The Use of an Hydrogen Peroxide Multipurpose Isolator for Inhouse Preparation of Human Embryo Culture Media: A Unique Successful Swiss Randomized Prospective Study

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Purpose: To develop inhouse made (IHM) embryo culture medium with a Multipurpose Isolator and compare the embryo development in a prospective randomized study with commercial media.

Methods: Fertilization by intracytoplasmic single sperm injection (ICSI) of Metaphase II oocytes obtained after 96 controlled ovarian hyperstimulation cycles in patients not older than 37 years. Transfer of zygotes to IHM or commercial Cook Sydney IVF Cleavage medium (SIC) immediately after pronucleus observation. Evaluation of embryo cleavage and score, pregnancy, and implantation rate.

Results: From 100 zygotes cultured in SIC, 61% were at the 4 cell stage 45 h after ICSI compared to 77% (78/101) in the IHM, $P < 0.05$. The mean embryo score with IHM was 3.9 ± 0.9 compared to 3.5 ± 1.2 with SIC, $P < 0.05$. The clinical pregnancy rate per transfer was 38.9% (37/95), the implantation rate was 23% (46/200), and no differences were observed between the groups.

KEY WORDS: Embryo score; hydrogen peroxide; inhouse media; Multipurpose Isolator; pregnancy rate.

INTRODUCTION

A stable culture system for human embryo culture which provides high implantation and live birth rates is, a quarter of a century after the birth of the first IVF baby (1), still one of the major concerns of clinical embryologists. Over the last decade there has been an evolution from simple monoculture media and co-culture toward sequential media more adapted to the dynamics of early embryo metabolism. Today a large variety of commercial media are available making

the right choice difficult. If some controlled comparisons between commercial media did not reveal differences in implantation potency (2) despite an earlier observed better embryo quality (3) others (4) have claimed the superiority of one medium system to another, based on implantation rates and cleavage kinetics. Despite this great availability of different media some laboratories (5) still produce their "inhouse" media today with similar effectiveness compared to the commercial ones. Such publications point out that it might be the quality in producing and managing a culture system rather than the type of medium, which is a key factor to successful human embryo culture.

The central pharmacy at the Kantonsspital, Baden, Switzerland (KSB) has developed in collaboration with Sintetica S.A., Mendrisio, Switzerland a Multipurpose Isolator, enabling the production of solutions

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Fig. 1. Sintetica Multipurpose Isolator. (a) Working area, (b) loading door, (c) outlet doors for finished products.

under ideal aseptic and nontoxic conditions. This unique opportunity stimulated the IVF laboratory to start a collaboration with the central pharmacy. It was decided to extend the monoculture system to a two-step sequential culture system and to develop, inhouse, the complete set of sequential media covering gamete handling from oocyte collection to embryo transfer at day 2. To evaluate the value of this extension as well as the overall performance of the system a randomized comparison was made with Sydney IVF cleavage medium (Cook IVF®, Queensland, Australia) as a commercial alternative. *This study was organized in the context of a global quality management project aiming to reduce the negative consequences of the restrictive Swiss law on reproductive medicine, which does not allow further culture of more than three fertilized oocytes, forbids embryo freezing and therefore does not allow embryo selection at day 2, 3 or at the blastocyst stage.*

MATERIAL AND METHODS

The Sintetica Multipurpose Isolator

The Multipurpose Isolator (MPI, Sintetica SA, Mendriso, Switzerland) consists of four elements.

A working cabinet with incorporated HEPA filters and laminar air stream, with temperature control

between 18 and 24°C and positive pressure of more than 60 Pa. The cabinet has two doors to access the inside (Fig. 1(a)). The doors incorporate two pairs of gloves that operators can use to work within the cabinet without compromising the sterility of the Class A environment. The cabinet is also equipped with one detachable flange for the process fluid inlet and with inlet for vaporized H₂O₂ (VHP) decontamination.

A decontamination cabinet with incorporated HEPA filter and one door in the front with a glove, to load the materials (Fig. 1(b)) and one door on the right side to transfer the materials after decontamination to the working area.

Two Egress pass-boxes (EPB) make up the system allowing the outlet of the finished products or rejects without affecting the Class A environment inside the cabinet (Fig. 1(C)). The EPB is equipped with two sequential chambers, connected between them and the isolator cabinet via automatic tight interlocked doors. The outer chamber is in overpressure condition, as an airlock, to protect the first chamber, and the Class A environment of the isolator cabinet. The air is HEPA filtered and overpressure monitored.

The VHP generator produces the vaporized H₂O₂ used to perform the decontamination cycles of the working area, the EPBs, and/or the decontamination cabinet. The connection between the generator and the MPI isolator is performed using a Tri-clamp port

with a pneumatic butterfly valve (6). The MPI is out-gassed for a validated period of time before use to ensure that no H_2O_2 residues remain in the working areas. All IHM solutions were sterilized with a 0.22- μm Meissner filter (Ref. GFSM0.2-33B, Skan AG, Allschwill, Switzerland.) and stored in Falcon tissue culture flasks (Ref. 353014, Becton Dickinson Labware Europe, Le Pont DE Claix France.)

Inhouse Media and Culture System

The Inhouse Media (IHM) are lactate free Earles Balanced Salt Solution, reconstituted from powdered Earles Balanced Salt Solution (Ref: Sigma, Fluka Chemie GmbH, Buchs, Switzerland) supplemented with 0.33-mMol sodium pyruvate (Ref. P5280, Sigma, Fluka Chemie GmbH, Buchs, Switzerland) and 25-mMol sodium bicarbonate (Ref. S5761, Sigma, Fluka Chemie GmbH, Buchs, Switzerland), the osmolality was adjusted to 285 mOsm/kg.

The IHM used for further culture after pronucleus assessment was also lactate free and the composition is detailed in Table I. The final osmolality of this medium is ± 275 mOsm/kg and was not adjusted. This medium was generated in the central pharmacy starting from powdered tissue cultured tested chemicals and Non Essential Amino Acid solution (Ref. 11140-035, Gibco, Basel, Switzerland).

The water used for medium preparation was obtained from a Milli Q Plus 185 system (Millipore AG, Volketswil, Switzerland). All IHM were supplemented with 8.25% (V/V) Serum Substitute Supplement (Ref: 99193, Santa Anna, CA, USA). For culture we prepared Falcon Petri dishes (Ref. 3001, Becton Dickinson Labware Europe, Le Pont DE Claix

France.) with five droplets of 40 μL of media covered by 3 mL liquid paraffin oil (Ref. No 101100125, MediCult, Stefan Gück, Berlin, Germany). The dishes were equilibrated 24 h before use in a 6% CO_2 -6% O_2 -88% N_2 atmosphere. Batches of Serum Substitute Supplement (SSS), paraffin oil, and powdered chemicals for Inhouse Medium preparation did not change during this study. The IHM had a shelf life of 1 month, pyruvate and SSS were only added the day before use.

Embryo Scoring

All embryos received 45 h after oocyte retrieval a score based on the regularity and the number of the blastomers and the presence of fragments. A maximum score of 6 was given to embryos that had four blastomers of equal size and no cytoplasm fragments (7).

Patients-Stimulation-Oocyte Collection

All patient not older than 37 years attending the Frauenklinik for ICSI with ejaculated semen, were included in this study if they had at least two fertilized oocytes (2PN). Controlled ovarian hyperstimulation was carried out using a standard long protocol, consisting of downregulation with Decapeptyl Retard (Ferring AG, CH8403 Wallisellen, Switzerland) and stimulation with Merional (IBSA S.A., CH6915 Pambio-Noranco, Switzerland). Oocytes were collected 36 h after 10000 I.U. HCG administration (Pregnyl, Organon, Oss, The Netherlands) by ultrasound guided transvaginal puncture under mild sedation with Ultiva (GlaxoSmithKline, CH30563 Münchenbuchsee, Switzerland). Cumulus cells were immediately removed after oocyte collection by exposure to 80 I.U. Hyaluronidase (Ref. H3757 Sigma, Sigma, Fluka Chemie GmbH, Buchs, Switzerland) for less than 1 min, remaining cumulus was mechanically removed with a 130- μm Flexipette (Cook, Queensland, Australia). All oocytes that reached the Metaphase II stage, by 1 p.m. were used for injection.

RESULTS

A total of 96 cultures were performed, on day 1 immediately after pronuclei (PN) observation and 48 cases were transferred into Sydney IVF Cleavage (SIC) medium and 48 into the Inhouse Medium (IHM). The mean female age was similar in both

Table I. Composition of the Inhouse Medium

Ingredient	Cleavage mM
NaCl	106.09
KCl	5.05
MgSO ₄	1.00
NaH ₂ PO ₄	0.50
Pénicilline	0.17
Na-pyruvate	0.47
NaHCO ₃	25.00
CaCl ₂ ·2H ₂ O	1.8
Glutamine	1.0
L-Alanine	0.1
L-Asparagine	0.1
L-Aspartic Acid	0.1
L-Glutamic Acid	0.1
Glycine	0.1
L-Proline	0.1
L-Serine	0.1

groups: 32 ± 2 years versus 32 ± 3 years. For the SIC group we initially had 303 fertilized oocytes (2 PN) of which 106 were placed in culture and for the IHM group of the 301 fertilized oocytes (2 PN) 102 were placed in culture, the remainder were frozen, with the exception of one embryo in a case of a high risk of multiple pregnancy. A single embryo was frozen in an "emergency" as described by the Swiss law, because of the high risk for a multiple pregnancy associated with high embryo quality in young patients (8). The two zygotes kept in culture for this patient cleaved into two 4-cell stage embryos with a maximum embryo score of 6 each. The transfer of one of these embryos resulted in an ongoing pregnancy. The number of 2 PN fertilized oocytes correspond in both groups to a fertilization rate of 78% of the oocytes that remained intact after ICSI. Oocyte damage during ICSI was 6.5% and did not differ between the groups. *In the SIC, six zygotes did not cleave versus one in the IHM. As a consequence one patient in the SIC group had no transfer.* The percentage of embryos that reached the 4-cell stage 24 h after pronucleus observation was only 61% (61/100) in SIC compared to 77% (78/101), $P < 0.05$, in IHM. As a consequence there was a significant difference ($P < 0.05$) in the mean embryo score of SIC group, 3.5 ± 1.2 , compared to the IHM group, 3.9 ± 0.9 . The number of transfers with 3, 2, and a single embryo were, respectively, for the SIC group: 9 (19.1%), 35 (74.5%), 3 (6.4%) and for the IHM group 5 (10.4%), 42 (87%), 1 (2%). From the four transfers with a single embryo only one was on request of the patient who achieved a clinical pregnancy, the others were caused by cleavage arrest. No triplet pregnancies were observed, however despite the fact that at least three quarters of the transfers were done with no more than two embryos. We observed a total number of 11 twin pregnancies (6 in SIC versus 5 in IHM), 29.7% (11/37) in total. The clinical pregnancy rate per transfer was 38.2% (18/47) for the SIC group and 39.6% (19/48) for the IHM group. A total of 100 embryos cultured in SIC were transferred on day 2 and 22 embryonic sacs were observed, giving an implantation rate of 22%, 101 embryos cultured in IHM from which 100 were replaced resulted in 24 embryonic sacs after transfer on day 2, corresponding to an implantation rate of 24%. Three abortions and two ectopic pregnancies were observed in the SIC group, bringing the ongoing pregnancy rate per transfer to 27.7%. In the IHM group no ectopic pregnancies occurred and three patients aborted, resulting in an ongoing pregnancy rate of 33.3%. An extra 12 singleton pregnancies were obtained from the frozen zygotes,

Table II.

	SIC	IHM
Number of patients	48	48
Patient age	32 ± 2	32 ± 3
Number of zygotes	303	301
Number of zygotes cultured	106	102
Number of zygotes frozen	197	199
Number of embryos ^a	100	101
Number of 4-cell embryos (%) ^a	61 (61)	78 (77)
Embryo score ^a	3.5 ± 1.2	3.9 ± 0.9
Number of embryo transfers	47	48
Number of embryos transferred ^b	100	100
Transfers with 3 embryos (%)	9 (19.1)	5 (10.4)
Transfers with 2 embryos (%)	35 (74.5)	42 (87.5)
Transfers with 1 embryo (%)	3 (6.4)	1 (2)
Pregnancy rate	38.2% (18/47)	39.6% (19/48)
Implantation rate	22% (22/100)	24% (24/100)
Spontaneous abortions	3	3
Ectopic pregnancies	2	0
Twin pregnancies	6	5
Singleton pregnancies	7	11
Ongoing pregnancy rate	27.7%	33.3%

^a6 Zygotes did not cleave in SIC and 1 did not cleave in IHM.

^b1 embryo was frozen in an emergency for a young patient at risk for multiple pregnancy. She became pregnant from the transfer of the remaining single embryo.

* $P < 0.05$.

all thawed zygotes were cultured in IHM, and this corresponds with an actual cumulative ongoing pregnancy rate per oocyte retrieval of 51% (49/96). *Results are summarized in Table II.*

DISCUSSION

Studies about embryo metabolism which revealed the preference for pyruvate at the early cleavage stage and glucose at the blastocyst stage (9–11) on one side and studies about the changes in the oviduct and the tubal fluids (12) during the menstrual cycle on the other hand, have led to the development of sequential media. These media offer without doubt a simple alternative to coculture on monolayers (13). The better harmony between these media and needs of the in vitro cultured preimplantation embryo and the commercial availability of these media under different formulations have brought us to reconsider our monoculture system and to extend it with a second step. A recent publication (14) showed that inhouse media even when used in a nonsequential system are able to give similar pregnancy rates and implantation rates as commercially available sequential media. In addition, the pharmacy of the Kantonsspital has a longstanding tradition of making media under optimal conditions in the Sintetica Isolator. It was therefore decided to

compare an inhouse formulated second medium with a commercially available medium. As the original inhouse prepared Earle's medium did not contain lactate it was decided to formulate the second medium also without lactate. It was shown that in the mouse the presence of lactate reduces the uptake of pyruvate (15), the preferential energy source during the three first days of most mammalian preimplantation embryos. This could explain why we observed more 4-cell stage embryos in our IHM system, because lactate is present in the SIC. A second reason for the omission of lactate was our previous successful experience with a lactate free medium for the study of the glycolytic activity of the human preimplantation embryo (16). Based on our previous work about possible effects of amino acids on preimplantation embryo development, no taurine but glutamine and MEM Non Essential Amino Acids were added (17–19). The presence of glutamine and Non Essential Amino Acids give faster cleavage rates in mouse zygotes in vitro (20). Glucose was omitted in the IHM because it is not essential (21), but even more so in a relatively high concentration of oxygen and phosphate it can induce a mitochondrial dysfunction and lead to preimplantation embryo developmental arrest. In the presence of glucose there is a possible accumulation of glycogen in the cytoplasm caused by metabolic blocks in the glucose pathways. Glycogen is able to absorb water and can lead to increase in cytoplasmic volume. The embryo will try to restore the correct ratio between cytoplasm volume and nuclear volume by extrusion of fragments during cleavage (22). This phenomenon is correlated with enhanced embryo quality with the use of glucose free media (23). The last consideration among those, which led to the final formulation of the inhouse medium, concerns the addition of a protein source. We opted for the Serum Substitute Supplement (SSS) because of the repeated positive reports about its use in preimplantation embryo culture systems even in high concentrations (24–27) and because we could reserve a single batch over a period of at least 1 year.

The above formulation can be prepared in any experienced IVF laboratory. However in most cases medium is prepared in a laminar airflow cabinet only. The use of the hydrogen peroxide vapor-phase decontaminated isolator has not only the advantage that no equipment has to suffer from adverse effects during sterilization, but is also known to be a safe and efficacious way of removing potentially virus-contaminated objects (28). Ambient air contamination and pollution have been described as having possible effects

on conception (29,30), this is avoided by the use of the isolator during medium conditioning.

To summarize: We were able to extend successfully our existing monoculture system to a sequential system either by the use of IHM or commercial SIC, with a similar clinical outcome for both systems. The higher proportion of 4-cell stage embryos observed in the IHM indicates that it might be inappropriate to use a single element out of a series of commercial sequential media to extend an existing culture system for human embryo culture. Instead a complete system of media are to be preferred and great care must be taken to work in conditions similar or close to the ones guaranteed by commercially produced media.

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