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ARTICLE

First births with a simplified culture system for clinical IVF and embryo transfer

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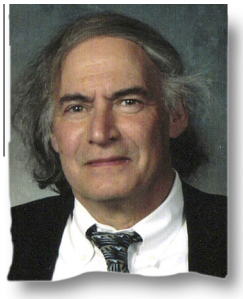

Jonathan Van Blerkom is a professor in the department of molecular, cellular and developmental biology at the University of Colorado and IVF Laboratory Director at Colorado Reproductive Endocrinology in Denver. His studies have focused on molecular and cellular aspects of early mammalian development including human follicles, oocytes and embryos and has published numerous research articles and reviews and coauthored or edited books dealing with early mammalian development, including the human. His current research centres on the role(s) of mitochondria in early development and the molecular organization of the oocyte and embryo plasma membrane as related to developmental competence.

Abstract This study reports the outcome results from a pilot clinical trial using a simplified laboratory method for human IVF. This system reproducibly generates *de novo* the atmospheric and culture conditions that support normal fertilization and preimplantation embryogenesis to the hatched blastocyst stage without the need for specialized medical-grade gases or equipment. Development from insemination to the hatched blastocyst stage occurs undisturbed in a completely closed system that enables timed performance assessments for embryo selection *in situ* that, in this study, involved single-embryo transfers on day 3. With the simplified culture system, 8/23 embryos implanted, one miscarried at 8 weeks of gestation and seven healthy babies have been born. The methodology and results are discussed with regard to how this simplified system can be adopted worldwide to meet the growing need for accessible and affordable IVF.

KEYWORDS: accessible IVF, infertility, low-cost IVF, simplified IVF, IVF in developing countires, IVF in developed word

Introduction

Current estimates of the worldwide prevalence of infertility (involuntary childlessness) indicate that 52.6–72.4 million couples will require or benefit from some form of medical intervention to achieve a pregnancy (Boivin et al., 2007; Mascarenthas et al., 2012). Infertility treatments using assisted reproduction technology that range from relatively simple pharmacological management of the menstrual cycle and intrauterine insemination to more invasive methods such as IVF using intracytoplasmic sperm injection (ICSI), are typically available to a relatively large segment of couples in the developed world. However, while the demand for IVF has shown no diminution since its formal introduction nearly 35 years ago (Steptoe and Edwards, 1978), neither

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have treatment costs, which have progressively increased in high-resource settings, especially where private programmes predominate (Boivin et al., 2007). Although access to IVF is largely commonplace in developed countries (Zhao et al., 2011), affordability remains the central issue for many in need of treatment.

When viewed from a worldwide perspective, a large majority of infertile couples are residents of the so-called 'low-resource' or developing countries (Boivin et al., 2007; Cates et al., 1985; Rutstein and Iqbal, 2004) where few, if any assisted reproduction programmes exist and where IVF costs at Western-style programmes make this treatment unavailable to the vast majority of those in need (Malpani and Malpani, 2002; Nachtigall, 2006; Ombelet and Campo, 2007; Van Balen and Gerrits, 2001; Vayena, 2009). Bilateral tubal occlusion is a common cause of infertility prevalent among young women in general and in developing countries in particular (Ombelet et al., 2008), and when a severe male factor is not present such that ICSI is required, it is effectively treated by conventional IVF. Low-cost programmes have been introduced in such settings but have mainly focused on reducing drug (e.g. low-stimulation protocols) and biochemical monitoring costs. However, the high laboratory costs associated with fertilization and embryo culture have not been addressed and can often represent over 50% of the financial outlay associated with an IVF attempt, including those using low stimulation protocols (Aleyamma et al., 2011). The present study was initiated to address this problem as part of a broader programme, the Walking Egg Project (Dhont, 2011; Ombelet, 2013, 2014), whose goal is to develop accessible and affordable reproductive health treatments worldwide, including assisted reproduction technology, with an emphasis on developing countries, where it is also much needed (Aleyamma et al., 2011; Boivin et al., 2007; Ombelet and Campo, 2007; Pilcher, 2006; Vayena, 2009; Vayena et al., 2009).

The specific intent of this research was to develop a simple culture system and methodology for human IVF to treat primarily bilateral tubal occlusions that could be set up in small centres and does not require specialized equipment common in high-resource IVF programmes such as microprocessor-controlled tissue culture incubators, large area air filtration systems and an infrastructure dependent upon medical-grade gases (N_2, O_2, CO_2), costly cultureware or the near immediate availability of replacement (e.g. electronic) components and the technical expertise to effect repairs.

Materials and methods

Development of a simplified culture system for clinical IVF

Beginning in 2009, a variety of commonly available and inexpensive vessels were tested for their suitability for IVF and embryo culture to the hatched blastocyst stage, first in the mouse model and then with donated human embryos, as described below. Systematic evaluations of different culture configurations showed that conditions for normal IVF and preimplantation embryogenesis could be reliably and consistently produced in a closed culture system using an inexpensive, disposable 10-ml plain glass vacutainer (366430; Becton Dickson). This single-tube method afforded adequate visualization of oocytes, pronuclear eggs and preimplantation embryos to the hatched blastocyst stage and enabled stage and performance assessments of sufficient detail to select single embryos for transfer. While the maintenance of sterility *in vitro* was critical for the clinical trial, it was also an underlying issue in anticipating use in IVF programmes in low-resource settings. However, the findings (as will be discussed) showed that sterility could be maintained by following several simple precautionary steps incorporated into the protocol.

Preparation, equilibration and verification of culture conditions

Prior to the addition of culture medium, disposable 10-ml plain glass sterile vacutainers for embryo culture were rinsed several times with sterile deionized water using a 3-ml syringe, followed by the complete removal of water with the same syringe; this operation also relieved the partial vacuum inherent in this type of tube. Pure CO_2 was produced in a sterile but unwashed vacutainer whose stopper had been removed and then replaced after the addition of the dry ingredients. A simple chemical reaction produced CO_2 *de novo*, namely the combination of a weak base (e.g. sodium bicarbonate, sodium carbonate), a weak acid (e.g. tartaric, ascorbic, citric acid) and water. After extensive studies with different acid and base combinations, citric acid and sodium bicarbonate were chosen because of their general availability and the rate at which a stable medium pH and atmospheric conditions were generated that were similar to those obtained using individual medical-grade gases and microprocessor-controlled tissue culture incubators.

The rate of pH and atmospheric stabilization was quantified for O_2 and CO_2 in the IVF vacutainer by titrating against varying amounts of dry ingredients and water, the diameter and length of the connecting tubing and the duration of connection between the two compartments. The proportion of sodium bicarbonate, citric acid and water were also titrated against pH and atmospheric composition for different bicarbonate buffered culture media commonly used in clinical IVF or for mouse studies. The percentage of CO_2 and O_2 were measured with corresponding microelectrodes calibrated according to the manufacturer's recommendations (O_2: DO-166MT-1; CO_2: GS-136C-1S; Lazar Research Laboratories, Los Angeles, CA, USA) that were inserted approximately midway into the vacutainer through small channels predrilled in its stopper prior to the introduction of culture medium and CO_2. pH measurements were made with an ion-sensitive field-effect transistor (ISFET) electrode (PH16-SS) and corresponding pH meter (IQ240; Hach Company, Loveland, CO, USA). For continuous long-term measurements (i.e. days), the same ISFET electrode and a longer O_2 electrode (ISO2; World Precision Instruments, Sarasota, FL, USA) were inserted midway into the culture medium. The air-tightness of the seal was confirmed by the absence of leakage when CO_2 was introduced, with both tubes submerged and continually maintained in a water bath at 37°C. Continuous recordings of pO_2, pCO_2 and pH were made at 10—30 min intervals with a microcomputer-based pH/mV meter (6230N; Jenco Electronics, San Diego, CA,

USA) and presented using GraphPad Prism version 5.04 (GraphPad Software).

For both mouse and human, any environment maintaining 37°C through the entire length of the culture tube was sufficient for normal fertilization and preimplantation embryogenesis, including a covered water bath, a thermos, a heating block or a battery-operated portable incubator. The standard protocol of vacutainer preparation for IVF described below retained a stable pH and atmosphere for a minimum of 2 weeks when stored between at 4–15°C. Upon warming to 37°C, sealed vacutainers tubes containing pre-equilibrated medium stored in this manner had the same pH, atmospheric composition and capacity to support fertilization and preimplantation embryogenesis in mouse and human as if freshly prepared.

Clinical IVF with the simplified culture system

As a result of preliminary studies, a standard protocol was developed for use with Global and Global Total IVF media (Global supplemented with 5% human serum albumin (HAS) or Global Total IVF; IVF Online Europe, Brussels, Belgium; Life Global, Guilford, CT, USA), which was used at the ZOL hospitals in Genk for routine IVF and embryo culture during the course of this study. CO_2 was generated by mixing 11.5 mg citric acid and 50 mg sodium bicarbonate in 3.0 ml water. Further simplification of the method involved using 3 ml of a 10× stock solution of citric acid. In the absence of an analytical balance, this amount of sodium bicarbonate can be closely approximated with a common 1/8-teaspoon measuring spoon that when the powder is levelled and 3.0 ml of the citric acid stock is added, generates the same pH and atmosphere described below. It should be noted that slight differences in the concentrations of these ingredients might be required with other types of culture medium used in clinical IVF. However, when a concentrated citric acid solution is used, it is injected into the tube containing the sodium bicarbonate, causing immediate effervescence and the liberation of CO_2. The routine culture system used 1 ml medium and, for this study, the same volume was added to a sterile washed vacutainer.

In Figure 1**A**, note the purplish tint of the pH indicator phenol red, which is consistent with a slightly basic condition (~pH 8.3; Figure 1**F**) prior to connection to the CO_2 generator (Figure 1**B1**). The vacutainer containing CO_2 under pressure was connected to the IVF tube by an 8-cm length of plastic tubing (Gynetics Medical Products, Lommel, Belgium) whose ends were previously sealed with 18 g 1.5-inch needles (Figure 1**B2**). After at least 5 h at 37°C, equilibration between the two tubes achieved the appropriate atmospheric conditions for IVF (Figure 1F) and the culture medium was ready for use or storage (Figure 1B3). After equilibration, the visible change in medium colour due to the presence of the phenol red indicator was consistent with pH 7.32–7.35 (Figure 1**C** and **F**). The same values were consistently obtained with culture medium that contained no colorimetric pH indicator dye (Figure 1**D**). This finding supported the reproducibility of the atmosphere and culture conditions generated by this method.

As discussed below, gamete addition (Figure 1**D**), IVF and embryo culture in the closed vacutainer occurred in the aluminium block (Figure 1**E**) in darkened environments that provided a constant 37°C, which included a covered water bath, a battery-operated portable incubator or a simple laboratory incubator not designed or intended for cell culture. Culture tubes could also be maintained in a high-quality thermos.

Validation of the simplified culture system

Mouse studies

Cumulus–oocyte complexes (COC) were collected from the ampullary region of the oviduct 12–14 h after ovulation induction with human chorionic gonadotrophin from outbred mice (Swiss-Webster) injected 48 h earlier with pregnant mare's serum gonadotrophin, as previously described (Van Blerkom and Davis, 2007). COC were randomly allocated to (i) the simplified culture system or (ii) an open system using a filter-topped vacutainer in a Billups-Rothenberg chamber. For IVF, as many as six COC were injected in a maximum of 50 μl culture medium into a pre-equilibrated vacutainer containing 1 ml KSOM-aa supplemented with 1.5% bovine serum albumin (fraction V; Sigma).

Motile spermatozoa extracted from the epididymis and processes, as previously described for IVF (Van Blerkom and Davis, 2007), were added in approximately 25 μl medium 1–2 h later and, for the open system, the chamber was regassed. Most cultures contained five or six COC and ~3000 motile spermatozoa (500–600/COC). The tubes were vortexed 16–18 h later to remove most if not all of the adherent cumulus and coronal cells, and the eggs were inspected *in situ* for fertilization (two pronuclei). After the pronuclear check, the simplified culture system and filter-top cultures were left undisturbed for ~4 days, scored for development to the expanded blastocyst stage and again after 18–26 h for hatching, which was the developmental endpoint of the mouse study.

Addition of spermatozoa and COC involved aspiration into an 18 g needle attached to sterile gas-tight 500-μl luer-lock syringe (type 1750; Hamilton Instruments, Reno, NV, USA) or the volume approximated with a 0.5-ml tuberculin syringe filled halfway with equilibrated medium. The needle was inserted with the tube held at about 45° and the COC were slowly expressed onto the glass surface (e.g. Figure 1**D**). Mouse COC and sperm collection and their introduction into the vacutainer occurred in room air. Prior to their insertion, 0.5 ml of 100% ethanol was placed in the depression of the vacutainer stopper for 5 min and then wiped dry with sterile gauze.

For insemination, the entire volume of spermatozoa was deposited directly into the culture medium with the tube kept at 37°C, where it remained except for timed assessments to determine fertilization and the progression of preimplantation embryogenesis. The primary endpoint of these studies was percentage hatched blastocysts in the simplified culture system compared with an open culture system of similar design, where the stopper of the vacutainer was replaced by a filter cap from a 50-ml tissue culture flask (CellStar; Greiner Bio One) and the medium equilibrated overnight in a Billups-Rothenberg cell culture chamber (Van Blerkom and Davis, 2007; Billups-Rothenberg, del Mar, CA, USA) using a premixed atmosphere of 5% O_2 and 6% CO_2, with the balance being N_2.

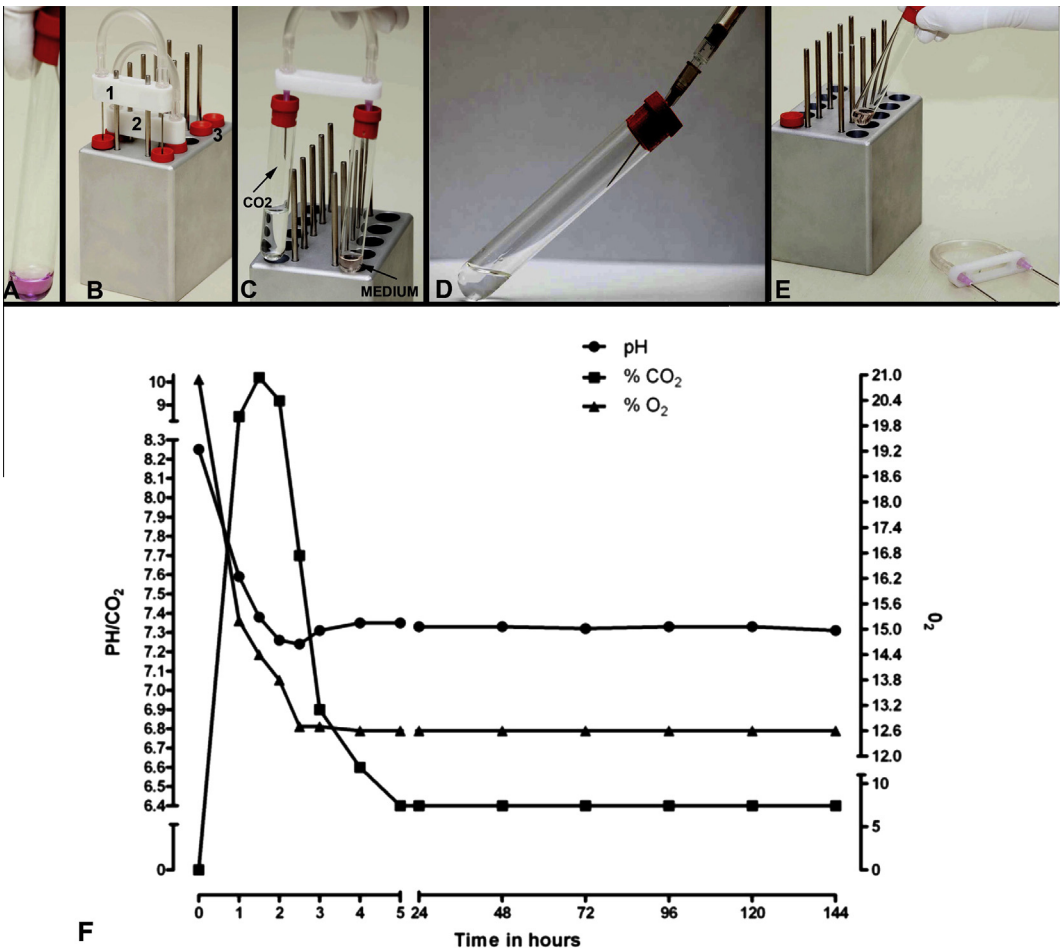

Figure 1 Set up of the simplified culture system and kinetics of atmospheric and pH formation. (A) Phenol red indicator in Global medium is consistent with a pH ~8.3 prior to equilibration. (B) 1, initial connection between CO_2 generator and culture tube; 2, equilibration of atmospheres; 3, culture tubes ready for IVF or storage. (C) Change in colour of culture medium after equilibration with CO_2 generator consistent with pH 7.35. (D) Insertion of spermatozoa and cumulus—oocyte complexes. (E) Aluminium block containing closed vacutainer tubes in which IVF and embryo culture took place. (F) Graphic demonstration of the kinetics by which stable O_2, CO_2 and pH conditions optimal for fertilization and embryogenesis in the simplified culture system were reproducibly generated. The aluminium tube holder shown in B allows simultaneous equilibration of medium, fertilization and embryo culture and storage. The equilibrated tubes are fully contained within the block and are ready for IVF that is performed in a dark environment that maintains 37°C throughout the length of the tube, such as a covered water bath.

Human studies

Insemination of human oocytes solely for the purpose of testing the simplified culture system without an expectation of transfer or cryopreservation was not acceptable and contrary to protocol. However, cryopreserved embryos, including bi- and tripronuclear eggs frozen by controlled-rate freezing 12—16 h after conventional IVF or ICSI and donated to the research programme of one of the authors (JVB) that had been cryopreserved before 2002, could be cultured in this system until the stage of developmental needed for unrelated studies was attained. Based on findings from the mouse, human studies followed the same protocol with the exception of the type of culture medium used.

After preparation and equilibration of the culture vacutainer (Figure 1C), intact embryos were injected in 50 μl medium and cultured in either a heated aluminium block, a covered water bath, high-quality thermos or the same tissue culture incubator routinely used for IVF (routine culture system), but only to maintain 37°C. As a control, thawed pronuclear and early-cleavage-stage embryos were cultured individually in the open (i.e. filter-capped) vacutainer system and atmosphere described above for mouse embryos. It is important to mention that, while IVF in the simplified culture system was demonstrated for the mouse during the development stage of this study, the clinical trial was the first time that human oocytes were inseminated *in vitro* by this method.

The progression of embryonic development was assessed at 12—24 h intervals up to day 6.5 using a dissecting microscope and imaging *in situ* (i.e. through the glass; e.g. Figure 3A). Typically, stage-specific assessments were performed in ambient conditions (22—26°C) and required between 15 and 30 s for day 1 (after vortexing, if necessary, to detect pronuclei) and day 2 (2—4 cell), and 15—45 s for day 3 (6—10 cell) and days 6—6.5 (expanding and hatched blastocyst, respectively). With experience and single

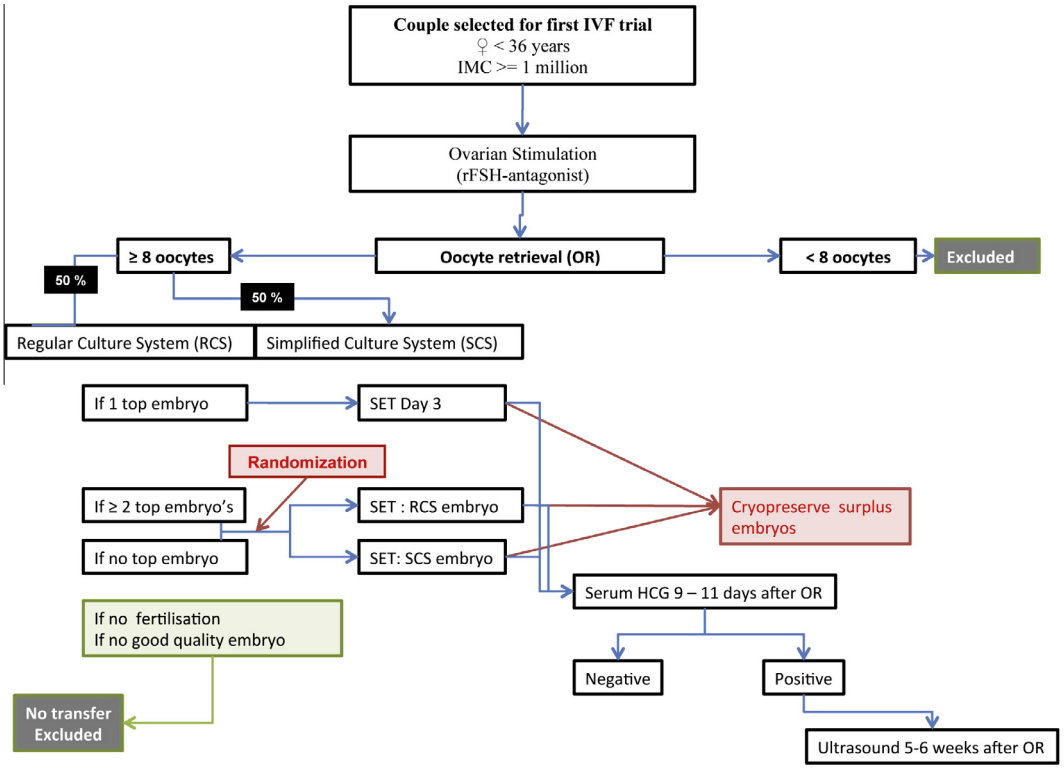

Figure 2 Flow chart of the Genk pilot study. HCG = human chorionic gonadotrophin; IMC = inseminating motile count after sperm preparation; OR = oocyte retrieval; rFSH, recombinant FSH; RCS = routine culture system; SET = single-embryo transfer; SCS = simplified culture system.

COC/embryo cultures, assessment times could be reduced. The resolution obtained through the glass was sufficient to determine stage and gross morphological characteristics of early development (e.g. uniformity of cleavage divisions and blastomere multinucleation) for embryo selection for transfer.

Because acute drops in temperature can adversely influence early human embryogenesis, this work examined the extent to which the temperature of the culture medium temperature changed during these assessments and the length of time required to return to 37°C. Temperatures were measured using a wire thermocouple inserted into culture medium through the stopper using an 18 g needle for the times required to perform the stage-related embryo evaluations noted above with a high-sensitivity digital thermometer (Omega Microprocessor HH21 Thermometer; Omega Engineering, Stamford, CT, USA). All embryo assessments were done with the microscope adjacent or in close proximity to a water bath, block heater, thermos or incubator.

For embryo cultures performed during the preclinical phase, all tubes were kept at 37°C for a minimum of 5 days after day 6.5 and the contents inspected microscopically for evidence of bacterial or fungal contamination.

Pilot clinical trial

Patient selection

In January 2012, a prospective non-inferiority study was begun at the ZOL hospitals in Genk, Belgium. The main outcome parameter of this study was embryo quality on day 3 (day of transfer) comparing two different culture methods: the simplified culture system) and the routine culture system. Secondary outcome parameters were embryo implantation rate, clinical pregnancy rate and live birth rate per transfer. Patients eligible for this study were couples requiring IVF as assessed by the standard protocol of infertility evaluation used at ZOL hospitals, which included the following indications: (i) first IVF attempt in women $<$36 years;(ii) bilateral tubal occlusion; and (iii) unexplained infertility or mild-to-moderate endometriosis with a minimum of three failed IUI with patterns characterized as normospermic or subfertile, the latter defined by the occurrence of $\sim$1 million motile spermatozoa separated from abnormal forms after low-speed centrifugation through a discontinuous gradient of colloidal silica, as described below. Couples with these indications were preferred because the cause of their infertility was likely to be representative of the target population in need of accessible and affordable conventional IVF. According to the Belgian law, single-embryo transfer (SET) is mandated in this group of patients (Ombelet et al., 2005). Therefore, the exact embryo implantation rate for both culture systems could be obtained because every pregnancy could be linked to only one method.

A flow diagram of the study protocol is shown in Figure 2. Only IVF cycles with at least eight oocytes recovered at follicular aspiration were included because the approved pilot study was comparative rather than randomized and, as such, had to maximize the potential for pregnancy with each IVF cycle. Consequently, the number of oocytes required for inclusion needed to allow allocation of similar numbers

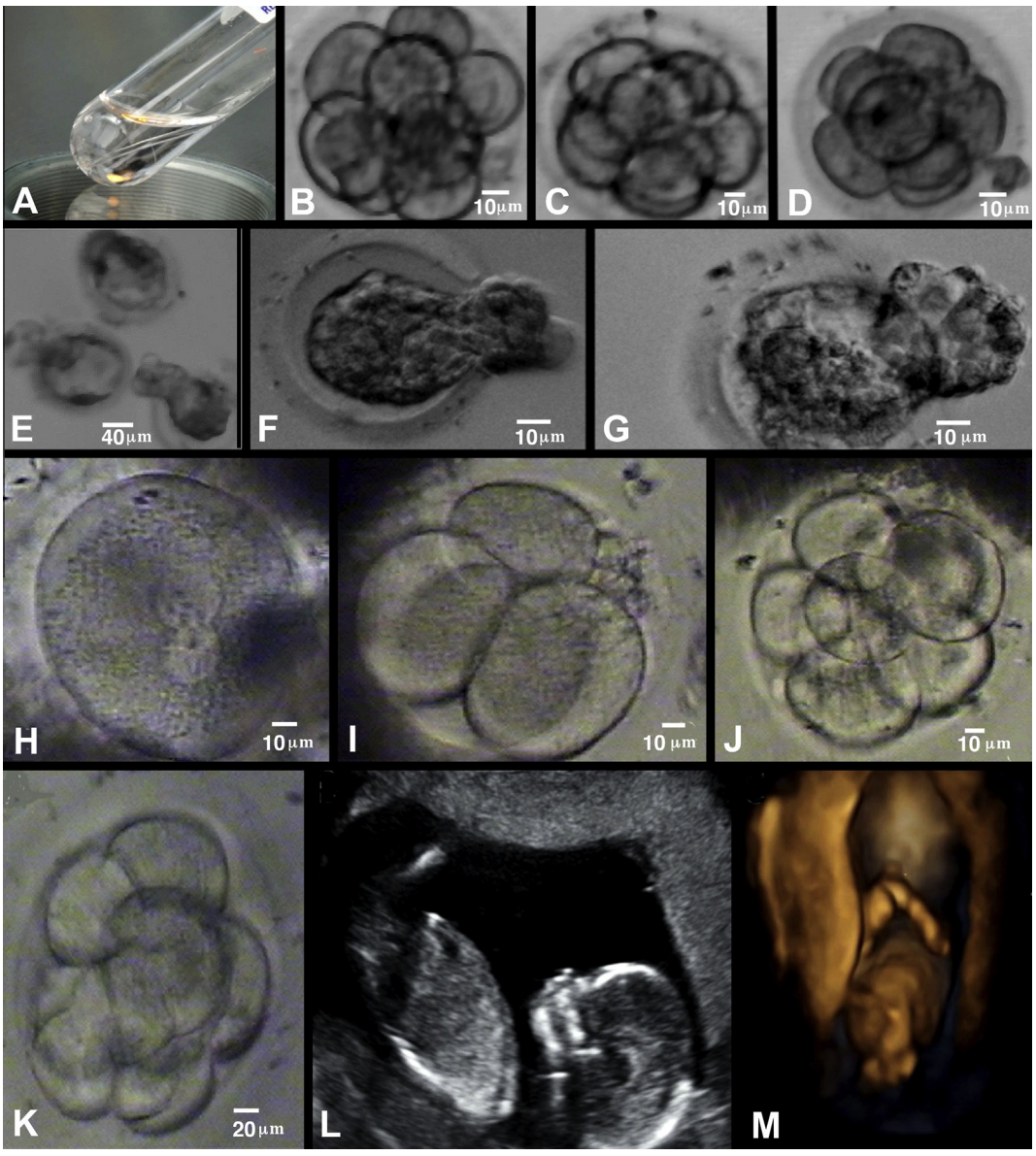

Figure 3 Light microscopic images of fertilization and human embryo development *in situ* in the simplified culture system taken through the glass vacutainer during the preclinical phase. (A) Typical position of the tube during inspection. (B—D) Representative images of cleavage-stage embryos on day 3 that developed from thawed 12—16 h tripronuclear (B) or bipronuclear embryos (C, D). (E—G) Three hatching day-6/6.5 blastocysts that developed from thawed pronuclear embryos as observed through the vacutainer (E) and after transfer to a standard tissue culture dish for higher resolution (F, G). (H—M) Typical light microscopic images of preimplantation development at the pronuclear (H, day 1), 4-cell (I, day 2), and 8-cell stage (J, day 3) obtained in the SCS during the clinical trial. The embryo that resulted in the first pregnancy and birth from this system is shown at transfer on day 3 (K), and as observed at 13 weeks of gestation by 2-D and 3-D ultrasound in L and M, respectively.

between the culture systems in order to take into account the possibility that, within randomly selected COC, not all corresponding oocytes would be meiotically mature (metaphase II at insemination). This strategy was found to be helpful for patients because at least four or more oocytes would be inseminated and cultured using the routine culture system.

Patients with the inclusion criteria were recruited after detailed discussions of how the new method compared with the routine culture system. However, to maximize the potential for a positive outcome, the embryo selected for transfer could occur from either system based on standard selection criteria at the ZOL hospitals' IVF programme, as described below. Participation in this first clinical trial required informed consent documentation. The approved method of ovarian stimulation for this first clinical trial was the standard protocol used at the IVF unit at the ZOL hospitals, namely a combination of recombinant FSH (Puregon; MSD) or purified urinary FSH (Menopur; Ferring) and GnRH antagonist (Orgalutran; MSD).

Without the application of any empirically based selection criteria on aspirated COC, IVF was performed on half using the routine culture system, with the remaining

oocytes allocated to the simplified culture system. When an odd COC number occurred, it was allocated to the routine culture system. Embryo transfers were performed in the early afternoon of day 3. Although embryo selection could be made through the glass culture tube, for this study digital images of individual embryos from the routine and simplified culture systems were taken 3 h prior to transfer with the selection for SET made by an independent embryologist who did not know the culture system used, nor could it be identified from the images. Criteria for SET used recognized morphological and performance characteristics for stage- and time-appropriate development (Istanbul consensus, 2011). Regardless of the IVF system used, if only one stage-appropriate embryo occurred, this top-quality embryo was transferred. If two or more top-quality embryos were available from both systems, the transferred embryo was selected by randomization using a computerized system (www.randomizer.org). Digital images of embryos were coded to identify the culture system but what the identifier signified was unknown to the embryologist performing the selection.

This pilot study was approved by the ethical committees of the ZOL hospitals in Genk and the ethical committee of the Free University of Brussels (reference no. 2011/011, approved 19 May 2011) and registered as B.U.N. 143201110348.

Routine culture system

Spermatozoa were prepared on the day of oocyte retrieval using 3-layer density-gradient centrifugation (90%–70%–40%) (PureSperm, Nidacon) followed by two washing steps in Earle's balanced salt solution + 5% HSA. Samples were resuspended in Global medium supplemented with 5% HSA and incubated at 36.5°C until fertilization. For fertilization, COC were placed in a loosely capped 5-ml tube with 1 ml pre-equilibrated medium, and a volume equivalent to 50,000 motile spermatozoa was added. Fertilization and embryo culture occurred in a tissue culture incubator in an atmosphere of 5% CO_2 in air, with pronuclear confirmation of fertilization made 16–20 h later after cumulus and coronal cell denudation by a standard protocol of repeated passage though an appropriately sized micropipette. Only embryos showing 2 pronuclei were transferred to a new culture tube containing fresh medium, with developmental performance assessed daily up to day 3.

Simplified culture system

Vacutainers containing the same culture medium were connected to a pressurized CO_2 generator after effervescence indicative of CO_2 production ceased (e.g. **Figure 1B1**). According to protocol, a simple aluminium heating block (**Figure 1C**) was kept in the same incubator (only to maintain 37°C), in which the other half of the patient's COC were inseminated and cultured according to the routine culture protocol. For the pilot study, the aluminium tube holder (**Figure 1B**, **C** and **E**) was designed to be used simultaneously for medium preparation, IVF and embryo culture in a water bath, heating block or incubator or for storage of pre-equilibrated vacutainers (Gynetics Medical Products).

After rinsing and drying the stopper with ethanol, individual COC were added to the culture tube by means of injection through a 18.5 g 1.5-inch bevelled needle with a common 0.5–1.0 ml tuberculin syringe, or a 500-μl gas-tight luer-lock syringe (**Figure 1D**). Each COC was picked up in approximately 25 μl medium, followed by a ~20 μl air buffer, and the entire length of the needle was inserted through the stopper so that the opening of the needle was on the surface of the tube, onto which the contents were expelled. Alternatively, COC could also be deposited directly into the centre of the tube and the needle examined for expulsion after withdrawal by repeated rinses and inspection under a dissecting microscope.

Insemination, fertilization confirmation and embryo performance

Insemination was performed 2–3 h after oocyte retrieval using ≤1000 motile spermatozoa per/ml prepared as described for the routine culture system. According to protocol, when sperm morphology was ≤5% (WHO criteria, Cooper et al., 2010), the insemination used ~5000 spermatozoa/ml if the motility was normal. If sperm parameters were found to be inconsistent for conventional IVF, ICSI was performed using the routine culture system; the results of these inseminations are not included in this study. After treating the stopper with ethanol, spermatozoa were added to the fertilization tube in 10–15 μl culture medium in the same manner as the COC and, following gentle swirling, the tubes were completely surrounded by a 37°C environment with only the top of the stopper visible (**Figure 1B3**). At the pronuclear check, the tubes were vortexed for up to 30 s in a standard vortexing instrument set between 5 and 6 on a typical scale of 1–10. This usually dislodged most residual cumulus and coronal cells such that pronuclei were detectable (e.g. **Figure 3H**), even if small fragments of the cumulus oophorus and corona radiate remained attached to the zona pellucida. In some instances, confirmation of fertilization was problematic because patches of cumulus or coronal cells were resistant to removal by vortexing. In these cases, oocytes were removed from the fertilization vacutainer, denuded mechanically and then returned (by injection) to a fresh culture tube. During the development phase of this study, it was noted that the inclusion of high-purity hyaluronidase (~200 IU/ml; chromatographically purified; Worthington Biochem, Lakewood, NJ, USA) in the IVF vacutainer followed by gentle vortexing at the pronuclear check could overcome the potential for difficult pronuclear visualization resulting from persistent cumulus/coronal cells, and the presence of this enzyme thereafter seemed to have no apparent adverse affects on fertilization or embryogenesis. It is worth mentioning that most oocytes requiring mechanical denudation were (meiotically) immature.

Embryos were observed through the glass vacutainer by holding the tube horizontally in either a dissecting microscope (e.g. **Figure 3A**) or inverted microscope using a long working distance ×10 objective lens. This permitted documentation of stage and morphology and facilitated preselection of candidate embryos for transfer. The simplified culture system protocol is intended to use a single COC/embryo per tube, so that selection for transfer can be done with the embryo undisturbed until transfer, which was accomplished by means of a catheter inserted directly

into the vacutainer through a bevelled needle with a size-appropriate gauge. The catheter was positioned above the embryo, which was easily withdrawn in ~10 μl medium. To reduce the possibility of pathogen contamination, the stopper was thoroughly treated (for the third time in this procedure) with ethanol prior to the insertion of the catheter. Each used culture tube was incubated for at least 5 days with periodic inspection for signs of contamination.

Results

Kinetics of atmospheric and pH equilibration

Systematic titrations of sodium bicarbonate, citric acid and water were made to create atmospheric conditions that would closely approximate those obtained at high-resource IVF centres either from certified, premixed medical-grade gases, or microprocessor-controlled CO_2 or triple-gas tissue culture incubators. The kinetics of medium equilibration with respect to pH and atmospheric composition shown in **Figure 1F** were those found to be optimal for fertilization and preimplantation embryogenesis in the mouse model and to support cleavage of tripronuclear eggs and development to the fully expanded blastocyst stage of thawed bipronuclear and early cleavage-stage human embryos. An initial sharp rise in CO_2 to ~10% when the medium and CO_2 generating tubes were first connected was followed by a progressive drop in pH from 8.3 to 7.3. Equilibration of atmospheres between tubes stabilized by ~5 h such that the following conditions were consistently obtained in the vacutainer used for IVF (mean ± SD): pH 7.33 ± 0.03; CO_2 6.5 ± 0.2%; O_2 12 ± 0.6. Equilibrated medium in sealed vacutainers could be used immediately or stored at 5–10°C for at least 2 weeks without loss of capacity to promote fertilization and normal preimplantation embryogenesis.

Preclinical studies

Mouse IVF and embryonic development

For this study, the endpoint was a fully expanded blastocyst with a clearly detectable inner cell mass that subsequently spontaneously hatched after IVF. The meiotic status of 378 intact COC was not determined when injected into the vacutainers. Overall, the fertilization rate was 83% (314/378), with 6% (22/378) at metaphase II but unfertilized when examined and 11% (42/378) meiotically immature or degenerate/fragmented. The fertilization rate in the two groups with oocytes presumed to be at metaphase II at insemination was similar: open, filter-top system in cell culture chamber ~81% (130/161); closed, simplified culture system ~84% (128/153). Rates of development to the expanded blastocyst stage were similar in the open (87%, 74/130) and closed systems (91%, 139/153), but with respect to hatching, higher in the simplified culture system (62%, 86/139) than in the open filter-top system (28%, 21/74), and this difference was significant ($P < 0.01$, Fisher's exact test).

Human preimplantation embryogenesis

Based on the ability of the simplified culture system to support development to the hatched blastocyst stage in the mouse, human studies were initiated using the same procedure to generate with Global medium, the same culture conditions shown in **Figure 1F**. Thawed, tripronucleate embryos ($n = 61$) that resulted from dispermic penetration, normally fertilized bipronucleate embryos ($n = 27$) and nascent 2-cell embryos ($n = 30$), were used to determine the efficacy of the simplified culture system in supporting preimplantation embryogenesis to day 3 and day 6–6.5, respectively.

Figure 3A shows the position of the culture tube during inspection for fertilization and staging of preimplantation embryogenesis. **Figure 3B–D** shows representative examples of day-3 cleavage-stage embryos from bipronucleate embryos imaged through the glass vacutainer. **Figure 3E** shows three hatching blastocysts on day 6.5 that developed from thawed pronuclear embryos. While hatching can be detected *in situ*, the resolution of the trophectoderm and inner cell mass detail was less distinct than for the cleavage stages. For comparative purposes, **Figure 3F** and **G** shows two of the embryos in **Figure 3E** after placement in a standard plastic issue culture dish. However, the imaging obtained *in situ* would be sufficient to determine stage and morphological characteristics (e.g. presence of an inner cell mass) for selective purposes for blastocyst transfers without prior removal of the embryo.

While the results showed that >80% of pronuclear and 2-cell embryos underwent successive cleavage divisions during 3 days of culture, for clinical trials to commence, spontaneous hatching of blastocysts (i.e. without assistance) in embryos that had been normally fertilized was considered to be a necessary and relevant developmental endpoint, despite lower frequencies of hatching that were expected with this protocol of cryopreservation. For thawed embryos, 32% (18/57) progressed to that expanded blastocyst stage, of which 55% (10/18) initiated hatching (similar to embryos in **Figure 3E**), of which 30% (3/10) were completely free of the zona pellucida by late day 6 (~162 h post conventional insemination).

Potential for temperature fluctuation and iatrogenic pathogen contamination

Timed, stage-specific evaluations were made *in situ* and were of sufficient quality to determine the normality of fertilization and to select embryos on day 3 for SET without their removal prior to transfer. Because of the potential for a drop in medium temperature during embryo assessments, temperatures were measured during the time(s) required for inspection from fertilization to the hatched blastocyst stage. The greatest potential for a drop in temperature was at the pronuclear and blastocyst stages, with −1.2°C being the greatest change. In contrast, the greatest change observed for cleavage stages on days 2 and 3 was −0.3°C. The greater temperature drops recorded for some pronuclear and blastocyst-stage embryos reflected increased times required to vortex and align the embryos to detect pronuclei and for the embryologist to position the embryo to reveal its inner cell mass. By comparison, the cleavage stages could be assessed rapidly. However, <3 min was required to return the temperature of culture medium to normal, which was likely due to the amount of surface area directly and uniformly exposed to 37°C in the heating block or water bath. It is worthwhile noting that during these evaluations, the dissecting microscope was in close proximity to an aluminium block (**Figure 1E**) kept at 37°C.

No detectable bacterial or fungal contamination was observed in any of the culture tubes after at least 5 days following development to the hatched blastocyst stage in the mouse (11—12 days after IVF) or with any of the thawed human embryos cultured under similar conditions and durations. It should be noted that mouse oocytes and spermatozoa were intentionally collected and introduced into the vacutainer in room air, as was thawing, rehydration and placement of human embryos in the vacutainer. Taken together, these results were considered sufficient proof of principle to initiate a clinical pilot study that compared outcomes from the routine and simplified IVF culture systems, which according to the protocol approved for this study, required both systems to be used for each patient.

Pilot clinical trial

Figure 2 shows the flow diagram of the clinical trial approved by the ethical committees that compared outcomes from an initial 40 patients, of which 35 underwent day-3 SET during an 8-month period in 2012, with embryos derived either from the simplified culture system or routine culture system methods.

In two cases (5%) no fertilization was observed: for one patient, oocyte quality was clearly impaired, while for the other, no apparent reason for failed fertilization was discernable at the light microscopic level.

For three patients, all embryos were cryopreserved because of a high risk for ovarian hyperstimulation syndrome after using the agonist Decapeptyl (0.2 mg) to induce the LH surge.

After being randomly divided between the simplified and routine systems, 493 COC aspirated from 40 women yielded 431 metaphase-II, 45 immature, and 17 degenerate or giant oocytes, identified as such at the fertilization check. The simplified and routine culture system groups contained 232 and 199 metaphase II oocytes, respectively. Rates of fertilization and cleavage to day 3 for the routine culture system (147/232, 63%; 130/147, 88%) and simplified culture system (138/199, 69/%; 119/138, 86%) were similar, and it was possible to perform SET on day 3 with an embryo originating from either system.

Thirty-five of the women enrolled in this study underwent SET on day 3. In nearly two-thirds of the cases (23/35), the embryo selected for transfer by an independent embryologist, unaware of the IVF system used, originated from the simplified culture system. In this group, clinical pregnancies occurred in eight of the 23 patients, with one miscarriage at 8 weeks of gestation. In 12 cases of SET with a top-quality embryo from the routine culture system group, two clinical pregnancies resulted.

The first baby conceived with the simplified culture system was a healthy boy born in November 2012, at 40 weeks of gestation (3500 g, 52 cm). The embryo resulting in this first pregnancy is shown at the pronuclear, 4-cell and 8-cell stages and at transfer on day 3 (10-cell) in Figure 3**H—K**, respectively. Figure 3**L** and **M** are ultrasound pictures of Figure 3**K** at 13 weeks of gestation. As of June 2013, seven healthy babies have been born from the simplified culture system cohort, four boys and three girls, while two healthy girls have been delivered using the routine culture system.

Discussion

Since the introduction of IVF as a successful clinical treatment for tubal factor infertility over three decades ago (Steptoe and Edwards, 1978), certain changes in the IVF laboratory can be considered landmark events because they markedly improved outcome: (i) preimplantation genetic diagnosis to eliminate oocytes and embryos that are aneuploid or carry pathogenic mutations (Handyside et al., 1990); (ii) ICSI for severe male factor infertility (Palermo et al., 1992); and (iii) cryopreservation (Trounson and Mohr,1983), including the recent and wide-spread adoption of the simpler vitrification method for oocytes and preimplantation-stage embryos (Kuwayama et al., 2005; Vanderzwalmen et al., 2006). In contrast, the basic procedures used for conventional IVF, and for embryo culture regardless of the means of insemination, have changed little from those used to produce the first IVF baby (Steptoe and Edwards, 1978) which, it is worth noting, even then used methods derived from earlier studies in experimental systems (e.g. mouse, rabbit, sheep).

If the goal of providing IVF for common aetiologies of infertility such as bilateral tubal occlusion or relatively low sperm count (for which IVF was originally indicated) is to be realized (Dhont, 2011; Ombelet, 2013; Ombelet and Campo, 2007; Pilcher, 2006), new methodologies and models will be needed to address two fundamental obstacles to treatment, namely affordability and accessibility. The current study describes how a methodology designed to address the laboratory cost of IVF treatment was tested in preclinical studies and, most importantly, validated by births in a pilot clinical trial. The result is a highly simplified culture system that consistently produces conditions very similar to those obtained at high-resource centres but without the attendant and costly infrastructure typically required by contemporary IVF programmes.

The introduction of this IVF method could have important implications both for the treatment of common aetiologies of infertility that require conventional IVF, such as bilateral tubal occlusion, as well as in reducing costs to make an advanced method of infertility treatment available to a wider segment of the population than is currently served in both developing and developed countries. Although use of the simplified culture system can be considered to be applicable worldwide, it is worth noting that the original focus was for developing countries (Dhont, 2011). Indeed, the first successful clinical application of this simplified IVF system at the ZOL hospitals in Genk, Belgium resulted from a systematic, multiyear approach that evaluated different ways by which IVF could be established and delivered in a challenging infrastructure often typical of low-resource settings. For example, the method had to be independent of logistical support (e.g. medical-grade gases, reliable electricity, rapid availability of replacement parts), complex equipment (e.g. microprocessor-controlled incubators including the triple-gas type), and costly disposable cultureware commonly used in high-resource settings. Three additional features considered necessary for this simple closed system to function properly and be clinically acceptable were: (i) it had to produce and maintain a defined atmosphere that was stable for prolonged periods so that

culture medium could be equilibrated well in advance of an IVF cycle, if desired; (ii) fertilization and preimplantation embryogenesis needed to remain undisturbed to maintain sterility and minimize or preclude potential changes in temperature, atmosphere or pH that could have adverse developmental effects; (iii) a uniform temperature had to be easily kept in a simple and inexpensive environment that could provide a constant and uniform 37°C. With respect to sterility, mouse oocytes and spermatozoa were intentionally collected and introduced into the vacutainer in room air, as was thawing, rehydration and insertion of human embryos. Precautionary ethanol treatments of the stopper prior to each operation and the presence of antibiotics in the culture medium may be sufficient to reduce the contamination potential, especially if a sterile field is unavailable. However, such a field is highly recommended for clinical application of this system.

During the course of this work, it became quite apparent that, in order for a new IVF method to gain acceptance where intended, it first had to be demonstrated that prospective patients in developing countries were not to be 'test subjects' and that the system could simply, repeatedly and inexpensively create conditions known to be consistent with normal fertilization and early human embryogenesis in high-resource IVF centres. Therefore, by necessity, outcomes with the simplified culture system needed to be demonstrated first in a developed country, in the present case Belgium, before application in most developing countries would likely be considered. The method finalized for clinical testing at the ZOL hospitals in Genk, Belgium created the necessary conditions for human IVF and preimplantation embryo culture in an inexpensive glass tube using a simple chemical process of CO_2 generation. Although performed at a high-resource facility, set up of the system for IVF, injection of gametes and assessments of fertilization and embryonic development followed a standard set of procedures designed to be used worldwide, including those necessary to maintain sterility and to minimize the possibility of temperature fluctuations during embryo evaluations for selective purposes. Parenthetically, this method is neither new nor particularly novel and had been used earlier for the transport animal oocytes and embryos for experimental purposes, although in this instance atmospheric composition in the transporting vessel was not quantified and pH was approximated by the tint of phenol red in culture medium (Van Blerkom and Manes, 1974). During the clinical-trial phase of the present study, a similar method of CO_2 generation from a common effervescent antacid was described for mouse embryo culture (Swain, 2011) but without IVF and precise quantification of the atmospheric conditions. These measurements were deemed to be essential for clinical adoption in order to assure both patients and medical providers that a system specifically designed for their treatment would be functionally equivalent to contemporary IVF in high-resource settings.

Based on embryo performance *in vitro* and outcome after SET, use of this simple IVF/embryo transfer culture system for the treatment of infertility resulting from bilateral tubal occlusions is highly feasible in general and has a realistic potential to increase accessibility for those in developing countries (Dhont, 2011; Ombelet, 2013). It is also worth noting that successful fertilization was obtained at sperm concentrations $\leq$1000/ml. This suggests that the simplified culture system can be effective when sperm counts are subnormal, but motility and morphology are largely normal. At present, typical treatment in such instances in many high-resource centres involves ICSI rather than conventional IVF, perhaps owing to the notion that low sperm numbers result in low fertilization rates. However, if outcome findings from ongoing and planned studies using low sperm numbers for insemination continue to support the simplified culture system method in mild male factor cases, it could have significant implications for treatment by offering a viable and more affordable and available alternative to ICSI.

The approved protocol required that the oocytes were allocated between the routine and simplified systems so as to maximize the potential for pregnancy and that, in all cases, the top-quality embryo be selected for SET by an embryologist who was unaware of the culture system and used the same widely accepted morphological and performance characteristics for both. Clinical pregnancies and live births that resulted from this trial indicate that the simplified culture system does not compromise fertilization, preimplantation development or outcome. Extrapolations regarding expected implantation, clinical pregnancy or live birth rates based on this small cohort of patients would be premature. However, it is worth mentioning the following findings, which have positive implications for further clinical trials of the simplified culture system method: (i) a clinical pregnancy rate of $\sim$34% occurred in a contemporaneous cohort of patients who, while eligible for inclusion, chose not to participate; and (ii) comparing the results of this pilot study with outcome data from the Belgian Registry for Assisted Procreation (www.belrap.be) in reports to the College of Physicians in Reproductive Medicine for the years 2007–2010, the results are almost identical to those reported here. For patients who met the same inclusion criteria ($<$36 years old, at least eight COC aspirated, first IVF attempt with day-3 SET), the clinical pregnancy and live birth rates in the registry were 34.2% (3403/9929) and 29.0% (2808/9680), respectively.

Additional studies will be needed to determine the following: (i) whether the outcomes of the simplified culture system can be improved by extending culture to the expanded blastocyst stage; (ii) how the simplified culture system can be implemented in high-resource settings where, for many prospective patients, conventional IVF is currently cost-prohibitive; (iii) how best to proceed in developing countries to make IVF assessable, affordable and acceptable as a treatment for common forms of infertility; and (iv) whether similar pregnancy rates can be achieved using natural-cycle IVF or mild/low-cost ovarian stimulation protocols, such as clomiphene citrate alone or in combination with purified urinary FSH. According to Belgian law, SET was used in this clinical trial and, while comparatively mild stimulation protocols have long been known to be effective in clinical IVF, they typically yield relatively small numbers of oocytes (Aleyamma et al., 2011; Kato et al., 2012; Nargund et al., 2007; Verberg et al., 2009). However, for the intended application of this methodology there may be a benefit to minimizing ovarian stimulation, especially if SET is favoured or required and in developing countries, where cryopreservation might not be an option.

While exact treatment costs are difficult to predict and are likely to be location specific, laboratory costs should be significantly reduced from those typical of high-resource programmes when conventional IVF is required for bilateral tubal occlusion (Ombelet, 2013).

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