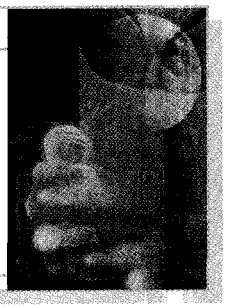
RESEARCH ARTICLE

A study of two sequential culture media – impact on embryo quality and pregnancy rates



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Objective. A comparative study of embryo quality and pregnancy outcome between Sydney IVF medium and Quinn's Advantage sequential culture media.

Design. A prospective randomised controlled trial and a retrospective study.

Setting. *In vitro* fertilisation clinic in an academic research environment.

Patients. All women < 38 years undergoing fresh embryo transfers.

Interventions. Use of clinic specific age, randomisation of patients and embryo score.

Main outcome measures. Fertilisation and cleavage rate, embryo quality (day 2 and day 3), blastulation rate and pregnancy rate.

Results. *Prospective randomised trial:* In this study the only significant difference was in day 3 embryo quality (33/79 (42%) v. 40/67 (60%) for Sydney IVF and Quinn's Advantage respectively, $p < 0.05$). *Retrospective study:* Significant difference ($p < 0.05$) for embryo development (early-dividing embryos 156/786 (20%) v. 263/919 (29%)), day 3 good quality (234/639 (37%) v. 378/795 (48%)) and pregnancy rate (ongoing pregnancy rate 31/179 (17%) v. 59/195 (30%)) between Sydney IVF v. Quinn's Advantage sequential culture media.

Conclusion. We conclude from these two studies that the range of Quinn's Advantage sequential culture media is more beneficial for *in vitro* embryo culture as each of the media in the range contribute collectively to more embryos with a better quality. The reason for the significant increase in embryo developmental parameters and pregnancy rate can possibly be attributed to the differences in composition between the two media.

Optimisation of culture media for the support of human embryos after *in vitro* fertilisation (IVF) and before embryo transfer has been a focus of considerable interest over the past decade. The first culture media used for human IVF, such as Ham's F10 and Earle's Balanced Salt Solution, were constructed to support the development of somatic cells and cell lines in culture. Other media, such as Tyrode's T6 and Whitten's WM1, have been used for IVF and embryo culture in laboratory animals. One of the first media specifically designed for human IVF was human tubal fluid (HTF).[1] Trying to imitate the *in vivo* environment to which the embryo is exposed, the formulation of HTF was based on the then known chemical composition of the fluids in human fallopian tubes.

IVF culture media can generally be categorised into either the simple balanced salt solutions containing energy sources (e.g. Earle's, Tyrode's T6, WM1) or the more complex mixtures of inorganic salts, energy sources, amino acids, vitamins and other substances (e.g. Ham's F10, Ménézo's B2 and B3 media, Eagle's MEM).[2] Before the mid-1990s, low production and accompanying low viability of blastocysts during human IVF due to the inability of any single culture medium to meet and sustain the changing nutritional requirements of preimplantation embryos resulted in blastocyst transfer being abandoned.[3] Recent development of sequential media designed for human embryo culture has led to improvements in human IVF outcomes. These sequential media were formulated according to

the carbohydrate composition of oviduct and uterine fluids and take into account the changing physiology and metabolic requirements of the human embryo.[3] The first stage of a sequential media system comprises a glucose-poor or even glucose-free medium designed to support the development of the zygote stage to the eight-cell stage, followed by a second, more complex, medium suitable for development, following activation of the embryonic genome, up to the blastocyst stage.

Several studies have confirmed that high implantation rates can be achieved after transfer of blastocysts obtained with this new generation of media.[4,5] In cases where patients have a good prognosis, implantation rates of up to 50% have been reported.[6,7] The percentage of blastocysts obtained as well as their viability is greater than that reported for culture systems using one medium throughout the culture, such as a mixture of Earle's and Ham's F-10[8,9] or minimal essential medium (MEM).[10] Overall, the results achieved with sequential medium appear to be in the same range as those reported when embryos are co-cultured with feeder cells.[4,11,12] However, culture in sequential media offers several advantages over co-culture, the risk of introducing pathogens is greatly reduced, culture handling is less time-consuming, and the composition of the culture medium is less uncertain.

Several of these sequential media are commercially available, including G1/G2 medium,[13-15] G1.2/G2.2 medium,[6,7] S1/S2 medium,[16] P1/Irvine Scientific Blastocyst medium,[17,18] IVF-50/G2,[15,19] IVF 50-S2,[5,11,17,19] Sydney IVF Cleavage/Blastocyst medium[20] and Quinn's Advantage sequential culture medium.[21] However, only a few studies have compared the proficiency of these media and assessed the viability of embryos cultured in sequential media.[22]

The aim of the present study was to assess pregnancy outcomes after transfer of day 3 embryos and day 5 blastocysts obtained in Sydney IVF medium and Quinn's Advantage sequential culture medium and to compare the *in vitro* development of embryos in these sequential media using developmental speed, embryo morphology (day 2 and 3) and blastocyst formation rate as end-points.

Materials and methods

The study was performed in two phases. In the first phase, both media were compared after conducting a prospective randomised control trial. The retrospective analysis comprises the second phase of the study.

Prospective randomised controlled trial (April - May 2003)

Inclusion criteria were as follows: female patient age < 38 years, day 3 FSH level < 12 mIU/ml,[23] and the presence of at least 2 follicles ≥ 18 mm in diameter on the day of human chorionic gonadotrophin (hCG) administration. At oocyte retrieval, patients were randomly (alternately) assigned to one of two groups. In group A (N = 20) zygotes were cultured in Sydney IVF Cleavage/Blastocyst Medium (Cook, Brisbane, Australia) and in group B (N = 19) culture was performed in Quinn's Advantage sequential culture medium (Sage In-Vitro Fertilization, a CooperSurgical Company, Trumbull, CT, USA). The composition of the two commercially available media is shown in Table I.

Table I. Composition of Sydney IVF (Cook, Australia) and Quinn's Advantage (CooperSurgical, USA) media

	Sydney IVF medium			Quinn's Advantage medium		
	Fertilisation stage	**Cleavage stage**	**Blastocyst stage**	**Fertilisation stage**	**Cleavage stage**	**Blastocyst stage**
pH	7.3 - 7.5	7.3 - 7.5	7.3 - 7.5	7.3	7.2	.3
Phosphate	Low*	Low*	Low*	0.01 mM	No	+*
$NaHCO_3$	25 mM	25 mM	25 mM	20.2 mM	15.6 mM	22.6 mM
Protein	1% (HSA)	1% (HSA)	1% (HSA)	3 mg/ml HSA	5 mg/ml SPS, α- & β-globulins	5 mg/ml SPS, α- & β-globulins
Glucose	+*	No		2.8 mM	0.1 mM	2.8 mM
Magnesium		Low*		0.2 mM	2 mM	2 mM
Na-pyruvate		0.33 mM		0.33 mM	0.33 mM	0.1 mM
Lactate		21.4 mM (Na-lactate)			2.04 mM (Ca-lactate)	
Ala-Gln		No, (Ala + Gln)*			1 mM	
Amino acids	Taurine, glutamine, glycine, non-ess AAs*			0.1 mM taurine + 0.5 mM non-ess AA (no Ala) + 1 mM ess AA†		
Phenol red		No			Yes	
Basic salts		Yes			Yes	

* Exact concentration not provided.

† Blastocyst Medium only.

HSA = human serum albumin; SPS = serum protein substitute; Ala = alanine; Gln = glutamine; ess AA = essential amino acids; non-ess AA = non-essential amino acids.

Retrospective study (January 2002 - March 2003 v. June 2003 - May 2004)

This study was conducted using our computer-based data system. For the abovementioned time periods the database was searched for all patients who had an intracytoplasmic sperm injection (ICSI) cycle, with the following inclusion criteria: female patient age < 38 years, and > 1 embryo transferred. For the period January 2002 - March 2003 Sydney IVF medium was used and 181 cycles adhered to the inclusion criteria (group A = Sydney IVF group). For the period June 2003 - May 2004 Quinn's Advantage medium was used and 203 cycles adhered to the inclusion criteria (group B = Quinn's Advantage group).

Ovarian stimulation

For both studies controlled ovarian stimulation was achieved using a long protocol of gonadotrophin-releasing hormone (GnRH) agonist (Synarel; Montosano South Africa, Searle) followed by human menopausal gonadotrophin (hMG) (Pergonal; Serono, South Africa (Pty) Ltd) and/or pure FSH (Metrodin; Serono) from cycle day 3. Patients were followed up by performing oestradiol determinations as well as serial ultrasonographic measurement of all the follicles. Ovulation was induced by the administration of hCG (Profasi; Serono) as soon as the leading follicle reached 18 mm in diameter.

Oocyte retrieval

Follicle aspiration was done under conscious sedation (Dormicum; Roche Products (Pty) Ltd, South Africa, or Diprovan; Zeneca Pharmaceuticals, South Africa). Oocytes were recovered by transvaginal ultrasound-guided follicle aspiration 34 - 36 hours after hCG administration.

The cumulus oocyte complexes were isolated into flushing medium with 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) (Medi-Cult, Copenhagen, Denmark) (group A), or Quinn's Advantage medium with HEPES (group B).

Semen preparation

In group A semen preparation was carried out in Medi-Cult sperm preparation medium with HEPES and in group B it was carried out in Quinn's sperm washing medium with HEPES. Where possible, motile sperm were isolated by a standard swim-up technique. This technique included two cycles of centrifugation (428 - 450 *g* for 10 minutes) and washing. Then 500 µl of the same medium was gently added to the resulting pellet and left in the incubator at 37°C for 60 minutes to allow swim-up. In cases of oligozoospermia, asthenozoospermia and testis biopsy samples, motile spermatozoa were isolated using a discontinuous (45, 70, 90%) Percoll (Sigma Chemical Co., St Louis, MO, USA) gradient made with the HEPES buffered medium used for sperm preparation in each group. Ejaculated, testicular biopsy, cryopreserved ejaculated and cryopreserved testicular biopsy semen specimens were all included in the study.

Insemination

Intracytoplasmic sperm injection (ICSI) was used to inseminate retrieved oocytes. Cumulus cells were removed 3 - 5 hours after retrieval using ~40 IU/ml hyaluronidase (Sigma) in group A and ~30 IU/ml hyaluronidase in HEPES-HTF (SAGE In-Vitro Fertilization) in group B and denuding pipettes (Flexi pet (Cook)). Denuded oocytes were rinsed three times in 1 ml buffered medium. Metaphase II oocytes were identified and incubated in 50 µl droplets covered with paraffin oil (group A: Paraffin Oil (Medi-Cult); group B: Paraffin Oil for Tissue Culture (SAGE In-Vitro Fertilization)) until injection (37°C, 5% CO_2). ICSI was performed as soon as possible after denuding, i.e. $\geq$ 3 hours but $\leq$ 5 hours after retrieval. A standard ICSI method was followed. Prerequisites for successful injection were immobilisation of the sperm cell in polyvinylpyrrolidone (PVP) (group A: PVP (Medi-Cult); group B: PVP (SAGE In-Vitro Fertilization)) and mild cytoplasmic aspiration.

ICSI was performed on a Nikon inverted microscope (Nikon Diaphot 300, Nikon Co., Tokyo, Japan) with Narishige micromanipulators (Narishige, Tokyo, Japan).

Embryo culture

For both studies and for all embryos, the culture conditions were identical. The only difference was the culture media used. Injected oocytes were rinsed and returned to the culture dish with either Sydney IVF Cleavage Medium (group A) or Quinn's Advantage Cleavage Medium (group B) for further incubation at 37°C. To ensure optimal culture conditions, manual temperature readings in the incubators (Forma 3164 (Forma Scientific, Marietty, Ohio, USA)) were performed every day, the pH of the media was checked every week and the CO_2 level was adjusted for the optimal pH range of 7.2 - 7.4. Using these criteria, the pH for both media during the study period was within the optimal range. None of the media used in this study underwent formulation changes during the course of the study. Oocytes were individually examined under a dissecting microscope at the following times: ~18 hours post insemination for the presence of two pronuclei and two polar bodies; 25 - 27 hours (~26 hours) post insemination for 2-cell division (early division); ~45 hours post insemination for 4-cell division; and ~72 hours post insemination for 6 - 8-cell division.

Embryo grading (modified from Veeck[24])

Embryo morphology was scored from 1 to 5 according to the shape of the blastomeres and the amount of

detached anuclear fragments. Embryos were regarded as of 'good quality' when they were at the 4-cell stage at ~45 hours post insemination or at the 6 - 8-cell stage, ~72 hours post insemination with equal-sized blastomeres and minor (< 20%) or no cytoplasmic anuclear fragments. Whenever there was uncertainty regarding the embryo score, it was double-checked by a second or third embryologist until consensus was reached. Embryologists assessing embryo grading and selecting embryos for transfer were blinded as to which culture media was being used in the prospective study.

Embryo transfer and pregnancy evaluation

Embryos with the highest score were selected for transfer either on day 3 or day 5. A day 3 transfer was performed when there were 3 or less good quality embryos available on this day; on the other hand a day 5 blastocyst transfer was performed when there were 4 or more good quality embryos on day 3. For day 3 transfer, 3 embryos were transferred per patient. For day 5 transfer, 1 or 2 blastocysts were transferred per patient. Pregnancies were reported as positive when βhCG serum levels were > 10 IU/ml 10 days post transfer and increased to four times that value on day 14 post transfer. Clinical pregnancy was defined by the presence of a gestational sac, crown rump length of 2 - 4 mm, and fetal heartbeat at ultrasound performed 6 - 7 weeks after embryo transfer (ET).

Statistical analysis

Results are expressed as mean ± SD. Data were analysed using the unpaired *t*-test and percentages were compared by χ^2 analysis. $p < 0.05$ was defined as statistically significant.

Results

First phase of study: Prospective randomised controlled trial

The study included 39 patients, who underwent 42 oocyte retrieval cycles. No statistically significant differences (Table II) were noted between the two groups with regard to age and number of previously failed cycles. In both the groups the indication for fertility treatment included tubal factor, male factor, idiopathic, anovulation, endometriosis and multiple factors.

Data on fertilisation, embryo quality and cleavage rates are presented in Table III. No significant difference was noted in the percentage of fertilised ova. Cleaving rates of embryos at 26 hours post insemination were not significantly different for the two groups. Embryo quality, as assessed by morphological parameters, were also similar at day 2 post fertilisation. However, at day 3 post fertilisation a significantly higher number of good quality embryos were noted in group B (group A: 33/79 (34%); group B: 40/67 (60%); $p < 0.05$). Blastulation rate included all embryos not transferred on day 3 that reached blastocyst stage; no significant difference between the two groups were noted.

Embryo transfer and pregnancy rates are also described in Table III. Normally cleaving embryos were available for transfer in all cycles in both groups. While the mean number of embryos transferred in both groups was similar, all measured outcome parameters were more favourable in group B, although this was not statistically significant. The rate of positive βhCG in group B was 32% compared with 17% in group A ($p = 0.477$); furthermore, both ectopic and ongoing pregnancy rates were not significantly different between the two groups.

Second phase of study: Retrospective study

The study included 300 patients who underwent 374 oocyte retrieval cycles. Patient demographic variables and characteristics were essentially similar (Table IV). In group B the patients were significantly older ($p < 0.0001$) with a mean age of 34.0 ± 3.1 years compared with 32.0 ± 3.4 years for group A. In both the groups the indication for fertility treatment included tubal factor, male factor, idiopathic, anovulation, endometriosis and multiple factors.

Data on fertilisation, embryo quality and cleavage rates are presented in Table V. Fertilisation did not differ significantly between the two groups (group A 786/1 201 (66%); group B 919/1 454 (63%); $p = 0.231$). However, there was a statistically significant increase ($p < 0.05$) for all embryo developmental parameters (early dividing embryos, good quality (day 2 and 3) and blastulation rate) in group B.

Embryo transfer and pregnancy rates are also described in Table V. There was no difference in the mean number of embryos transferred in both groups. The rate of positive βhCG in group B (70/195 (36%)) was significantly increased ($p < 0.05$) compared with that in group A (46/179 (26%)). This significance was reflected in the ongoing pregnancy rate, which was significantly increased ($p < 0.05$) in group B (59/195 (30%)) compared with that in group A (31/179 (17%)). Only 1 ectopic pregnancy was noted in this study; it occurred in group A.

Discussion

Sydney IVF medium has previously been compared with Ménézo B2 medium,[25] G1.2 medium[22] and P1 medium.[26] All these studies found a delay in early embryonic development when culture took place in Sydney IVF medium compared with another medium in question.[24-26] Slow-cleaving embryos play an important role in the success of the IVF cycle, as it is well known that early-dividing (2-cell stage at 25 - 27 hours post insemination/injection) embryos significantly improve pregnancy rates when they are

Table II. Indication for fertility treatment in the prospective study for the control (group A) and trial (group B) groups.

	Group A	Group B	*p*-value
Cycles	23	19	
Repeat cycles	3	0	$p = 0.102$
Age (yrs) (mean ± SD)	33 ± 5.0	35 ± 3.5	$p = 0.158$
Number of previously failed IVF cycles (mean ± SD)	2.0 ± 1.6	2.5 ± 1.5	$p = 0.321$
Indication for IVF			
Tubal factor	1/20 (5.0%)	5/19 (26.3%)	$p = 0.168$
Male factor	6/20 (30.0%)	9/19 (47.4%)	$p = 0.446$
Idiopathic	2/20 (10.0%)	2/19 (10.5%)	$p = 0.674$
Anovulation	4/20 (20.0%)	2/19 (10.5%)	$p = 0.661$
Endometriosis	3/20 (15.0%)	1/19 (5.3%)	$p = 0.605$
Multiple factors	4/20 (20.0%)	0	$p = 0.126$

Table III. Prospective randomised study: Fertilisation, embryo development, embryo transfer and pregnancy rate

	Group A	Group B	*p*-value
Cycles	23	19	
Retrieved oocytes	134	122	
Number of oocytes (mean ± SD)	5.8 ± 3.2	6.4 ± 3.2	
Fertilisation	95/134 (70.9%)	84/122 (69%)	$p = 0.826$
Early-dividing embryos	26/95 (27.4%)	20/84 (23.8%)	$p = 0.705$
Embryo quality			
Day 2 good quality	38/95 (40.0%)	39/84 (46.4%)	$p = 0.476$
Day 3 good quality	33/79 (41.8%)	40/67 (59.7%)	$p = 0.031$
Blastulation rate	11/32 (34.4%)	14/31 (45.2%)	$p = 0.536$
Embryo transfer (ET)			
Embryos transferred/cycle	2.5 ± 0.8	2.5 ± 0.9	$p = 1.000$
Pregnancy rate (% per ET cycle)			
Positive βhCG/ET	4/23 (17.4%)	6/19 (31.6%)	$p = 0.477$
Ectopic pregnancy	None	1/19 (5.3%)	$p = 0.452$
Ongoing pregnancy	2/23 (8.7%)	4/19 (21.1%)	$p = 0.384$

Table IV. Indication for fertility treatment in the retrospective study for the control (group A) and trial (group B) groups.

	Group A	Group B	*p*-value
Cycles	179	195	
Repeat cycles	30	31	$p = 0.822$
Age (mean ± SD)	32 ± 3.4	34 ± 3.1	$p = 0.000$
Number of previously failed IVF cycles (mean ± SD)	2.0 ± 1.5	2.2 ± 1.7	$p = 0.282$
Indication for IVF			
Tubal factor	17/146 (11.6%)	10/154 (6.5%)	$p = 0.180$
Male factor	68/146 (46.6%)	65/154 (42.2%)	$p = 0.515$
Idiopathic	12/146 (8.3%)	17/154 (11.0%)	$p = 0.532$
Anovulation	7/146 (4.8%)	18/154 (11.7%)	$p = 0.051$
Endometriosis	4/146 (2.7%)	8/154 (5.2%)	$p = 0.430$
Multiple factors	38/146 (26.0%)	36/154 (23.4%)	$p = 0.698$

selected and transferred.[27,28] Furthermore, fast-cleaving human embryos have a greater potential to develop to blastocysts *in vitro*.[29] In the prospective randomised study we found a higher, although not significant, blastulation and pregnancy rate in group B. The study cohort in the prospective randomised study is small (39 patients); this is because an interim analysis 3 months into the study showed that there was a significant increase in day 3 embryo quality for embryos cultured in Quinn's Advantage sequential culture medium. Furthermore, the pregnancy rate was better for embryos cultured in this medium (31.6% (group B) v. 17.4% (group A)). Although it was not significant a trend was observed, and these observations prompted an immediate changeover in our culture system.

The retrospective study reflected the observations of the prospective trial. In the retrospective study patients in group B (34.0 ± 3.1) were significantly older ($p < 0.0001$) than those in group A (32 ± 3.4). It is therefore

Table V. Retrospective study: Fertilisation, embryo development, embryo transfer and pregnancy rate

	Group A	Group B	*p*-value
Cycles	179	195	
Retrieved oocytes	1 201	1 454	
Number of oocytes (mean ± SD)	6.7 ± 3.6	7.5 ± 3.8	
Fertilisation	786/1 201 (65.5%)	919/1 454 (63.2%)	$p = 0.231$
Early-dividing embryos	156/786 (19.8%)	263/919 (28.6%)	$p < 0.05$
Embryo quality			
Day 2 good quality	275/786 (35.0%)	416/909 (45.3%)	$p < 0.05$
Day 3 good quality	234/639 (36.6%)	378/795 (47.5%)	$p < 0.05$
Blastulation rate	74/267 (27.7%)	175/384 (45.6%)	$p < 0.05$
Embryo transfer (ET)			
Embryos transferred/cycle	2.8 ± 0.7	2.7 ± 0.6	$p = 1.000$
Pregnancy rate (% per ET cycle)			
Positive βhCG/ET	46/179 (25.7%)	70/195 (35.9%)	$p < 0.05$
Ectopic pregnancy	1/179 (1.1%)	None	
Ongoing pregnancy	31/179 (17.3%)	59/195 (30.3%)	$p < 0.05$

of considerable interest that we not only found a higher incidence of early-dividing embryos (EDE) in group B, but that all other embryo development parameters as well as pregnancy rates were significantly increased. In an attempt to explain this one should be aware that *in vitro* embryo development and survival is affected by various factors. These can collectively be categorised as firstly, the immediate environment of the embryo (the culture system) and secondly, the DNA composition of the embryo.

The culture systems employed in this study differed only in the sequential culture medium; all other culture conditions were kept identical. The two culture media used in this study differ in various ways; we can only speculate that these differences have a cumulative beneficial effect for embryo development in the one medium. Differences (Table I) include the absence of labile glutamine and free alanine in Quinn's Advantage medium, and conversely, the presence of stable alanyl-glutamine. The absence of alanine in this medium promotes pyruvate transamination. In turn, the process of pyruvate transamination inhibits the build-up of toxic ammonia (NH_3) in the medium. Furthermore, it is possible that the absence of inorganic phosphate in the Quinn's Advantage Cleavage Medium is beneficial for early embryo development, as glycolysis is then not promoted and the co-factors and other metabolites involved in glycolysis may be utilised for more energy-productive processes, such as oxidative phosphorylation. An additional difference between these media occurs between the blastocyst media, where there is a higher Mg^{2+} concentration in the Sage product, which lowers the uptake of Ca^{2+} from the medium, which has, in turn, a beneficial effect on maintaining mitochondrial activity.[30]

The specific sequential growth medium used, as previously mentioned, can influence embryo quality and development. However, it is well known that the DNA composition of the embryo also plays a major role.[31] The DNA composition of the embryo is influenced by an array of factors, including chromosomal abnormalities,[31-34] DNA fragmentation, reactive oxygen species (ROS) and apoptosis.[35-38]

However, because randomisation of patients occurred in the prospective study and patients were matched in the retrospective study, the types of patients studied in the two groups are essentially similar. Furthermore, it should also be noted that during the course of this study variables such as embryologist, embryo transfer techniques and physicians were all constant. Five embryologists work on a rotational basis that was implemented before the onset of the study. Every rotation includes two embryologists in the IVF laboratory. The embryo transfer technique used did not change over the period of this study (January 2002 - May 2004). We can therefore speculate that the impact on embryo quality and pregnancy rate that we noted in both groups is mainly due to the sequential culture media used.

In conclusion, in the randomised prospective trial we noted a significant increase in day 3 embryo quality in group B (Quinn's Advantage sequential medium). Retrospectively, we found a significant increase in early-dividing embryos, good-quality embryos (day 2 and 3), blastulation rate and pregnancy rate in Quinn's Advantage medium compared with Sydney IVF medium. Although the randomised controlled trial is a small study, the trends observed are reflected by the results from the retrospective study. However, to prove unequivocally that one medium is superior to the other, a larger prospective randomised control trial would have to be undertaken. At present, taking all results into account, the embryo culture medium of choice in our laboratory is Quinn's Advantage sequential culture medium.

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