divided into two groups and two different culture systems were used. So far, in previous studies, randomisation procedures have been performed in relation to weekdays [9] or other randomisation procedures have been used [10], increasing the possibility that the randomisation procedure might influence the final result. By using sibling oocytes where each patient serves as her own control, we were able to control for confounding factors that are inherent to the above mentioned study designs.

The number of oocytes was higher in group A, which was a result of the fact that the media system used in this group was standard in the clinic at the time and when an uneven number of oocytes was retrieved, the additional oocyte was allocated to group A.

Detailed composition of the two media is unknown—the ingredients are to some extent known, as shown in Table 1, but their concentrations are company secrets. Therefore it is difficult to determine which ingredients are the most important for efficient embryo culture. It is noticeable that the majority of studies carried out to determine the effects of media additives on embryo development have been performed using murine or bovine embryos and the conclusions drawn from these studies might not be valid for human embryos.

The fertilization rate was significantly higher in group A than in group B. The fertilization media differ, among other things, as regards the serum component, sodium citrate, energy sources, antibiotics, EDTA and taurine. Fertilization medium A contains human serum albumin and a number of defined amino acids, while fertilization medium B contains a synthetic serum replacement component that contains serum albumin plus other ingredients. It is not known if this

serum replacement component also contains amino acids—the exact composition is kept secret.

Fertilization medium A contains sodium citrate, which is not known to be included in fertilization medium B. The impact of sodium citrate on fertilization is not clearly understood. It is known that citrate does not seem to improve sperm motility after freezing [11]. Addition of citrate to bovine embryo culture appears to enhance morula and blastocyst development, and hatching [12–14], although in one study no impact on bovine blastocyst formation was found [15]. The present results indicate that it could be beneficial for early human embryo development to have citrate in the fertilization medium.

Energy is needed for fertilization. Fertilization medium A contains four sources of sugar—fructose, glucose, pyruvate and lactate—while fertilization medium B contains only glucose and pyruvate. Fructose and glucose are both energy sources and it has been shown that fructose can replace glucose, at least as regards bovine embryos [16].

Both lactate and pyruvate are favourable energy sources for spermatids [17]. For pig embryo development, lactate and pyruvate for the first 48 h resulted in significantly higher cell numbers than in those cultured with glucose only [18]. It was recently shown that lactate increases the *in vitro* fertilization rate in mice [19] and this could to some extent explain why the fertilization rate was higher in group A. Lactate can not be replaced by pyruvate for energy requirements, as their mechanisms of action are different. Pyruvate up-regulates ATP production in sperm and oocytes, while lactate regulates the redox process instead of increasing ATP production [20]. Lactate is present in culture medium B, but this might be too late as regards optimal early embryo development.

Insulin is used in cell culture systems to promote cell proliferation [21]. It was recently shown that insulin enhances sperm motility and the acrosome reaction, as well as nitric oxide production [22]. Only fertilization medium B contained insulin, which did not enhance fertilization rate in comparison with medium A. However, it is important to keep in mind that these media are complex and it is difficult to identify one factor as being more important than any other.

The types of antibiotic used in culture media formulations could be of importance for embryo development. Follicle bioassay has been found to reveal a concentration-

dependent decrease in mouse oocyte nuclear maturation during continuous exposure to streptomycin and a significantly reduced rate of polar body formation. A lower fertilization rate was also seen after exposure to streptomycin. Additionally, it has been reported that exposure of hamster embryos to penicillin and streptomycin reduced the percentage of 8-cell embryos and blastocysts versus controls and in comparison with gentamycin alone [23]. This could be one explanation as to why the fertilization and cleavage rates were lower in media system B, containing both streptomycin and penicillin.

Chelating agents such as EDTA improve bovine embryo development [24], but the effect on fertilization is not known. Only fertilization medium A contains EDTA, although it is included in both culture media.

Addition of glutamine and hypotaurine has been suggested to improve embryo development by improvement of intracellular oxidative status [25]. Taurine has also been shown to improve bovine embryo culture by increasing total cell number and the rate of blastocyst formation [26]. As far as we know only fertilisation medium B contains taurine, although it is included in both culture media.

Culture medium A contain hyaluronan, which is absent in culture medium B. Mouse embryos produce hyaluronan, and its receptor RHAMM is present throughout embryo development, suggesting that hyaluronan plays a role at this stage [27]. Several investigators have shown that hyaluronan-enriched medium can improve implantation and pregnancy rates [28–30], but in a recent study we found that although the implantation rate of human embryos was improved when adding hyaluronan to the transfer medium, the delivery rate was not affected, as a result of an increased number of early pregnancy losses [31].

Embryos cultured in vitro are constantly exposed to stress factors such as reactive oxygen species (ROS). These are partly formed during embryo metabolism and are associated with suboptimal composition of plastics used in embryo culture. Other sources of ROS are UV light, volatile organic compounds and oxygen in the air. Culture medium A contains lipoic acid as an antioxidant. This is a potent antioxidant which protects embryos in culture [32]. The presence of this acid in culture medium could explain the superiority of medium A over medium B.

The percentage of embryos transferred was higher after fertilization and culture in media system A. Three embryologists decided together on the embryos that were of the highest quality and thereby the best to transfer. The decision was a professional one with the aim of achieving the best possible chance of pregnancy for the individual couple. The embryologists were not blinded to the media in which the embryos had been cultured. However, the fact that a higher proportion of embryos cultured in system A reached the 4 and 8-cell stages is likely to mirror the quality

of the embryos. This is supported by the significantly higher fertilization and embryo usage rates in group A.

In summary, fertilization medium A contains citrate, lactate, EDTA and taurine, all favourable for fertilization. In addition, it contains gentamycin, which is less toxic to oocytes than streptomycin, which is included in fertilization medium B. Fertilization medium B on the other hand, contains insulin, which is thought to be favourable. Taken together, the combination of nutrients in fertilization medium A enhances fertilization rate. Culture medium A is richer and contains hyaluronan, citrate which improves early embryo development, and a less toxic antibiotic, in combination with (protective) lipoic acid, which could be the reason for better early embryo development after culture in this medium.

In conclusion, the most interesting finding in the present study is that early embryonic development appears to be improved, and may even be sped up (ie. more embryos are at cleaved stage in group A), but since there is no blastocyst data, chances are that these differences may be minimised or overcome by day 5 or day 6, and that the outcomes as relates to implantation and delivery are no different (non randomized outcomes in this study). The data show the importance of culture media and the fact that increased effort is needed to improve them further.

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