QUALITY CONTROL (QC) TESTING PROCEDURE USING THE CULTURE OF MOUSE ZYGOTES

PRINCIPLE AND CLINICAL SIGNIFICANCE:

The mouse has proven to be a good model for many human IVF procedures, including embryo culture. The culture of mouse embryos has been shown to be a sensitive quality control test of medium and equipment used for the incubation and culture of human gametes. A regular QC assay using mouse embryos should help to maintain optimal culture conditions in a human Assisted Reproductive Technology (ART) laboratory. The IVF Laboratory must be enrolled in any proficiency testing program offered to be in compliance with government regulations; the culture of mouse zygotes, as outlined in this procedure, can be used to partake in this proficiency test.

EQUIPMENT AND MATERIALS:

Equipment:

1. Incubator - CO_2 incubator at 37°C with optimally, a static or flow-through humidified atmosphere of 6% CO_2: 5% O_2: 89% N_2. A regular 5% CO_2 in air incubator will also work but is not optimal.

2. Dissecting scissors and watchmakers forceps for collection of mouse reproductive tracts.

3. Tissue culture grade syringes, pipettes, tubes, culture dishes and filtering devices. The recommended syringes are the all plastic, non-pyrogenic type with no lubricant from Air-Tite, Virginia Beach, VA. Pipettes and culture dishes are from Falcon (1 ml pipette cat. no. 7521; 5 ml pipette cat. no. 7543; 10 ml pipette cat. no. 7551; tissue culture dish 60 x 15 mm cat. no. 3002; petri dish 60 x 15 mm cat. no. 1007; organ culture dish cat. no. 3037). Tubes are from Falcon (14 ml - Falcon #2057: 4 ml - Falcon #2054). Flasks from Falcon (70 ml, cat. no. 3082; 250 ml, cat. no. 3083). Filters are from Gelman Sciences (Acrodisc, pore size 0.2 um, cat. no. 4192). A mechanical or, preferably, electrically powered pipette aid (eg Drummond Scientific, model no. 400100) is used with the 1, 5, and 10 ml pipettes.

Materials:

1. Pregnant mare serum gonadotropin (PMSG; Gestyl, Diosynth, Chicago or Sigma Chemical Co. although Sigma's product can have a lower bioactivity). A serum gonadotropin preparation called P.G. 600, marketed by Intervet Inc, Millsboro, DE 19966, and distributed by local veterinary supply companies, eg. JRG Supply, Stratford, Iowa 50249 (Telephone 800-354-7433 or 515-838-2414) is also effective. Make up to 50 U/ml in 0.9% (w/v) saline and store frozen in 3 ml aliquots in 4 ml Falcon #2054 tubes.

2. Human chorionic gonadotropin (hCG; Sigma or equivalent clinical supplier). Make up to 50 U/ml in saline and store frozen in appropriate aliquots.

3. Commercially prepared IVF medium or equivalent in-house produced product.

4. Bovine serum albumin (BSA; Sigma Chem Co, Product No. A9647).

5. Hyaluronidase (Sigma Chem Co, Type I-S, Product No. H-3506). Made up to 200 U/ml in Hepes-buffered HTF medium containing 5 mg/ml BSA, filter sterilized (Acrodisc, 0.2 mm pore

size, Gelman Sciences, Product No. 4192) and stored frozen in 2.5 ml aliquots in 4 ml Falcon #2054 tubes.

6. Paraffin oil **[EACH LOT HAS TO BE QC TESTED WITH MOUSE EMBRYOS** . Equilibrate with 10% (vol/vol) HCO3-buffered HTF, place in 6% CO_2 in air incubator overnight, replace medium with fresh batch the next morning and store in CO_2 incubator with lid of container loosely in place to allow gas equilibration.
Other sources of oil are Squibb, Fisher Scientific and Sigma Chemical Co.

7. F1 female hybrid mice, 4-5 weeks old. The strain currently being used is B6C3 F1 from Simonsen Laboratory or Charles River Labs. The strains B6CB F1 and (SJL x DBA) F1 have also been used. Other F1 hybrids also work, as do mice from other commercial suppliers.
F1 stud males, 8 - 10 weeks old are purchased, housed one per box and used over the following 18 months. Each male should have at least two days' abstinence between matings for optimal results.

8. Frozen mouse embryos are also available from commercial sources. Follow the suppliers instructions for thawing and washing the embryos.

Preparation:
1. F1 female mice are superovulated by giving intraperitoneal (ip) injection of 10 U (0.2 ml) PMSG or P.G. 600, followed 48 hours later by 10 U (0.2 ml) hCG. These injections are given between 12.00 noon and 6.00 pm, usually around 3.00 pm. After the hCG injection, each female is placed individually with a stud male and checked the following morning for successful mating as evidenced by the presence of a vaginal copulation plug. The plugged females are brought to the laboratory for oocyte collection.

2. Medium or Device under test
If a device, such as a catheter, dish etc, is under test, it is rinsed with test medium or treated with medium in an appropriate fashion and the resulting treated medium tested. This medium will normally contain 5 mg/ml BSA. Protein-free medium may also be tested if deemed appropriate.

Medium for testing is prepared the day before zygotes will be cultured by dissolving 5 mg/ml BSA into the medium, filter sterilizing the medium, placing 10 ul aliquots of the medium using a 10 ul Pipetman pipettor (Gilson) in a 35 mm diameter tissue culture dish (eg Corning Tissue Culture Dish, Product No. 25000) and covering the 10 ul drops of medium with 2.5 - 3.0 ml of equilibrated paraffin oil. The culture dish is then placed in the incubator under an humidified atmosphere of 6% CO_2: 5% O_2: 89% N_2 at 37^{o}C and allowed to equilibrate overnight. A similar culture dish containing droplets of protein-free medium may also be prepared. The culture dishes are placed with their lids displaced on the dishes to allow for adequate gas exchange.
The medium is usually prepared in 10 ml lots in capped polystyrene tubes (Falcon, Product No. 2057).

Performance Parameters:
In medium containing 5 mg/ml BSA, at least 80% of the embryos which have progressed through the 2-cell stage (this is evidence of fertilization) should reach the fully expanded blastocyst (FEB) stage after 96 - 108 hours of culture. In protein-free medium, at least 50% of the zygotes should reach the FEB stage.

Storage Requirements:
When not in use, all media not containing protein is stored at 2-8^{o}C for up to four weeks before being discarded. Once BSA has been added, medium is kept in the incubator at 37^{o}C under humidified 6% CO_2 in air for up to four days.

Any labels put on containers or culture dishes should be self-adhesive, removable labels (eg Avery Dennison, Product No. S-812) and all writing should be with a lead pencil. Marking pens are never to be used. The tubes of medium and culture dishes are labelled with contents, lot number and date of preparation.

CALIBRATION:

Standard Preparation:

Two samples of medium, one known to have given a positive result within the past 50 days, and one known to have given a negative result shall be prepared for testing in the same manner as the sample media under test.

Calibration Procedure:

The standard positive and a negative culture medium (or one that has been adulterated with formalin to produce negative results) need to be prepared and run each time a sample is tested.
The positive control medium needs to have a result of at least 80% FEB development and the negative control should have a FEB development rate of between 0 and 65%.
If these levels are not met, the run needs to be repeated using new positive and negative controls. If the controls in the repeat run again fail, the incubator needs to be cleaned and other appropriate remedial action with other equipment should be undertaken.

QUALITY CONTROL:

A known positive and a known negative control medium, must be run every test period.
The results for the controls and test specimens can be entered in an appropriate data base.

PROCEDURE - STEPWISE:

1. Plugged females are killed the morning the copulation plug is found for the collection of 1-cell zygotes. The animals are sacrificed between 9.00 am and 12.00 noon by cervical dislocation, killing between one and three animals at a time. The fallopian tubes are dissected out free of connective tissue using dissecting scissors and watchmakers forceps which have been soaked in 70% alcohol and flame sterilized. The fallopian tubes are placed in a 35 mm culture dish containing between 0.5 and 1.5 ml of hyaluronidase solution which has been warmed to about 35^{o}C on a warming plate. The oocyte-cumulus complexes are dissected out of the ampulla region of the fallopian tube and into the hyaluronidase using a disposable, sterile 26 gauge needle connected to a 1 ml syringe and a pair of watchmakers forceps.

2. After all the oocytes have been collected or within 20 minutes, those oocytes considered to be viable zygotes, as judged by morphological features such as a second polar body, pronuclei and reasonably narrow perivitelline space, are collected from the hyaluronidase solution using a finely drawn glass pipette controlled by mouth suction. The zygotes are then washed through three changes of Hepes-HTF medium warmed to about 35^{o}C and containing 5 mg/ml BSA (3 ml/wash) and stored in a 50-100 ul drop of similar medium under paraffin oil in a 35 mm culture dish on a warming plate at 32-37^{o}C. The washing medium in the dishes is covered with 1.5 ml paraffin oil.

3. When all the washed zygotes have been collected, the number chosen for culture in each culture dish are collected and washed through three 100 ul drops of the equilibrated medium in which they are to be cultured. These drops are set up under oil in a 35 mm culture dish, quickly covered with oil because the medium is usually bicarbonate buffered and will lose CO_2 if left exposed to air for more than 2 minutes, and taken to the dissecting microscope along with the culture dish containing the medium

under test. The zygotes are then collected, thoroughly washed through the three 100 ul drops of medium and placed in the culture drops at a density of 10 zygotes per 10 ul. The culture dish is then returned to the incubator as soon as possible.
A minimum of 40 zygotes per test should be incubated.

4. If the zygotes are to be cultured in protein-free medium, they need to be washed through five changes (2.5 ml/wash) of Hepes-HTF containing 1 mg/ml polyvinylpyrrolidone (Sigma Chem Co., Product No. PVP-40). The embryos are then placed into the culture drops of protein-free medium at an embryonic density of 20 per 10 ul.

5. Scoring of Development The day that culture is initiated is assigned the term Day 0 (D0). On the morning of D1, the embryos are scored for cleavage to the 2-cell stage. Those that reach this stage are considered to be fertilized. Those oocytes that do not cleave are invariably unfertilized.
On D4 (96 hours of culture), most embryos will have reached the fully expanded blastocyst stage and the number reaching this stage should be recorded. The next morning on D5, a few slow developing (<5%) embryos may also have reached the FEB stage and these can be added to the final score if desired but this rarely makes any difference to the final outcome if the final score is taken at 96 hours of culture. Complete hatching of FEBs from the zona pellucida begins on D4 and is completed by D8.

CALCULATIONS:

The percentage of zygotes which develop to the fully expanded blastocyst stage is calculated by taking the number of embryos which reach the FEB stage, dividing by the number of embryos which reached the 2-cell stage and multiplying by 100. Thus if 50 oocytes are cultured, 45 reach the 2-cell stage and 40 develop into FEBs at 96 hours of culture, then the % blastocyst rate is 40/45 x 100 = 89%.

REPORTING RESULTS:
Use the formula given in the CALCULATIONS section above.
All QC results must be retained for at least two years.

PROCEDURE NOTES:
Some of the pitfalls and precautions in the procedure include the following:

1. Female mice are too old; F1 females older than six weeks respond poorly to superovulation. Also, general good animal health is important to obtain good results.

2. Poor pH control in the incubator gives the biggest problem. Frequent opening and closing of the incubator door is deleterious to adequate CO_2 equilibration. The opening and closing of the incubator door should be kept to a minimum or the culture dishes should be placed in a separate incubator in which the doors are only opened to place in dishes containing embryos. The dish is not removed again that day. The lid may seal to the culture dish with oil, thus limiting the exchange of gases, hence the lids are placed askew on the dishes or not put on at all.

3. The particular batch of oil being used may be embryotoxic, particularly if most of the zygotes fail to cleave and undergo degeneration. If this occurs, change the lot number of oil the oil being used, or only use oil that has been previously checked in a mouse embryo bioassay and found to be non-toxic; such oil is available from several commercial sources, eg Advanced Reproductive Technologies, Inc and Sigma Chemical Co.

Reference Ranges:
Positive control with BSA: ≥ 80% FEB rate
Positive control with no protein: ≥ 50% FEB rate
Negative control with BSA: ≤ 65% FEB rate
.

SPECIMEN DISPOSAL:

All discarded specimen samples and disposable laboratory supplies should be disposed of properly in a container marked BIOLOGICAL HAZARD and disposed of accordingly. A contract with a commercial company for the pickup of dead animal carcasses should be established. The carcasses can be stored in a freezer until the day of pickup.

LIMITATIONS OF THE PROCEDURE:

For adequate development of F1 mouse zygotes to the FEB stage, the medium must contain at least 0.1 mM sodium pyruvate and 2.0 mM sodium lactate. Excessive amounts of some vitamins and amino acids should be avoided eg nicotinamide.

REFERENCES

1. Quinn P, Whittingham DG. Effect of fatty acids on fertilization and development of mouse embryos in vitro. J Androl 1982; 3:440-444.

2. Quinn P, Warnes GM, Kerin JF, Kirby C. Culture factors in relation to the success of human in vitro fertilization and embryo transfer. Fertil Steril 1984; 41:202-209.
3. Hogan B, Costantini F, Lacy E. Manipulating the mouse embryo. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986.