In vitro fertilization

SECOND EDITION

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Contents

7

The clinical in vitro fertilization laboratory

Introduction

In the armoury of medical technology that now exists for the alleviation of disease and improvement in the quality of life, there is nothing to match the unique contribution of assisted reproductive technology. There is no other life experience that matches the birth of a baby in significance and importance. The responsibility of nurturing and watching children grow and develop alters the appreciation of life and health, with a resulting long-term impact upon individuals, families and, ultimately, society. Thus, the combination of oocyte and sperm to create an embryo with the potential to develop into a unique individual cannot be regarded lightly, as merely another form of invasive medical technology, but must be treated with the respect and responsibility due to the most fundamental areas of human life.

Successful assisted reproduction involves the careful co-ordination of both a medical and a scientific approach to each couple who undertake a treatment cycle, with close collaboration between doctors, scientists, nurses and counsellors. Only meticulous attention to detail at every step of each patient's treatment can optimize their chance of delivering a healthy baby. Appropriate patient selection, ovarian stimulation, monitoring and timing of oocyte retrieval should provide the in vitro fertilization (IVF) laboratory with viable gametes capable of producing healthy embryos. It is the responsibility of the IVF laboratory to ensure a stable, nontoxic, pathogen-free environment with optimum parameters for oocyte fertilization and embryo development. The first part of this book reveals the complexity of variables involved in assuring successful fertilization and embryo development in animal systems, together with the fascinating and elegant systems of control which have been elucidated at the molecular level. It goes without saying that human in vitro fertilization must of necessity involve systems of at least equal, if not greater, complexity,

The Assisted Conception Treatment Cycle

- Consultation: history, examination, investigation, counselling
- Pituitary downregulation with GnRH agonist
- Baseline assessment
- Gonadotrophin stimulation
- Follicular phase monitoring
- Ultrasound + endocrinology
- HCG administration to induce ovulation
- Oocyte retrieval (OCR)
- In vitro fertilization
- Embryo transfer
- Supernumerary embryo cryopreservation
- Luteal phase support
- Day 15 pregnancy test
- Follow-up pregnancy tests: day 20, 25, 30
- Day 35 ultrasound assessment

and it is essential for the clinical biologist to be aware that control mechanisms exist which are exquisitely sensitive to even apparently minor changes in the environment of gametes and embryos, in particular, temperature, pH and any other factors which potentially affect cells at the molecular level. Multiple variables are involved, so the basic science of each step must be carefully controlled, while allowing for individual variation between patients and between treatment cycles. In addition, in this current era of rapidly evolving technology, the success of new innovations in technique and technology can only be gauged by comparison with a standard of efficient and reproducible established procedures. The IVF laboratory therefore has a duty and responsibility not only to ensure that a strict discipline of cleanliness and sterile technique is adhered to throughout all procedures, but also to produce and maintain daily records, with systematic data analysis and reports.

Setting up a laboratory: equipment and facilities

The design of an IVF laboratory should provide a distraction- and accident-free environment in which concentrated attention can be comfortably and

safely dedicated to each manipulation, with sensible and logical planning of work stations which are practical and easy to clean. Priority must be given to minimizing the potential for introducing infection or contamination from any source, and therefore the tissue culture area should allow for the highest standards of sterile technique, with all floors, surfaces and components easy to clean on a daily basis. Ideally, the space should be designated as a restricted access area, with facilities for changing into clean operating theatre dress and shoes before entry.

Ambient air quality

The importance of ambient air and the possible consequences of chemical air contamination have been reviewed by Cohen et al. (1997). Whereas most organisms and species are protected to some extent from hazards in their ambient environment through their immune, digestive and epithelial systems, oocytes and embryos in vitro have no such protection, and their active and passive absorption mechanisms are largely indiscriminate. IVF laboratories set up in buildings within polluted areas, or close to industrial manufacturing sites, may be subject to serious chemical air contamination which may be reflected by inadequate pregnancy and live birth rates. Incubators obtain their ambient air directly from the laboratory room; CO_2 is supplied in gas bottles, which may well be contaminated with organic compounds or metallic contaminants. Pressurized rooms using high efficiency particulate air (HEPA) filtration are used by many IVF laboratories, with standards applied to pharmaceutical clean rooms; however, HEPA filtration cannot effectively retain gaseous low molecular weight organic and inorganic molecules.

The four most common air pollutants are:

1. In urban and dense suburban areas, volatile organic compounds (VOC) produced by industry, a variety of cleaning procedures and vehicle and heating exhausts. Instruments such as microscopes, television monitors or furniture (as a result of manufacturing processes) may also produce VOCs.
2. Small inorganic molecules such as N_2O, SO_2, CO.
3. Substances derived from building materials, e.g. aldehydes from flooring adhesives, substituted benzenes, phenol and *n*-decane released from vinyl floor tiles – flooring adhesives have been found to be particularly aggressive in arresting embryo development! Newly painted surfaces frequently present a hazard, as many paints contain substances that are highly toxic in the IVF lab.
4. Other polluting compounds which may be released by pesticides or by aerosols containing butane or iso-butane as a propellant. Liquids such as

floor waxes may contain heavy metals, which have a drastic effect on embryo implantation potential.

Cohen and colleagues conducted a detailed study of chemical air contamination in all areas of their IVF laboratory, which revealed dynamic interactive processes between air-handling systems, spaces, tools, disposable materials and other items unique to their laboratory. Anaesthetic gases, refrigerants, cleaning agents, hydrocarbons and aromatic compounds were found, and some accumulated specifically in incubators. They suggest that there may be an interaction between water-soluble and lipid-soluble solid phases such as those in incubators: whereas some contaminants may be absorbed by culture media, this may be counteracted by providing a larger sink such as a humidification pan in the incubator. Mineral oil may act as a sink for other components. Unfiltered outside air may be cleaner than HEPA filtered laboratory air or air obtained from incubators, due to accumulation of VOCs derived from adjacent spaces or specific laboratory products, including sterile Petri dishes. Manufacturers of compressed air and incubators have no concern for the specific clean air needs of IVF: standards for supplies of compressed gases are based upon criteria which are not designed for cultured and unprotected cells. Testing a new incubator revealed concentrations of VOC $>$ 100-fold higher than those obtained from testing used incubators from the same manufacturer – allowing the emission of gases from new laboratory products is crucial. In view of the fact that it appears impossible to prevent pollution inside the laboratory from the surroundings with current air-conditioning technologies, primary consideration should be given to design of culture spaces and adjacent areas. In order to circumvent this problem of potential hazards in ambient air and culture systems, active filtration units with activated carbon filters and oxidizing material have now been developed specifically for IVF laboratories. These can be placed inside cell-culture incubators (CoDa, GenX, Connecticut, MA, USA) or in the laboratory spaces themselves (Eco-Care, New York, USA).

Laboratory space layout

Careful consideration should be given to the physical manoeuvres involved, ensuring ease and safety of movement between areas, in order to minimize the possibility of accidents. Bench height, adjustable chairs, microscope eye height and efficient use of space and surfaces all contribute to a working environment that minimizes distraction and fatigue. The location of storage areas and

equipment such as incubators and centrifuges should be logically planned for efficiency and safety within each working area; the use of mobile laboratory components allows flexibility to meet changing requirements.

Basic essential equipment required for routine IVF includes:

1. Dissecting, inverted, and light microscopes
2. Incubator with accurately regulated temperature and CO_2
3. Centrifuge for sperm preparation
4. Warmed stages or surfaces for culture manipulations
5. Refrigerator/freezer
6. Dry heat oven for drying and sterilizing

A video camera system is also recommended for teaching, assessment and records (patients receive enormous satisfaction and psychological support from observing their oocytes and embryos on a video screen).

When choosing these expensive items of equipment, ensure that not only is each easy to use and maintain, but that servicing and repairs can be quickly and efficiently obtained. Routine schedules of cleaning, maintenance, and servicing must be established for each item of equipment, and checklist records maintained for daily, weekly, monthly and annual schedules of cleaning and maintenance of all items used, together with checks for restocking and expiry dates of supplies.

Incubators

Two types of gas phase have been successfully used: 5% CO_2 in air, and the triple gas mixture of 5% CO_2, 5% O_2, 90% N_2. An atmosphere of 5% CO_2 is required to maintain correct pH in bicarbonate buffered culture media systems in order to maintain a physiological pH of just under 7.5. This equilibrium depends upon the composition of the media used, and is affected by both temperature and atmospheric pressures: some conditions may require a 6% CO_2 in air mixture in order to maintain the correct pH.

Carefully calibrated and accurately controlled CO_2 incubators are critical to successful IVF. The choice of humidified or nonhumidified incubator depends upon the type of tissue culture system used: whereas humidity is required for standard 4-well 'open' culture, the use of an equilibrated humidified overlay of mineral oil allows the use of incubators without humidity. Dry incubators carry less risk of fungal contamination, and are easier to clean. The incubator must be regularly monitored, and readings of the LED display checked and calibrated against independent recordings of temperature and pH

monitored by probes placed in a standard 'test' culture system. Temperature stability can be monitored with 24-hour thermocouple readings as part of the standard maintenance schedule.

The inside walls and doors should be washed with sterile water weekly, and a yearly inspection and general servicing by the supplier is recommended. Repeated opening and closing of the incubator affects the stability of the tissue culture environment, and the use of a small benchtop mini-incubator during oocyte retrievals and manipulations helps to minimize disturbance of the storage incubator. Modern mini-incubators specific for IVF culture have recently been introduced designed to overcome problems of pH and temperature consistency and stability encountered with traditional large incubator systems.

Water quality

A reliable source of ultrapure water is a critical factor in the laboratory, particularly if media is prepared 'in house'. Weimer et al. (1998) carried out a complete analysis of impurities that can be found in water: this universal solvent provides a medium for most biological and chemical reactions, and is more susceptible to contamination by other substances than any other common solvent. Both surface and ground water are contaminated with a wide range of substances, including fertilizers, pesticides, herbicides, detergents, industrial waste effluent and waste solvents, with seasonal fluctuations in temperature and precipitation affecting the levels of contamination. Four categories of contaminants are present: inorganics (dissolved cationic and anionic species), organics, particles and microorganisms such as bacteria, algae, mold and fungi. Chlorine, chloramines, polyionic substrates, ozone and fluorine may be added to water during treatment processes, and must be removed from water for cell culture media preparation. In water purification, analysis of the feed water source is crucial to determine the proper filtration steps required, and water-processing protocols should be adapted to meet regional requirements. Processing systems include particulate filtration, activated carbon cartridge filtration, reverse osmosis (RO) and electrodeionization (EDI), an ultraviolet oxidation system, followed by a Milli-Q PF Plus purification before final filtration through a 0.22 μm filter to scavenge any trace particles and prevent reverse bacterial contamination from the environment.

IVF laboratory personnel should be familiar with any subtle variations in

their water source, as well as the capabilities of their water purification system, and develop protocols to ensure consistently high-quality ultrapure water supplies. Weimer et al. recommend the following water maintenance schedule for the system that they use in purifying water for media preparation:

Replace carbon and depth filters monthly
Monitor chlorine every 21 days
Chlorine sanitization of RO and EDI system bimonthly
Mock sanitization of RO and EDI system bimonthly
Replace RO pretreatment pack every 3 months
UV recirculation of storage tank
NaOH sanitization storage tank quarterly
NaOH sanitization Milli-Q PF Plus bimonthly
Replace resin purification pack quarterly
Replace 0.22 μm final filter unit monthly
Monitor silica and Total Organic Carbon (TOC) at final product daily.

Supplies

A basic list of supplies is outlined at the end of this chapter; the exact combination required will depend upon the tissue culture system and techniques of manipulation used. Disposable supplies are used whenever possible and must be guaranteed nontoxic tissue culture grade, in particular the culture vessels, needles, collecting system and catheters for oocyte aspiration and embryo transfer.

Disposable high-quality glass pipettes are required for gamete and embryo manipulations; these must be soaked and rinsed with tissue culture grade sterile water and dry heat sterilized before use. In preparing to handle gametes or embryos, examine each pipette and rinse with sterile medium to ensure that it is clean and residue-free.

Daily cleaning routine

During the course of procedures any spillage should be immediately cleaned with dry tissue. No detergent or alcohol should be used whilst oocytes/embryos are being handled. Should it be necessary to use either of the above, allow residual traces to evaporate for a period of at least 20 minutes before removing oocytes/embryos from incubators.

At the end of each day:

1. Heat seal, double bag, and dispose of all waste from procedures.
2. Remove all pipette holders for washing and sterilizing before reuse.
3. Reseal and resterilize pipette canisters.
4. Clean flow hoods, work benches, and all equipment by washing with a solution of distilled water and 7X laboratory detergent (Flow Laboratories), followed by wiping with 70% methylated spirit.
5. Prepare each work station for the following day's work, with clean rubbish bags, pipette holder and Pasteur pipettes.

Washing procedures

If the laboratory has a system for preparation of ultrapure water, particular attention must be paid to instructions for maintenance and chemical cleaning. Water purity is essential for washing procedures, and the system should be periodically checked for organic contamination and endotoxins.

Pipettes

1. Soak new pipettes overnight in fresh Analar or Milli-Q water, ensuring that they are completely covered.
2. Drain the pipettes and rinse with fresh water.
3. Drain again and dry at 100°C for 1–2 hours.
4. Place in a clean pipette canister (tips forward), and dry heat sterilize for 3 hours at 180°C.
5. After cooling, record date and use within 1 month of sterilization.

Non-disposable items (handle with non-powered gloves, rinsed in purified water)

1. Soak in distilled water containing 3–5% 7X (flow Laboratories).
2. Sonicate small items for 5–10 minutes in an ultrasonic cleaning bath.
3. Rinse eight times with distilled water, then twice with Analar or Milli-Q water.
4. Dry, seal in aluminium foil or double wrap in autoclave bags as appropriate.
5. Autoclave, or dry heat sterilize at 180°C for 3 hours.
6. Record date of sterilization, and store in a clean, dust-free area.

Tissue culture media

A great deal of scientific research and analysis has been applied to the development of media which will successfully support the growth and development of human embryos. Many controlled studies have shown fertilization and cleavage to be satisfactory in a variety of simple and complex media (comprehensive reviews are published by Bavister, 1995, and Edwards & Brody, 1995). Metabolic and nutritional requirements of mammalian embryos are complex, stage specific, and, in many cases, species specific: several decades of research in laboratory and livestock animal systems have shown that, although there are some basic similarities, culture requirements of different species must be considered independently. Understanding metabolic pathways of embryos and their substrate and nutrient preferences has led to major advances in the ability to support embryo development in vitro. Rigorous quality control is essential in media preparation, including the source of all ingredients, especially the water, which must be endotoxin-free, low in ion content, and guaranteed free of organic molecules and microorganisms. Each batch of culture media prepared must be checked for osmolality (285 ± 2 mOsm/kg) and pH (7.35–7.45), and subjected to quality-control procedures with sperm survival or mouse embryo toxicity before use. Furthermore, culture media can rapidly deteriorate during storage, with decrease in its ability to support embryo development, and careful attention must be paid to storage conditions and manufacturers' recommended expiry dates. Commercially prepared, pretested high-quality culture media is now available for purchase from a number of suppliers world-wide, so that media preparation for routine use in the laboratory is not necessary, and may not be a cost-effective exercise when time and quality control are taken into account. A list of available commercial culture media is supplied at the end of this chapter. There is so far no firm scientific evidence that any is superior to another in routine IVF, and choice should depend upon considerations such as quality control and testing procedures applied in its manufacture, cost and, in particular, guaranteed efficient supply delivery in relation to shelf-life. After delivery, the medium may be aliquoted in suitable small volumes, such that one aliquot can be used for a single patient's gamete preparation and culture (including sperm preparation). Media containing HEPES, which maintains a stable pH in the bicarbonate-buffered system, can be used for sperm preparation and oocyte harvesting and washing; however, HEPES is known to alter ion channel activity in the plasma membrane and may well be embryotoxic. The gametes must therefore subsequently be washed in HEPES-free culture medium before insemination and overnight culture.

Media specially designed for 'sperm washing' is also commercially available.

Prior to 1997, single media formulations were used for all stages of IVF. However, during the 1990s, a great deal of research in animal systems led to elucidation of the metabolic biochemistry and molecular mechanisms involved in gamete maturation, activation, fertilization, genomic activation, cleavage, compaction and blastocyst formation. This drew attention to the fact that nutrient and ionic requirements differ during all these different stages. Inappropriate culture conditions expose embryos to cellular stress which could result in retarded cleavage, cleavage arrest, cytoplasmic blebbing, impaired energy production, inadequate genome activation and gene transcription. Blastocyst formation is followed by an exponential increase in protein synthesis, with neosynthesis of glycoproteins, histones and new surface antigens.

Although the specific needs of embryos during their preimplantation development have by no means been completely defined, sequential, stage-specific and chemically defined media are now being developed for use in IVF systems. These endeavour to mimic the natural in vivo situation and take into account the significant changes in embryo physiology and metabolism that occur during the preimplantation period.

Fertilization is invariably successful in very simple media such as Earle's, or a TALP-based formulation, but the situation becomes more complex thereafter (see chapter 4 for details of embryo metabolism).

Serum

Prepared and filtered maternal serum has traditionally been added to IVF culture media, and preparations of human (or bovine) serum albumin were found to be effective replacements during fertilization and cleavage (although blastocyst development was apparently impaired in the absence of maternal serum). Maternal serum is routinely heat-treated to inactivate potentially harmful complement, but this might also destroy beneficial factors and/or create embryotoxic compounds.

Albumin is the major protein constituent of the embryo's environment; it can be incorporated by the embryo, it binds lipids and may help to bind and stabilize growth factors. Theoretically it may not be indispensable, but it does effectively replace serum, and has a major role both in maintaining embryo quality and in preventing gametes and embryos from sticking to glass or plastic surfaces, facilitating their manipulation. Commercial preparations can be highly variable in quality, and recent concerns about blood product transmission of pathogenic agents such as hepatitis and HIV viruses and

Creutzfeld–Jacob disease has made reliable sources of human serum albumin (HSA) increasingly difficult to find. Recombinant technology is now increasingly applied to commercial production of a number of physiological proteins, and recombinant HSA may be available in the future.

In domestic animals, serum has been found to induce a wide range of abnormalities in vitro, and its presence has been associated with the development of abnormally large fetuses (Thompson et al., 1995). Although the mechanisms involved are unresolved, the findings have led to further concerns about the use of serum in human IVF (see chapter 6).

Serum-free media have been introduced, using substitutes such as polyvinyl alcohol (PVA) and hyaluronate to replace albumin. PVA is a synthetic polymer, and, although it has been shown to support human IVF culture (Rinehart et al., 1998), it is a nonphysiological compound, and any long-term effects or consequences may remain undetected for some time. Hyaluronate is a glycosaminoglycan found at increased levels in the uterus around the time of implantation (Zorn et al., 1995), and the human embryo expresses its receptor throughout preimplantation development (Campbell et al., 1995). It is also known to form an antiviral and anti-immunogenic layer around the embryo, to increase angiogenesis, and to facilitate the rapid diffusion of transfer medium into the viscous uterine fluid environment. It may also be involved in the initial phases of attachment of the blastocyst to the endometrium. Hyaluronate has no protein moieties, and can therefore be synthesized and isolated in a pure form. Preliminary trials indicated that its presence not only replaced albumin in culture, but also significantly increased the implantation rate of resultant mouse blastocysts (Gardner, 1999).

Growth factors

Growth factors play a key role in growth and differentiation from the time of morula/blastocyst transition. However, defining their precise role and potential for improving in vitro preimplantation development is complicated by factors such as gene expression both of the factors and their receptors. There is also the potential of ascribing positive effects to specific factors when the result may in fact be due to a combination of a myriad of other causes. The mammalian blastocyst expresses ligands and receptors for several growth factors, many of which can cross-react, making it difficult to interpret the effects of single factors added to a medium. Insulin, LIF, EGF/TGFα, TGFβ, PDGG, HB-EGF have all been studied in IVF culture, and, although it is clear that these and other growth factors can show an influence on in vitro

blastocyst development and hatching, further assessment remains an area of research – a comprehensive review was published by Kane et al. in 1997. It has been suggested that the mechanism whereby serum induces abnormalities in domestic animal systems may involve the overexpression of certain growth factors – there is no doubt that complex and delicate regulatory systems are involved.

Culture of embryos in 'groups' rather than singly has been found to improve viability and implantation in some systems: it is possible that autocrine/paracrine effects or 'trophic' factors exist between embryos. However, observed effects of 'group' culture will inevitably be related to the composition of the culture medium and the precise physical conditions used for embryo culture, especially the ratio of embryo: medium volume.

Follicular flushing

Ideally, if a patient has responded well to follicular phase stimulation with appropriate monitoring and timing of ovulation induction by human chorionic gonadotrophin (hCG) injection, the oocyte retrieval may proceed smoothly with efficient recovery of oocytes without flushing the follicles. If the number of follicles is low or the procedure is difficult for technical reasons, follicles may be flushed with a physiological solution to assist recovery of all the oocytes present. Balanced salt solutions such as Earle's (EBSS) may be used for follicular flushing, and heparin may be added at a concentration of 2 units/ml. HEPES buffered media can also be used for flushing. Temperature and pH of flushing media must be carefully controlled, and the oocytes recovered from flushing media subsequently washed in culture media before transfer to their final culture droplet or well.

Quality control procedures

IVF laboratories should have an effective Total Quality Management (TQM) system that can monitor all procedures and components of the laboratory. This must include not only pregnancy and implantation rates, but also a systematic check and survey of all laboratory material, supplies, equipment and instruments. Standard Operating Procedures should be available to all laboratory personnel, which describe not only the detailed method to be applied to each procedure, but also products, equipment and relevant quality control measures, with quality standards or specifications for each aspect of the testing procedure. All equipment must have a clear and readily accessible

manual, together with a log-book of maintenance, servicing, and routine checks. A system that monitors individual performance of members of the team, with regular appraisal, is also helpful in maintaining an optimal standard of results.

The ultimate test of quality control must rest with pregnancy and live birth rates per IVF treatment cycle. An ongoing record of the results of fertilization, cleavage, and embryo development provide the best short-term evidence of good quality control (QC). Daily records in the form of a laboratory log-book are essential, summarizing details of patients and outcome of laboratory procedures: age, cause of infertility, stimulation protocol, number of oocytes retrieved, semen analysis, sperm preparation details, insemination time, fertilization, cleavage, embryo transfer and cryopreservation. It is also essential to record details of media and oil batches for reference, along with the introduction of any new methods or materials used. A range of bioassays to detect toxicity and suboptimal culture conditions have been tried, such as human sperm survival, hamster sperm survival, somatic cell lines, and the culture of mouse embryos from either the 1-cell or 2-cell stage. The validity of a mouse embryo bioassay has been questioned as a reliable assay for extrapolation to clinical IVF: it assumes that the requirements of human and mouse embryos are the same, and we know that this is a false assumption. The mouse embryo cannot regulate its endogenous metabolic pool before the late 2-cell stage; this is not the case for human or bovine embryos. Mouse embryos will develop from the 2-cell stage onwards in a very wide variety of cell culture media, without discrimination. Whilst none of the systems currently available can guarantee the detection of subtle levels of toxicity, they can be helpful in identifying specific problems. Any bioassay done routinely and frequently with baseline data for comparing deviations from the norm will be helpful in minimizing the random introduction of contaminants into the system, and is a useful investment of time and resources in an IVF lab.

New batches of media, oil, material or supplies used in the culture system, if not pretested, should be tested before use, and in routine IVF culture a normal fertilization rate in the order of $>70\%$ and cleavage rate of $>95\%$ is expected. The cleavage rate is important, as a block at the 2PN stage indicates a serious problem. At least 65% of inseminated oocytes should result in cleaved embryos on Day 2. The physico-chemical limits of culture media testing are also crucial: osmolarity must be within the limits of 275 and 305 mOsm, with a total variation of no more than 30 mOsm. pH must be within the limits of 7.2–7.5, with a maximal variation of 0.4 units of pH. Larger variations in either parameter indicate poor technique/technology and

inadequate controls during manufacture, leading to poor reproducibility.

Tissue culture plastics have on occasion been found to be subject to variation in quality: even within a single 4-well plate, well to well variations have been observed, and rinsing plates with media before use may be a useful precaution. Studies have shown that oil can interact with different plastic supports, and this can affect embryo development. Manufacturers of plasticware used for tissue culture may change the chemical formulation of their products without notification, and such changes in manufacture of syringes, filters and culture dishes may sometimes be embryotoxic. Embryos are very sensitive quality control indicators: firstly, in any block at the 2PN stage, and, secondly, in the appearance of the blastomeres and the presence of fragmentation. The early human embryo should be bright and clear, without granules in the cytoplasm. During cell division, the nucleocytoplasmic ratio is important. If osmolarity of the culture medium is low, the size of the embryo increases relative to the volume of the cytoplasm, and cytoplasmic blebs are formed in order to compensate and reach the adequate N/C ratio for entering mitosis. However, in doing so, the embryo loses not only cytoplasm but also mRNA and proteins, which are necessary for further development.

Suggested useful routine QC procedures include the following:

1. *Sperm survival test*

Select a normal sample of washed prepared spermatozoa and assess for count, motility and progression. Divide the selected sample into 4 aliquots: add test material to 2 aliquots, and equivalent control material (in current use) to 2 aliquots. Incubate one control and one test sample at 37°C, and one of each at room temperature. Assess each sample for count, motility and progression after 24 and 48 hours (a computer-aided system can be used if available). Test and control samples should show equivalent survival. If there is any doubt, repeat the test.

2. *Culture of surplus oocytes*

Surplus oocytes from patients who have large numbers of oocytes retrieved may be used to test new culture material. Culture at least 6 oocytes in the control media, and a maximum of 4 in test media.

3. *Multipronucleate embryo culture*

Oocytes which show abnormal fertilization on day 1 after insemination can be

used for testing new batches of material. Observe, score and assess each embryo daily until Day 6 after insemination.

4. Culture of 'spare' embryos

Surplus embryos after embryo transfer which are not suitable for freezing can also be used for testing new culture material. Observe, score, and assess each embryo daily as above. Embryo development to the blastocyst stage is regarded as evidence of adequate culture conditions, and blastocysts assessed to be of good quality may be cryopreserved.

Tissue culture systems

Vessels successfully used for in vitro fertilization include test tubes, 4-well culture dishes, organ culture dishes and Petri dishes containing microdroplets of culture medium under a layer of paraffin or mineral oil. Whatever the system employed, it must be capable of rigidly maintaining fixed stable parameters of temperature, pH and osmolarity. Human oocytes are extremely sensitive to transient cooling in vitro, and modest reductions in temperature can cause irreversible disruption of the meiotic spindle, with possible chromosome dispersal. Analyses of embryos produced by IVF have shown that a high proportion are chromosomally abnormal, and it is possible that temperature-induced chromosome disruption may contribute to the high rates of preclinical and spontaneous abortion that follow IVF and gamete intrafallopian transfer (GIFT). Therefore, it is essential to control temperature fluctuation from the moment of follicle aspiration, and during all oocyte and embryo manipulations, by using heated microscope stages and heating blocks or platforms.

An overlay of equilibrated oil as part of the tissue culture system confers specific advantages:

1. The oil acts as a physical barrier, separating droplets of medium from the atmosphere and airborne particles or pathogens.
2. Oil prevents evaporation and delays gas diffusion, thereby keeping pH, temperature and osmolality of the medium stable during gamete manipulations, protecting the embryos from significant fluctuations in their microenvironment.
3. Oil prevents evaporation: humidified and pre-equilibrated oil allows the use of nonhumidified incubators, which are easier to clean and maintain.

It has been suggested that oil could enhance embryo development by removing lipid-soluble toxins from the medium; on the other hand, an oil overlay prevents free diffusion of metabolic by-products such as ammonia,

and accumulation of ammonia in culture media is toxic to the embryo. The use of an oil overlay also influences oxygen concentration in the medium, with resulting effects on the delicate balance of embryo metabolism; as mentioned previously, it can absorb and concentrate harmful volatile organic compounds.

Oil preparation

Mineral, paraffin or silicone oil should be sterile as supplied, and does not require sterilization or filtration. High temperature for sterilization may be detrimental to the oil itself, and the procedure may also 'leach' potential toxins from the container. Provo and Herr (1998) reported that exposure of mineral oil to direct sunlight for a period of 4 hours resulted in a highly embryotoxic overlay, and they recommend that washed oil should be shielded from light and treated as a photoreactive compound. Contaminants have been reported in certain types of oil. Washing procedures remove water-soluble toxins, but non water-soluble toxins may also be present which will not be removed by washing. Therefore it is prudent not only to wash, but to test every batch of oil before use with, at the very least, a sperm survival test as a quality control procedure. In 1995, Erbach et al. suggested that zinc might be a contaminant in silicone oil, and found that washing the oil with EDTA removed a toxicity factor that may have been due to the presence of zinc. Some mineral oil products may also contain preservatives such as alpha-tocopherol. Oil can be carefully washed in sterile disposable tissue culture flasks (without vigorous shaking) with either Milli-Q water, sterile saline solution or a simple culture medium without protein or lipid-soluble components, in a ratio of 5:1 oil:aq. The oil can be further 'equilibrated' by bubbling 5% CO_2 through the mixture before allowing the phases to separate and settle. Washed oil can be stored either at room temperature or at 4°C in equilibrium with the aqueous layer, or separated before storage, but should be prepared at least 2 days prior to its use. Oil overlays must be further equilibrated in the CO_2 incubator for several hours (or overnight) before introducing media/gametes/embryos.

Serum supplements

Commercially prepared media are supplied complete, and do not require the addition of any supplements; most contain a serum substitute such as

'albuminar', human serum albumin. If maternal serum is used in the culture system, it must be homologous; pooled or donor sera are not recommended, even after thorough viral screening.

Preparation of maternal serum

Collect 20 ml of the patient's blood by venepuncture, maintain in an ice bucket, and spin immediately, before the sample clots. Remove the supernatant serum, and leave it to clot for approximately 30–60 minutes. Remove the clot by compressing it around a Pasteur pipette, and heat inactivate the serum at 56 °C for 45 minutes. Cool, and then filter through two millipore filters of 0.45 μm and 0.22 μm in sequence. Store at 4 °C. Maternal serum must not be used in cases of immunological or idiopathic infertility, or in cases with a previous history of unexplained failed fertilization.

Basic equipment required for the IVF laboratory

Embryology

CO_2 incubator
Dissecting microscope
Inverted microscope
Heated surfaces for microscope and manipulation areas
Heating block for test tubes
Laminar flow cabinet
Oven for heat-sterilizing
Small autoclave
Water bath
Pipette 10–100 μl Eppendorf
Pipette 20–1000 μl Eppendorf
Refrigerator
Supply of medical grade CO_2

Supply of 5% CO_2 in air
Wash bottle + Millex filter for gas
Rubber tubing
Pipette canisters
Mineral or paraffin oil
Culture media

Glassware for media preparation
Osmometer (for media preparation)
Weighing balance
Millipore Bell filter unit for filtering media

Tissue culture plastics: (Nunc, Corning, Sterilin)
Flasks for media and oil: 50 ml, 175 ml
Culture dishes: 60, 35 mm
OCR (oocyte retrieval) needles
Test-tubes for OCR: 17 ml disposable
Transfer catheters: embryo, GIFT, IUI
Syringes
Needles
Disposable pipettes: 1, 5, 10, 25 ml
'Pipetus' pipetting device
Eppendorf tips, small and large
Millipore filters: 0.22, 0.8 μm
Glass Pasteur pipettes (Volac)
Pipette bulbs
Test-tube racks
Spirit burners + methanol or gas Bunsen burner
Rubbish bags
Tissues
Tape for labelling
7X detergent (Flow)
70% ethanol
Sterile gloves

Oil: Boots, Squibb, Sigma, Medicult
Supply of purified water: Milli-Q system or Analar
Glassware for making culture media: beakers, flasks, measuring cylinder

Details of IVF media can be obtained from the following manufacturing companies:

Medicult: Møllehaven 12, DK-4040 Jyllinge, Denmark
Scandinavian IVF Science AB, Mölndalsvägen 30A, PO Box 14105, Göthenburg, Sweden
Ham's F-10, EBSS: Flow Laboratories, UK
HTF: Irvine Scientific, USA

Cook IVF: 12 Electronics Street, Brisbane Technology Park, Eight Mile Plains, Queensland 4113, Australia
Sage BioPharma, Inc. 944 Calle Amanecer, Suite L., San Clemente, CA 92673, USA

Further reading

Almeida, P.A. & Bolton, V.N. (1996) The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote* **3**:357–65.

Angell, R.R., Templeton, A.A. & Aitken, R.J. (1986) Chromosome studies in human in vitro fertilisation *Human Genetics* **72**:333–9.

Ashwood-Smith, M.J., Hollands, P. & Edwards, R.G. (1989) The use of Albuminar (TM) as a medium supplement in clinical IVF. *Human Reproduction* **4**:702–5.

Augustus, D. (1996) Cell culture incubators: tips for successful routine maintenance. *Alpha Newsletter* vol. 4, April 1996.

Bavister, B.D. (1995) Culture of preimplantation embryos: facts and artifacts. *Human Reproduction update* **1(2)**: 91–148.

Bavister, B.D. & Andrews, J.C. (1988) A rapid sperm motility bioassay procedure for quality control testing of water and culture media. *Journal of In Vitro Fertilization and Embryo Transfer* **5**:67–8.

Boone, W.R., Johnson, J.E., Locke, A.J., Crane, M.M. & Price, T.M. (1999) Control of air quality in an assisted reproductive technology laboratory. *Fertility and Sterility* **71**:150–4.

Campbell, S., Swann, H.R., Aplin, J.D., Seif, M.W., Kimber, S.J. & Elstein, M. (1995) CD44 is expressed throughout pre-implantation human embryo development. *Human Reproduction* **10**:425–30.

Cohen, J., Gilligan, A., Esposito, W., Schimmel, T. & Dale, B. (1997) Ambient air and its potential effects on conception *in vitro*. *Human Reproduction* **12(8)**:1742–9.

Danforth, R.A., Piana, S.D. & Smith, M. (1987) HIgh purity water: an important component for success in in vitro fertilization. *American Biotechnology Laboratory* **5**:58–60.

Davidson, A., Vermesh, M., Lobo, R.A. & Paulsen, R.J. (1988) Mouse embryo culture as quality control for human in vitro fertilisation: the one-cell versus two-cell model. *Fertility and Sterility* **49**:516–21.

Dumolin, J.S., Menheere, P.P., Evers, J.L., Kleukers, A.P., Oueters, M.H., Bras, M. & Geraedts, J.P. (1991) The effects of endotoxins on gametes and preimplantation embryos cultured in vitro. *Human Reproduction* **6**:730–4.

Edwards, R.G. & Brody, S.A. (1995) Human fertilization in the labortory. In: *Principles and Practice of Assisted Human Reproduction*. W.B. Saunders & Co., Philadelphia, Pennsylvania, 351–413.

Elder, K. & Elliott, T., (1998) Troubleshooting and Problem Solving in IVF. *Worldwide Conferences on Reproductive Biology*. Ladybrook Publishing, Australia.

Elliott, T. & Elder, K. (eds.) (1997) Blastocyst culture, transfer, and freezing. *Worldwide Conferences on Reproductive Biology*. Ladybrook Publishing, Australia.

Erbach, G.T., Bhatnagar.,P., Baltz, J.,M. & Biggers, J.D. (1995) Zinc is a possible toxic contaminant of silicone oil in microdrop cultures of preimplantation mouse embryos. *Human Reproduction* **10**:3248–54.

Fleetham, J. & Mahadevan, M.M. (1988) Purification of water for in vitro fertilization and embryo transfer. *Journal of In Vitro Fertilization and Embryo Transfer* **5**:171–4.

Fleming, T.P., Pratt, H.P.M. & Braude, P.R. (1987) The use of mouse preimplantation embryos

for quality control of culture reagents in human in vitro fertilisation programs: a cautionary note. *Fertility and Sterility* **47**:858–60.

Gardner, D. (1999) Development of new serum-free media for the culture and transfer of human blastocysts. *Human Reproduction* **13**, **Suppl. 4**:218–25.

George, M.A., Braude, P.R., Johnson, M.H. & Sweetnam, D.G. (1989) Quality control in the IVF laboratory: in vitro and in vivo development of mouse embryos is unaffected by the quality of water used in culture media. *Human Reproduction* **4**:826–31.

Johnson, C., Hofmann, G. & Scott, R. (1994) The use of oil overlay for in vitro fertilisation and culture. *Assisted Reproduction Review* **4**:198–201.

Kane, M.T., Morgan, P.M. & Coonan, C. (1997) Peptide growth factors and preimplantation development. *Human Reproduction Update* **3(2)**: 137–57.

Ma, S., Kalousek, D.K., Zouves, C., Yuen, B.H., Gomel, V. & Moon, Y.S. (1990) The chromosomal complements of cleaved human embryos resulting from in vitro fertilisation. *Journal of In Vitro Fertilization and Embryo Transfer* **7**:16–21.

Marrs, R.P., Saito, H., Yee, B., Sato, F. & Brown, J. (1984) Effect of variation of in vitro culture techniques upon oocyte fertilization and embryo development in human in vitro fertilization procedures. *Fertility and Sterility* **41**:519–23.

Matson, P.L. (1998) Internal and external quality assurance in the IVF laboratory. *Human Reproduction Supplement* **13**, Suppl. 4:156–65.

Ménézo, Y. (1998) Why culture media should mimic nature. *Alpha Newsletter* **13**:8.

Mortimer, D. & Quinn, P. (1996) Bicarbonate-buffered media and CO_2. *Alpha Newsletter* vol. 4, April 1996.

Naaktgeboren, N. (1987) Quality control of culture media for in vitro fertilisation. *In Vitro Fertilisation Program*, Academic Hospital, Vrije Universiteit, Brussels, Belgium. Annal Biologie Clinique (Paris) **45**:368–72.

Pearson, F.C. (1985) *Pyrogens*: *Endotoxins*, *LAL Testing*, *and Depyrogenation.* Marcel Dekker, Inc., pp. 98–100, 206–11.

Pellestor, F., Girardet, A., Andreo, B., Anal, F. & Humeau, C. (1994) Relationship between morphology and chromosomal constitution in human preimplantation embryos. *Molecular and Reproductive Development* **39**:141–6.

Pickering, S.J., Braude, P.R., Johnson, M.H., Cant, A. & Currie, J. (1990) Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertility and Sterility* **54**:102–8.

Plachot, M., De Grouchy, J., Montagut, J., Lepetre, S., Carle, E., Veiga, A., Calderon, G., Santalo, J. (1987) Multicentric study of chromosome analysis in human oocytes and embryos in an IVF programme *Human Reproduction* **2**:29.

Provo, M.C. & Herr, C. (1998) Washed paraffin oil becomes toxic to mouse embryos upon exposure to sunlight. *Theriogenology* **49(1)**:214.

Purdy, J. (1982) Methods for fertilization and embryo culture in vitro. In: (Edwards, R.G. & Purdy, J.M. eds.) *Human Conception In Vitro*. Academic Press, London, p. 135.

Quinn, P., Warner, G.M., Klein, J.F. & Kirby, C. (1985) Culture factors affecting the success rate of in vitro fertilization and embryo transfer. *Annals of the New York Academy of Sciences* **412**:195.

Rinehart, J.S., Bavister, B.D. & Gerrity, M. (1988) Quality control in the in vitro fertilization laboratory: comparison of bioassay systems for water quality. *Journal of In Vitro Fertilization and Embryo Transfer* **5**:335–42.

Rinehart, J., Chapman, C., McKiernan, S. & Bavister, B. (1998) A protein-free chemically defined embryo culture medium produces pregnancy rates similar to human tubal fluid (HTF) supplemented with 10% synthetic serum substitute (SSS). Abstracts of the 14th Annual

meeting of the ESHRE, Göteborg 1998. *Human Reproduction* **13**:59.

Staessen, C., Van den Abbeel, E., Carle, M., Khan, I., Devroey, P. & Van Steirteghen, A.C. (1990) Comparison between human serum and Albuminar-20(TM) supplement for in vitro fertilization. *Human Reproduction* **5**:336–41.

Thompson, J.G., Gardner, D.K., Pugh, A., MacMillan, W. & Teruit, J.J.R. (1995) Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biology of Reproduction* **53**:1385–91.

Wales, R.G. (1970) Effect of ions on the development of preimplantation mouse embryos in vitro. *Australian Journal of Biological Sciences* **23**:421–9.

Weimer, K.E., Anderson, A. & Stewart, B. (1998) The importance of water quality for media preparation. *Human Reproduction Supplement* **13, Suppl. 4**:166–72.

Yovich, J.L., Edirisinghe, W., Yovich, J.M., Stanger, J. & Matson, P. (1988) Methods of water purification for the preparation of culture media in an IVF-ET programme. *Human Reproduction* **3**:245–8.

Zorn, T.M.T., Pinhal, M.A.S., Nader, H.B., Carvalho, J.J., Abrahamsohn, P.A. & Diedrich, C.P. (1995) Biosynthesis of glucosaminoglycans in the endometrium during the initial stages of pregnancy of the mouse. *Cell Molecular Biology* **41**:97–106.