Human Reproduction vol.15 no.10 pp.2241–2246, 2000

ESHRE guidelines for good practice in IVF laboratories

Luca Gianaroli[1], Michelle Plachot, Roelof van Kooij, Safaa Al-Hasani, Karin Dawson, Anick DeVos, M.Cristina Magli, Jacqueline Mandelbaum, Jacqueline Selva and Wouter van Inzen (Committee of the Special Interest Group on Embryology)

[1]To whom correspondence should be addressed at: SISMER Reproductive Medicine Unit, V. Mazzini 12, 40138 Bologna, Italy

Education has always been a priority for the European Society of Human Reproduction and Embryology (ESHRE). Many efforts have been dedicated to promoting knowledge of techniques, procedures and strategies in order to ensure use of the highest quality practices in reproductive medicine. The need to develop a set of guidelines was a logical consequence that found its first expression in 1990, when *Focus on Reproduction* (vol. 1, pp. 10–38) published the first guidelines which were distributed among the membership. Five years later a new, more complete edition with several novel techniques and developments appeared in *Human Reproduction* (vol. 10, pp. 1246–1271). Both have proved to be invaluable references. Five more years have now passed. The necessity to produce current guidelines for good IVF laboratory practice has provided the strongest motivation. This originated from the increasing awareness that embryologists have a duty to prevent unintentional incidents that might result from poor practice in the laboratory. Therefore, the Embryology Special Interest Group (SIG) undertook to draw up guidelines aimed at giving support and guidance to the laboratory staff. All the aspects required to provide a safe working system were taken into consideration by members of the SIG and their effort produced this document. We hope that it will assist staff in achieving the best clinical outcome for their patients.

Key words: assisted reproduction techniques/handling gametes and embryos/laboratory safety/quality control

Introductory remarks

Clinical embryology has put into practice in the laboratories for assisted reproduction techniques (ART) the recent discoveries regarding the physiology of early events in the development of human life. The embryologist is responsible for the correct and justified application of ART in these laboratories.

The European Society of Human Reproduction and Embryology (ESHRE) wishes to give guidelines which constitute the minimal requirements for any laboratory offering ART, with the aim of implementing a quality control system for all the embryologists of ESHRE. The strict application and further development of these guidelines will benefit all patients attending ART clinics.

A laboratory safety programme depends on every employee's participation and cooperation. Non-compliance with safety precautions not only endangers the individual, but often compromises the health and safety of fellow workers or patients and may result in damage or loss of gametes and/or embryos. Relevant patient consent should be verified before commencing any procedure.

1. Staffing and direction

1.1. The laboratory is directed by an appropriately qualified and experienced scientist or medical doctor according to national rules.
1.2. There are appropriate numbers of staff with the required experience to undertake the workload of the laboratory.
1.3. All new staff are given a comprehensive orientation and introduction programme.
1.4. Continuing medical education (CME) is recommended for all personnel involved.
1.5. Individual responsibility of each member of staff and line of responsibilities are indicated in a written procedure.

2. Policies and procedures

2.1. All laboratory procedures must include provision for unique patient identification whilst retaining patient confidentiality.
2.2. Laboratory results should be reported according to a written procedure. They should be validated, dated and include unique patient identity. Any interpretation of results should be accurate, comprehensive and clinically relevant.
2.3. There is a record of all reagents, calibration and quality control material.
2.4. There is a written, signed and dated protocol for every procedure, written transmission of results and regular maintenance of equipment.
2.5. All the procedures should be gathered in a manual kept in the laboratory and available for consultation.
2.6. A log book should be maintained to permit a regular evaluation of the results.

3. Laboratory safety

3.1. Laboratory design

The embryology laboratory should have adequate space to follow good laboratory practice. More specifically:

- The construction of the laboratory should ensure aseptic and optimal handling of gametes and pre-embryos during all phases of the treatment.
- The location of storage areas and equipment such as incubators, centrifuges and cryo equipment should be logically planned for efficiency and safety within each working area.
- Separate office space should be provided for administrative work, such as record keeping and data entry.
- A general wet area in which washing of equipment, sterilization, etc., is performed, should be separate from the embryo laboratory. Moreover, if fixatives are applied, these analyses should be performed in a separate room in a fume-hood.

When commissioning the laboratory, thought should be given to the most recent developments in equipment and facilities. Bench height, adjustable chairs, microscope eye height, efficient use of space and surfaces, sufficient air-condition and the amount of daylight, all contribute to a working environment that minimizes distraction and fatigue. Consideration should also be given to local health and safety requirements.

3.2. Laboratory equipment

- The laboratory equipment used should be adequate for laboratory work and easy to clean and disinfect.
- Critical items of equipment, including incubators and frozen embryo storage facilities, should be appropriately alarmed and monitored.
- All embryo laboratories should have an automatic emergency generator backup in the event of power failure.
- A minimum number of two incubators is recommended. Gas cylinders should be placed outside or in a separate room with an automatic backup system.
- Incubators should be frequently cleaned and sterilized. Nitrogen tanks should be cleaned and sanitized at least every year.

3.3. Infectious agents

All assisted reproductive technologies involve handling biological material, and pose a potential hazard of transmitting diseases to personnel. Sources of infection in this system are compounds and follicle fluid of animal origin. Each unit should establish procedures and policies for the safety of personnel, taking national and/or local safety regulations into consideration. Therefore:

- Vaccination of the personnel against hepatitis B or other available viral disease is recommended.
- Screening patients and gamete donors for HIV, hepatitis B/C and other sexually transmitted diseases before treatment or gamete cryopreservation should be routinely adopted.

Although patients' admittance to IVF treatment cycles is regulated by physicians, the laboratory staff must be informed about the risks of handling potentially infected biological material. The following recommendations should be considered:

- If one or both partners are HIV-positive, IVF and embryo transfer should not be attempted, unless local rules give authorization after extensive examination. Donor semen is advised for a seronegative woman if her partner is seropositive, as HIV-1 viral particles may be present in the cytoplasmic compartment of motile spermatozoa (Baccetti *et al*., 1994). Therefore, the possibility exists that viral particles enter the oocyte during fertilization either with conventional IVF or intracytoplasmic sperm injection (ICSI). The fate of these particles in the resulting embryo is unknown.
- If the male partner is infected by the virus of hepatitis B, the seronegative woman must be vaccinated before IVF.
- If the woman is infected by the virus of hepatitis B, IVF is feasible but the couple must be warned about the necessity of specific vaccination of the baby at birth. It has been recently suggested that the use of ICSI in hepatitis B-positive patients could increase the risk of bringing the virus into the oocyte from the surrounding fluid, possibly adhered to the sperm membrane.
- If one partner is infected by the virus of hepatitis C, IVF may be performed after extensive counselling.
- In case of seropositivity for syphilis, appropriate treatment must be given before IVF.

3.4. Protective measures

The purpose of the protective measures is also to ensure aseptic conditions for gamete and embryos. The procedures should deal with, but not be limited to, the following:

- Use of laboratory clothing.
- Use of non-toxic (non-powdered) gloves and masks.
- Use of eye and face protection if cryogenic materials are handled.
- Use of vertical laminar-flow benches.
- Use of mechanical pipetting devices.
- Use of fume-hood in case of fixatives.
- Disinfection and sterilization of potentially infected equipment.
- Use of disposable material; after usage, it must be discarded immediately in the proper waste containers. Potential infectious materials must be disposed of in a manner that protects laboratory workers and maintenance, service, and housekeeping staff from exposure to infectious materials in the course of their work.
- Needles and other sharps should be handled with extreme caution and discarded in special containers. If possible, glassware should be omitted in the laboratory, otherwise the Pasteur pipettes and broken glassware should be discarded in special containers.

Cross-contamination with infectious material from one patient to another could still occur during the cryopreservation procedure when straws with semen or embryos are filled by dipping the straw in patient medium with semen or embryos, sealing it and passing it into liquid nitrogen without external disinfection (Tedder *et al*., 1995). Material stored in the cryopreservation tanks should be kept in a way that avoids contact of the liquid nitrogen phase with the biological substances. Safety cryo straws have been conceived in order to fulfil this requirement. Specimen known to be contaminated should be stored in such high security straws.

4. Correct handling and identification of patients and their gametes and embryos

Written procedures should be present describing the various phases of IVF techniques. Rules concerning the correct handling and identification of gametes and embryo samples should be established by a system of checks and, where needed, double-checks. Proper training of all the laboratory staff according to these procedures is mandatory.

- All material obtained from the patients, i.e. tubes with blood, follicular fluid and spermatozoa, should bear unique identification of the treated couple.
- Incubators should be organized in order to facilitate identification of embryos, oocytes and spermatozoa.
- Verification of patients' identity should be performed at critical steps: before ovum retrieval, at semen recovery and embryo transfer procedures.
- Double checks need to be considered at least at: insemination of oocytes, replacement of embryos, embryo freezing and thawing.

Written validation of all critical steps in each patient's file is essential.

5. Culture media preparation and quality control testing

5.1. Disposables and reagents.

- Glassware and disposable items used for preparation of culture media should be of tissue culture grade and dedicated for this purpose alone.
- Culture media, or reagents used in their preparation, should be of a purity appropriate for the purpose.

5.2. Laboratory protocols should include in-house quality control procedures for culture media.

- All tissue culture media prepared in the laboratory should undergo quality control using an appropriate bioassay system.
- When commercially produced media are used, integrity of the packages and appropriate delivery conditions should be controlled.
- Documentation of quality control testing should be supplied by the manufacturer for any commercially produced media employed. Correspondence with the delivered batch should be verified.
- Reagents and media should always be used prior to the manufacturer's expiry date.

5.3. Water used for the preparation of culture media should be of appropriate purity.

- If the water is purified on site, protocols must be in place for quality control of the water system, including sanitization of the system, regular cartridge changes and replacement of other components, endotoxin and bacterial screening.
- If culture grade water is purchased there must be strict control over storage and use in relation to temperature and shelf-life.

5.4. There should be a systematic record of all batch numbers of reagents, media and disposables used in the preparation of media with the date of their introduction to the system.

- Reagents should be designated exclusively for use in culture media.

5.5. Donor serum or follicular fluid are not recommended. If they are used in culture media they should be screened according to the local rules for blood donors.

- Human serum albumin (HSA) should come from a source screened as above. Commercial suppliers of HSA or media containing a serum derived protein source should supply evidence of such screening.
- Appropriate quarantining arrangements should be in place for donor use.

5.6. Oocytes may be cultured under equilibrated mineral oil. The oil maintains temperature, osmotic pressure and pH during short-term manipulations of the oocytes. Before use, it requires pre-equilibration in the incubator.

6. Handling of oocytes and spermatozoa

The laboratory procedures regarding the handling of gametes for assisted reproduction techniques have been standardized to a large extent. The procedures should be easy, simple and effective and must be performed in a laminar flow hood equipped with stages and heating blocks pre-warmed at 37°C.

Class II hoods should be preferred as they give protection to the operator. Disposable items used in the laboratory preparation of culture media should be of tissue culture grade.

6.1. Aseptic technique should be used at all times.

6.2. Appropriate measures should be taken to ensure that oocytes and embryos are maintained at 37°C during handling/observation using hot plates or other systems.

6.3. Tissue culture grade disposables should be used for handling body fluids/cells.

6.4. Pipetting devices (pasteurs, drawn pipettes, tips etc.) should be used for one procedure only, should never be used for more than one patient, and should be disposed of immediately after use.

6.5. Simultaneous treatment of more than one patient should never be done in the same working place.

6.6. Identifying information marked on the culture dish/tube should be cross-referenced to the patient and the patient's documentation.

- Procedures should be in place which ensure correct patient identification at all stages.
- Labelling of dishes/tubes containing oocytes, embryos, or spermatozoa should be permanent. (Strong odour markers should be avoided.)
- Incubators should be organized in order to facilitate identification of embryos, oocytes and spermatozoa.

6.7. At each stage of the procedure the identity of the embryologist should be recorded.

7. Oocyte retrieval

7.1. Oocytes need to be kept at body temperature (36–37°C) as much as possible. There should also be appropriate equipment in use to maintain this temperature when laboratory and egg collection areas are on different sites. Scanning Petri dishes, collection tubes and heating blocks are pre-warmed at 37°C. Follicular aspirates are checked for the presence of oocyte-cumulus complexes under a stereo dissecting micro-

scope with transmitted illumination base and heated stage. Exposure of oocytes to light should be minimized where possible.

7.2. Where donor oocytes are to be used they should be labelled with the donor's name, or reference code, at the time of oocyte retrieval, and only identified with the recipient's name at the time of allocation.

8. Sperm preparation

Before starting a treatment cycle, semen analysis is performed according to the protocols described in the World Health Organization manual (WHO, 1999).

The semen sample is collected in a sterile, plastic container without using condoms, creams or lubricants. The container should be clearly labelled with the name of the couple.

8.1. Records should be kept of the period of abstinence, the type of container used (if this differs from the norm), time and place of collection (with particular reference to samples produced outside clinic facilities), and the time interval between collection and preparation.

8.2. Where donor spermatozoa is used, the necessary identifying information (donor code/clinic code) must be recorded.

8.3. The method of sperm preparation should be recorded, including details of any variation on the standard laboratory protocol.

8.4. A record should be kept of pre- and post-preparation sperm parameters and of any dilution carried out prior to insemination.

8.5. In case of surgically retrieved spermatozoa, exceeding spermatozoa after insemination should be cryopreserved for further assisted reproductive cycles; this will avoid repeated surgery.

9. Insemination of oocytes

9.1. Preparation of spermatozoa for insemination.

The method of preparation is designed according to individual samples. A trial preparation prior to the treatment cycle may be advisable in order to choose the most adequate technique. Sperm preparation aims to:

- concentrate and select the active and motile spermatozoa;
- get rid of seminal plasma, debris and contaminants;
- select against abnormal forms.

A frozen backup sample may be requested for those patients who anticipate the possibility of collection difficulty.

Different methods have been proposed for sperm preparation. Among them, the swim-up technique and the discontinuous density–gradient centrifugation are the most used (Burkman *et al*., 1984; McClure *et al*., 1989; Van der Zwalmen *et al*., 1991).

As a general rule excessive centrifugation should be avoided especially in oligospermic samples in order to avoid increasing the concentration of reactive oxygen species.

Since January 1997 the Percoll that was used in the discontinuous density–gradient centrifugation procedure has been prohibited for clinical purposes.

The preparation of spermatozoa retrieved by microsurgical operations requires specific protocols (Devroey *et al*., 1994; Silber *et al*., 1994, 1995; Oates *et al*., 1997; Kupker *et al*., 1998).

9.2. A record should be kept of the time of insemination and the sperm concentration used. For conventional insemination, the number of spermatozoa must be high enough to yield the best fertilization rate without compromising embryo development.

9.3. For the intracytoplasmic sperm injection (ICSI) procedure, it is important to select vital spermatozoa, as evaluated by their motility. Where only non-vital sperm cells are present in the ejaculate, the use of testicular spermatozoa may be proposed.

9.4. Preparation of oocytes for ICSI. Removal of cumulus–corona (CC) cells.

Oocytes are denuded from the surrounding cumulus and corona cells using an enzymatic procedure with hyaluronidase, followed by mechanical denudation using a pipette with an inner diameter of ± 150 μm. Both the enzyme concentration and the duration of exposure to the enzyme should be limited (Van de Velde *et al*., 1998). Care needs to be taken in order to avoid damage to the oocytes, which can result from too vigorous pipetting or from a pipette diameter which is too small. After rinsing several times in washing medium drops, the denuded oocytes are observed under the inverted microscope at $\times 200$ magnification and cultured until the time of injection.

9.5. The injection procedure.

During ICSI, the following points are important:

- the selection and immobilization of a viable sperm cell;
- the correct positioning of the oocyte prior to injection;
- the rupture of the oolemma prior to the release of the sperm cell into the oocyte.

Polyvinylpyrrolidone (PVP) can be used to facilitate the manipulation of spermatozoa and to control the fluid in the injection pipette, limiting the volume injected into the oocyte.

10. Scoring for fertilization

10.1. All oocytes that have been inseminated or micro-injected should be examined for the presence of pronuclei at 16–20 h post-insemination.

10.2. The status of each oocyte should be recorded.

10.3. Oocytes with one or more than two pronuclei should be isolated from normally fertilized oocytes (Plachot *et al*., 1992).

10.4. Oocytes showing no signs of fertilization should be maintained in culture and observed for late appearance of pronuclei.

10.5. The normally fertilized oocytes are removed from the insemination medium and transferred into new dishes with pre-equilibrated fresh growth medium.

11. Embryo culture and embryo transfer

The scoring of embryos at regular intervals provides the possibility to the embryologist of tracking embryo development.

11.1. The stage of embryo development at the time of transfer should be documented. Embryos can be grown to day 5 or 6 for a transfer at the blastocyst stage. Sequential media (one medium from days 1–3 and a second one from days 3–5 or 6) allow the growth of blastocysts with a high implantation rate replacing the need for co-culture systems (Guerin and Nicollet, 1997; Gardner *et al*., 1998).

11.2. In some countries the maximum number of embryos transferred is established by the local legislation. As a general recommendation, it is advisable not to exceed two. In cases where more than two embryos are replaced, the couple has to be extensively informed on the risks of multiple gestations. When blastocysts are available, the number of blastocysts transferred should be limited to two. The supernumerary embryos may be cryopreserved, donated to research or discarded, according to the couple's wishes and to the national legislation.

11.3. The patient records for embryo transfer should include details of:

- batch number and type of media used for transfer;
- time from oocyte retrieval to transfer;
- time from oocyte insemination to transfer;
- the number and developmental stage of embryos at transfer;
- fate of excess embryos;
- type of catheter used for transfer.

11.4. If the laboratory is some distance from the embryo transfer room, arrangements should be made to maintain temperature and pH by the use of mobile equipment whilst transporting embryos.

11.5. Sterile disposable catheters should be used for transfer.

12. Cryopreservation of embryos

Embryo freezing can be performed at different stages: zygotes, embryos in early cleavage (day 2 or day 3), blastocysts. Embryos displaying a high degree of fragmentation or very slow or blocked cleavage rate should be discarded from storage procedures due to the low survival rate reported after thawing.

In some countries, the application of cryopreservation procedures is regulated by law.

12.1. Techniques and facilities for cryopreservation of embryos should be available in each IVF centre with the aim of:

- cryopreserving spare embryos after transfer;
- delaying embryo transfer in a subsequent cycle if the patient becomes ill or develops ovarian hyperstimulation syndrome (OHSS);
- storing the embryos generated from donated oocytes in order to allow a 6 month quarantine so that the potential donors may be controlled for infectious diseases prior to embryo transfer.

12.2. Several protocols have been formulated depending on the embryo cellular stage, type of cryoprotectant, and speed of cooling (Testart *et al*., 1986; Ménézo *et al*., 1992; Veeck *et al*., 1993).

12.3. In order to minimize any risk of transmission of infection via liquid nitrogen, embryos should be stored in specific receptacles (i.e. straws, vials etc.) that can be sealed effectively.

- Transfer of embryos to receptacles should be by a method which avoids contamination of the external surface.
- Sealing should be checked carefully before freezing.

12.4. Patients having transfer of thawed embryos ideally should be screened for hepatitis B and C, and HIV.

- When a patient is known to be a source of infection risk, a system of separate storage should be considered.

12.5. Documentation on stored embryos should include:

- the type and batch number of cryoprotectant used;
- the stage of embryo development;
- the number of embryos in each straw/vial, which should not exceed two;
- the number of straws/vials stored per patient.

12.6. Straws/vials containing embryos must be clearly and permanently labelled with reference to patient details and their unique identification code.

12.7. Storage records should be kept in both the patient's individual records and the storage records for individual nitrogen banks.

12.8. An annual audit of stored gametes and embryos must be carried out, cross referencing contents with storage records.

12.9. Storage records should include precise details of the location of the vials/straws.

12.10. Documentation of thawing procedures should include morphological changes seen during thawing and the time period of culture prior to transfer.

13. Assisted hatching

This technique has been designed with the aim of improving embryo hatching and possibly implantation. However there are conflicting reports about its clinical efficacy.

13.1. Three methods are being used; the mechanical technique, that is partial zona dissection with glass microneedles (Cohen *et al*., 1990), the chemical assisted hatching using acidic Tyrode's (Cohen *et al*., 1992), and the laser assisted hatching (Strohmer and Feichtinger, 1992).

13.2. Special care should be taken to avoid damage to the embryo during the procedure.

14. Preimplantation genetic diagnosis

The purpose of the procedure is to identify embryos, generated *in vitro*, which carry genetic or chromosomal abnormalities and exclude them from transfer.

14.1. Genetic counselling should be available to all 'at risk' couples.

14.2. The biopsy procedure is carried out by:

- polar body removal (Verlinsky *et al*., 1996);
- single or double blastomere biopsy at the day 3 stage (Tarin and Handyside, 1993);
- trophectoderm biopsy at the blastocyst stage (still under investigation).

14.3. The cells destined to genetic investigation are removed in the IVF laboratory using glass microtools on a micromanipulation set. All cells and embryos for genetic investigation are individually handled, carefully identified and labelled, and tracked during the whole procedure.

14.4. Special care should be taken to avoid damage to the embryo during the procedure. In addition, when blastomere biopsy is performed, integrity of the removed cell is extremely important for the correctness of the genetic analysis.

14.5. The biopsy sample should be subjected to diagnostic procedures in a separate specialist laboratory.

Although applied in a few centres world-wide and not allowed by law in some countries, the results obtained demonstrate that the procedure does not negatively compromise embryo development and implantation despite its invasiveness (Gianaroli *et al*., 1999). The advantages derived from its application are:

- an alternative to therapeutic abortion due to a minimized risk of transferring affected embryos;
- an increased take-home baby rate associated to the transfer of euploid embryos.

15. Quality control using evaluation of results

Results should be evaluated on a regular basis. In order to prevent bias due to patient variation for the purpose of quality control, a group should be selected where certain criteria can be established. The following should be regularly analysed:

- fertilization rates;
- embryo quality;
- pregnancy rates;
- multiple pregnancy rates;
- implantation rates.

For a complete evaluation of the results, this analysis should be performed in collaboration with the clinical staff.

Future prospects

Novel techniques have been proposed during the last few years aimed at expanding ART indications: freezing of oocytes and ovaries, in-vitro maturation of oocytes, intracytoplasmic transfer, spermatid injection, embryo implantation. Reports continue to accumulate from animal studies and preliminary data. However, additional studies are necessary before admitting these procedures to the standard technique protocols which are routinely performed in IVF laboratories.

Acknowledgements

Some sections of these guidelines follow those originally published by the UK Association of Clinical Embryologists, in conjunction with Clinical Pathology Accreditation (UK), and we are happy to acknowledge their value to us in drawing up the ESHRE guidelines.

References

Baccetti, B., Benedetto, A., Burrini, A.G. *et al.* (1994) HIV-particles in spermatozoa of patients with AIDS and their transfer into the oocyte. *J. Cell. Biol*., **127**, 903–914.

Burkman, L.J. (1984) Characterisation of hyperactivated motility by human spermatozoa during capacitation: comparison of fertile and oligozoospermic sperm population. *Arch. Androl.,* **13**, 152–165.

Cohen, J. (1992) Zona pellucida micromanipulation and consequences for embryonic development and implantation. In Cohen, J., Malter, H.E., Talansky, B.E. and Grifo, J. (eds) *Micromanipulation of human gametes and embryos*. Raven Press, New York, pp. 191–222.

Cohen, J., Elsner, C., Kort, H. *et al*. (1990) Impairment of the hatching process following IVF in the human and improvement of implantation by assisted hatching using micromanipulation. *Hum. Reprod*., **5**, 7–13.

Devroey, P., Liu, J., Nagy, Z. *et al.* (1994) Normal fertilization of human oocytes after testicular sperm extraction and intracytoplasmic sperm injection. *Fertil. Steril.*, **62**, 639–641.

Gardner, D.K., Schoolcraft, W.B., Wagley, L. *et al*. (1998) A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum. Reprod*., **13**, 3434–3440.

Gianaroli, L., Magli, M.C., Munné, S. *et al*. (1999) Advantages of day four embryo transfer in patients undergoing preimplantation genetic diagnosis of aneuploidy. *J. Assist. Reprod. Genet.*, **16**, 148–152.

Guerin, J.F. and Nicollet, B. (1997) Interest of co-cultures for embryos obtained by in-vitro fertilization: a French collaborative study. *Hum. Reprod*., **12**, 1043–1046.

Kupker, W., Al Hasani, S., Bals-Pratsch, M. *et al.* (1998) Cryo TESE – the use of frozen–thawed testicular sperm for ICSI. *16th World Congress on fertility and sterility*, October 4–9, San Francisco, USA.

McClure, R.D., Nunes, L. and Tom, R. (1989) Semen manipulation: improved sperm recovery and function with a two layer Percoll gradient. *Fertil. Steril.*, **51**, 874–877.

Ménézo, Y., Nicollet, B., Herbaut, N. *et al.* (1992) Freezing co-cultured human blastocysts. *Fertil. Steril.*, **58**, 977–980.

Oates, R.D., Mulhall, J., Burgess, C. *et al*. (1997) Fertilization and pregnancy using intentionally cryopreserved testicular tissue as the sperm source for ICSI in 10 men with non-obstructive azoospermia. *Hum. Reprod*., **12**, 734–739.

Plachot, M. and Crozet, N. (1992) Fertilization abnormalities in human IVF. *Hum. Reprod*., **7** (suppl.), 1, 89–94.

Silber, S.J., Nagy, Z.P., Liu, J. *et al*. (1994) Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. *Hum. Reprod.,* **9**, 1705–1709.

Silber, S.J., Van Steirteghem, A.C., Liu, J. *et al.* (1995) High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum. Reprod.,* **10**, 148–152.

Strohmer, H. and Feichtinger, W. (1992) Successful clinical application of laser for micromanipulation in an *in vitro* fertilisation program. *Fertil. Steril*., **58**, 212–214.

Tarin, J.J. and Handyside, A.H. (1993) Embryo biopsy strategies for preimplantatation diagnosis. *Fertil. Steril*., **59**, 943–952.

Tedder, R.S., Zuckerman, M.A., Goldstone, A.H. *et al*. (1995) Hepatitis B transmission from contaminated cryopreservation tank. *Lancet*, **346**, 137–139.

Testart, J., Lasalle, B., Belaïsch-Allart, J. *et al.* (1986) High pregnancy rate after early human embryo freezing. *Fertil. Steril.*, **46**, 268–272.

Vanderzwalmen, P., Bertin-Segal, G., Geerts, L. *et al.* (1991) Sperm morphology and IVF pregnancy rate: comparison between Percoll gradient centrifugation and swim up procedures. *Hum. Reprod.*, **6**, 581–588.

Van de Velde, H., De Vos, A., Joris, H. *et al*. (1998) Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. *Hum. Reprod*., **13**, 3160–3164.

Veeck, L.L., Amundson, C.H., Brothman, L.J. *et al.* (1993) Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. *Fertil. Steril.*, **59**, 1202–1207.

Verlinsky, Y., Cieslak, J., Freidine M. *et al*. (1996) Polar body diagnosis of common aneuploidies by FISH. *J. Assist. Reprod. Genet*., **13**, 157–162.

World Health Organization (1999) *WHO laboratory manual for the examination of human semen and sperm–cervical mucus interaction*. Cambridge University Press, Cambridge.

Received on April 20, 2000; accepted on June 26, 2000