II) MATERIAL AND METHODS

II. 1) Introduction and Objectives

This study aimed to determine whether the selection of embryos on the basis of morphology at day 2 (around the 4-cell stage), a usual practice in human IVF clinics, is a good predictor of embryo viability at day 7. The hypothesis is that such selection is a good predictor of subsequent embryo viability.

To achieve this goal, and given that provision of human embryos was not possible for regulatory and ethical reasons, bovine eggs were used in a "human model" context. Embryos were provided for the study in independent groups of ten, each group of ten representing a different "patient", 10 eggs being a reasonable yield to expect to get in a human clinic. The embryos were on day 2 according to the criteria used in a human IVF clinic, the Aberdeen Fertility Centre. Of these, 5 good or viable eggs were "selected". Five was the chosen number because, in a real human situation, from among eggs collected from a patient, normally two would have been transferred to the mother (in the Aberdeen Fertility Centre, as in most clinics in the UK, no more than 2 embryos are transferred to avoid multiple pregnancies) and three would have been cryopreserved for a future two-egg transfer (cryopreserved embryos have a lower survival and pregnancy rate because of the procedure) (Maureen Wood, personal communication). On day 7, according to an adaptation of the criteria used by Lindner and Wright (1983) for bovine embryo morphology and evaluation, a verdict of viability on developmental failure was recorded. Gardner' s recommended criteria, referred to in Section I.1 of the literature review was to be used originally but bovine embryos are darker than human eggs and therefore Lindner and Wright' s criteria gave a more appropriate verdict on each embryo' s morphological status.

Preliminary studies were carried out with two preliminary batches of embryos to determine the time when most of the embryos would be at the 4-cell stage, to ensure

that timing of egg “selection” in the subsequent experiment would be appropriate and, in so doing to account for any differences between human and bovine embryo growth.

II. 2) Experimental Procedures

All reagents and media were obtained from Sigma Chemical Co Ltd, Poole, Dorset, UK; all 30 mm petri dishes were Sterilin (Fisher Scientific, UK); all 60 mm petri dishes were NUNC Surface (Fisher Scientific, UK) and all pipettes supplied by GILSON (Anachem, Luton, UK) unless otherwise stated.

II.2.A) Collection, *in vitro* maturation and insemination of oocytes

Bovine oocytes were obtained from slaughterhouse-derived ovaries, collected at an abattoir (20 minutes from laboratory) and transported to the laboratory at approximately 32 - 33 °C in PBS (phosphate buffered saline, Oxoid Basingstoke, UK) containing 50mg/L kanamycin. Ovaries originated from a variety of beef and dairy heifers. Cumulus-oocyte complexes were recovered by aspiration from 2 to 8 mm diameter follicles using an 18 g needle and 5 mL syringe. These were put in 4 ml Search Medium buffered with HEPES (TCM-199 + HEPES buffer (cat No M-7528) and 5 % v/v FCS (Fetal Calf Serum, cat. No F9665) and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin, cat No P-4333), pH 7.3, 290-300 mOsm). Dishes were searched (in Search Medium) under x10 magnification in stereo microscope and all oocyte-cumulus complexes (OCC) were transferred into a standard sterile 30 mm petri dish containing 2 ml Search Medium. OCC were evaluated morphologically under stereo microscope (same magnification) and good quality OCC were put into clean standard 30 mm petri dishes containing 2 ml Search Medium. Good OCC were picked up in groups of between 30 and 200 and placed in sterile 30 mm petri dishes containing 2 ml of Maturation Medium (TCM 199 without HEPES (cat No M-2154), and supplemented with 10% FCS (cat. No F9665), antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin, cat No P-4333) and 10μg/mL LH (cat No L-9773) and 10 μg/mL FSH (cat No F-2293), pH 7.3-7.4, 290-300 mOSm). The following criteria had to be met by a good oocyte:

- surrounded by at least 4 to 5 layers of cumulus cells
- bright, ungranulated
- even cytoplasm
- cumulus cells unexpanded
- compact and even at this stage.

Cryopreserved semen from a tested bull was thawed at 35°C and layered (0.13 mL) under 1 mL of capacitation medium (mTALP: modified calcium-free Tyrode' s Albumin Lactate Pyruvate (TALP) made with 6mg/mL BSA fraction V (cat. No A3311), 50 IU/mL penicillin and 50 μg/mL streptomycin (cat No P-4333), pH 7.4) in a swim-up tube. Tubes were incubated (38.5°C, 5% CO_2 in air at maximum humidity) for 30 minutes. 0.6-0.7 mL were removed from the top of each tube using a 1 mL pipette and centrifuged in a IEC Centra 4R Centrifuge at 1500 rpm for 10 minutes. Supernatant was discarded leaving a 200 μl pellet. The pellet was resuspended, motile spermatozoa were counted in a haemacytometer and added ($1x10^6$ sperm/ml) to 50 μl fertilisation drops (modified TALP with 0.2 μmol/L penicillamine (cat No P-4875), 0.1 μmol/L hypotaurine (cat No H-1384), 0.02 μmol/L epinephrine (cat No E-1635), 6 mg/mL fatty-acid free BSA (cat. No A8806), 30 μg/mL heparin (cat No H-3149), 50 IU/mL penicillin and 50 μg/mL streptomycin, pH 7.8 (cat No P-4333)) containing the oocytes. The oocytes had previously been transferred from maturation dishes to a petri dish containing 2 mL Wash Medium (Hepes-buffered TALP supplemented with 3 mg/mL BSA fraction V (cat. No A3311), 50 IU/mL penicillin and 50 μg/mL streptomycin (cat No P-4333)). Cumulus and corona cells were reduced to 4-5 layers by careful pipetting. The partially stripped oocytes were washed in Wash Medium and 10 to 20 oocytes were added to each fertilisation droplet (46 μl) in 4 μl medium (to make a 50 μl droplet). Oocytes and spermatozoa were incubated together (38.5°C, 5% CO_2 in air; maximum humidity) under mineral oil for 18-20 hours.

II.2. B) Embryo culture and experimental design for two preliminary study batches (Experiment 1).

At 18-20 hours after insemination (Day 1), zygotes were transferred to 60 mm petri dishes containing 50 μl droplets of SOFaaBSA (see Appendix 3), after being washed

in the same medium 2-3 times, and incubated at 38.5°C, 5% CO_2, 5% O_2, 90% N_2. Starting at 36 hours after insemination and then every 12 hours until Day 7 for the first batch, embryos were taken out of the incubator, examined under stereomicroscope (x20 magnification) and their stage and cell number noted. The same procedure was undertaken for the second batch, but observations every 12 hrs were restricted to between 36 hours and 68 hours post-insemination to avoid exposing the embryos to harmful conditions out of the incubator too often.

On day 2 (48 –50 hours post insemination), embryos were allocated randomly to any one of three different treatments, washed in the same type of medium in which they were to be cultured, and then placed in 50 μl droplets in groups of 10 in separate 60 mm petri dishes for each treatment.

Treatment 1: Synthetic Oviduct Fluid (SOFaaBSA) until day 7

Treatment 2: Synthetic Oviduct Fluid (SOFaaBSA) until day 2 and then change to SOFaaBSA plus 2% v/v lipoproteins (bovine plasma) (Sigma, cat No L - 3626)

Treatment 3: Synthetic Oviduct Fluid (SOFaaBSA) until day 4 and then change to SOF+ 10% v/v adult bovine serum (ABS)

The embryos were placed in fresh drops every two days (if they were to be placed in a different medium, they were previously washed in 1 ml of that next medium in which they were to be cultured).

The incubation conditions were always the same:

- in 50 μl droplets in 60 mm NUNC Surface dishes under mineral oil
- in atmospheres of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C.

The Treatment 1 medium was prepared as follows:
SOFaaBSA: See composition in Appendix 3

The Treatment 2 medium was prepared as follows:
SOFaaBSA: See composition in Appendix 3
SOFaaBSA plus 2% v/v lipoproteins (bovine plasma) (Sigma, cat No L - 3626)

The Treatment 3 medium was prepared as follows:

SOFaaBSA: See composition in Appendix 3

SOF + 10% v/v ABS: Basic SOF (as shown in Appendix 3) to which 10% v/v ABS (cat. No B-2771) was added 1h 30 mins before use.

II.2.C) Blastocyst assessment and Cell counts.

Blastocysts only from SOFaaBSA/ SOF+ 10% ABS (Treatment 3) (the other embryos were destined for other independent experiments) were separated on day 7 post-insemination (after being identified under a x20 stereo microscope) and put in new 60 mm dishes (preincubated under same conditions for 1.5 hour) containing several (between 6 and 8) 50 μl drops of fresh SOF+10% ABS under mineral oil. Each blastocyst was placed in a different drop to be followed and identified individually under an inverted microscope (x320 magnification). The assessment criteria were an adaptation of those described by Lindner and Wright (1983) and included developmental stage, shape, colour, number, size, compactness of cells, state of inner cell mass and blastocoele and percentage mass of extrusions (see below). The diameters of the blastocysts also were measured under an inverted microscope (x320 magnification).

Blastocyst grades (modified from Lindner and Wright, 1983)

Grade 1 = excellent
Grade 1.5 = very good
Grade 2= good
Grade 2.5= fair
Grade 3= poor
Grade 4 = degenerate

Parameters that counted in evaluation:

- colour: light (preferably) / dark
- texture
- symmetry
- % of extrusions (internal and external)
- size of inner cell mass (when more intrusions, smaller size, a mid-blastocyst is supposed to have 50% ICM, 50% blastocoele)
- characteristics of inner cell mass: whether it is diffuse / distinct (better)

- thickness of zona (the more advanced the blastocyst, the thinner it should be to allow hatching)
- number of degenerate cells
- cells of varying sizes
- vesiculation

Developmental stages from morula to hatched blastocyst (Lindner and Wright, 1983):

- Morula: ball of cells. Individual blastomeres (embryo cells) are hard to discern one from another.
- Compact morula: Individual blastomeres have coalesced forming a compact mass.
- Early blastocyst: Embryo that forms a blastocoele (fluid-filled cavity). Embryo occupies 70 to 80% of perivitelline space.
- Blastocyst: Embryo occupies most of perivitelline space, outer trophoblast differentiates and inner cell mass darker and more evident.
- Expanded blastocyst: Overall diameter of embryo increases significantly (1.2 to 1.5 X). Zona pellucida thins.
- Hatched blastocyst: Can either be hatching or already hatched. Many embryos are collapsed at this stage.

The fact that an embryo was or was not at the correct developmental stage at the right time was also important in the grading. See Figure II.1 below.

Fig II.1. Timing of development of bovine embryos (modified from Lindner and Wright, 1983)

Cell stage	Developmental age in vivo
Morula	5 days
Compact morula	6 days
Early blastocyst	7 days
Blastocyst	7 days
Expanded blastocyst	8 days
Hatched blastocyst	9 days

After individual assessment, the blastocysts were fixed in individual wells containing 600 μl of a solution of ethanol:acetic acid (3:1, v:v) for a minimum of 24 hours. This arrested growth and dissolved the zona pellucida. Then, each blastocyst was placed on a glass slide, dried, stained with 14 μl of 10 μg/mL Hoechst stain 33342 in an aqueous solution of 5μl Hoechst (1 mg stain/1 ml H_2O) (cat No B-2261) in 500 μl Na Citrate (cat No S-4641), then covered with a coverslip. Total cell number in each blastocyst was determined by counting stained nuclei with the aid of a binocular fluorescence microscope (400x). Cells were counted twice and a note was taken of the average of both cell counts.
Non-blastocysts were discarded after morphological evaluation.

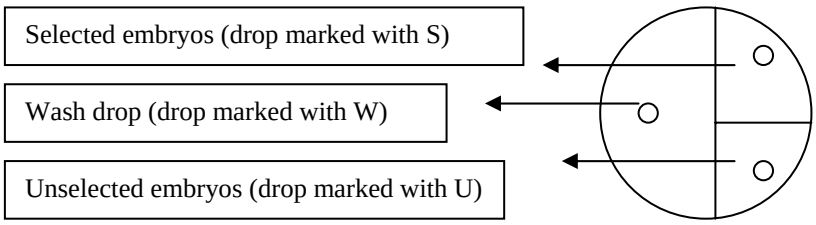
II.2.D) Embryo culture and experimental design for Experiment 2 (5 replicates)

At 18 to 20 hours after insemination (Day 1), zygotes generated from IVM and IVF of OCC from 5 batches of ovaries were transferred to 60 mm petri dishes containing 50 μl droplets of SOFaaBSA (see Appendix 3), after being washed in the same medium 2 or 3 times, and were incubated at 38.5°C (5% CO_2, 5% O_2, 90% N_2). On day 2, around 48 to 50 hours after insemination, embryos were taken out of the incubator and assigned randomly, in groups of 10, into 50 μl droplets of SOFaaBSA under mineral oil in 60 mm petri dishes (5 drops/dish; dishes had previously been incubated for at least 1 hour under standard conditions). Each group of 10 embryos was examined under a stereomicroscope (x20 magnification), their cell numbers were noted and they were classified according to the classification system used by the Aberdeen Fertility Clinic (see Appendix 2) and then transferred to a new 60 mm petri dish (incubated in standard conditions for at least 1 hour), where five "selected" embryos (see earlier description) were placed in a 50 μl drop of SOFaaBSA, marked S (for Selected), under mineral oil. The other five embryos were placed in the same dish in another 50 μl drop of SOFaaBSA, marked U (for Unselected) and then the embryos were incubated. Note: the "S" and "U" marks were written in permanent ink on the base of the dish (outer surface).

On Day 4, embryos were taken out of the incubator, examined under a stereomicroscope (x 20 magnification) and their stages and cell numbers were noted.

Each group of 5 embryos was examined separately and identified by dish and drop. Embryos were then changed to fresh preincubated (standard conditions, at least 1 hour) 60 mm dishes containing 50 μl drops of SOF + Foetal Calf Serum + Vitamin E (SOF+FCS+Vitamin E, see below) medium under mineral oil. There were 3 drops per dish: one for washing the embryos, one for the S group and one for the U group, all marked accordingly (see Figure II.2 below). As part of the changeover procedure, embryos were washed first in 1 ml of the new medium (preincubated for 1 hour) and then placed in the new dishes to be incubated.

Figure II.2: Dish layout as prepared for every group of ten embryos: they were all washed in one drop (W) (in SOF+FCS+Vitamin E) and then the 5 that were selected went to the drop marked S and the rest to the drop marked U (unselected), both drops made of of SOF+FCS+Vitamin E.



On day 6, embryos were taken out of incubator, examined under a stereomicroscope (x 20 magnification) and their stage and/or cell numbers noted. Each group of 5 embryos was examined separately and data noted by dish and drop. Embryos were then changed to fresh preincubated (standard conditions, at least 1 hour) 60 mm dishes with 50 μl drops of SOF+FCS+Vitamin E medium under mineral oil. There were 2 drops per dish: one for the S group and one for the U group, marked accordingly. Embryos were then reincubated.

The standard incubation conditions used were always the same:

- embryos in 50 μl droplets in 60 mm dishes under mineral oil
- dishes in humidified atmospheres of 5% CO_2, 5% O_2, 90% N_2 at 38.5°C.

The SOF + FCS + Vitamin E medium was prepared as follows:

Basic SOF (see Appendix 3) + (10% FCS+vit E), that is Vitamin E (alpha tocopherol, FLUKA, Biochemica, US; cat. No 95240) dissolved in 95% ethanol, giving a 200 mM stock solution, which was added to FCS (cat. No F-9665) to give a concentration of 1 mM in less than 0.5 % ethanol and therefore a final concentration of 100 μM vitamin E in SOF. A 10% v/v solution of "FCS + vit E" (aliquoted in freezer) was added to the basic SOF 1h 30 mins before use.

II.2.E) Blastocyst assessment

On day 7, embryos were taken out of the incubator, examined under a stereomicroscope (x20 magnification) and their stage and/or cell numbers noted. Each group of 5 embryos was examined separately and identified by dish and drop. Blastocysts were separated post-examination (after being identified under a x20 stereo microscope) and put into new dishes (preincubated under same conditions for 1.5 hours) containing several (between 6 and 8) 50 μl drops of fresh SOF+FCS+vitamin E medium under mineral oil. Each blastocyst was placed into a different drop to be measured, followed and identified individually under an inverted microscope (x320 magnification). The assessment criteria were an adaptation of those described by Lindner and Wright (1983), as already recorded above. Non-blastocysts were discarded after morphological evaluation.

II.2.F) Pyruvate metabolism

After individual assessment, blastocysts (from batches 2, 3, 4 and 5) underwent a pyruvate uptake assay prior to their fixation for cell counts. These were washed in mECM+p (see below) (previously warmed) two or three times. Then individual embryos were picked up singly (individually, 1 per tube) in a 3 μl drop of that medium and placed alone on the lid of a micro tube (1.5 ml sterile micro tube containing 1 ml 0.1 M NaOH warmed, with lid detached). Then 3 μl of "hot" ECM (m-ECM-p plus radiolabelled substrate, [2-^{14}C] Pyruvate (American Radiolabeled Chemicals, Sp. act. 5.5 mCi/mmol), see below) were added and mixed well. Lids were put back on micro tubes containing 1 ml 0.1 M NaOH. Blanks were prepared with 3 μl mECM+p and 3 μl "hot" ECM (mECM-p plus radiolabelled pyruvate) but without an embryo. Blanks were placed every 7 or 8 embryos. Micro tubes (containing embryos or serving as blanks) were incubated for 3 hours at 38.5°C in air.

During incubation time, scintillation tubes were prepared by adding 4 ml scintillation fluid, Emulsifier-Safe (Packard, The Netherlands), a scintillation liquid that facilitates detection of beta particle emissions, to each vial (2 vials prepared per micro tube used). A 4-well dish with mECM+p was prepared and kept warm. At the end of the incubation, lids were removed and embryos transferred in a minimum volume (< 1 μl) to the 4-well dish and washed three times. Thereafter each embryo was individually fixed in ethanol:acetic acid (3:1, v:v) for cell counts as described below.

The contents of the incubated micro tubes were transferred into 2 scintillation vials (500 μl of 0.1 M NaOH in each). To avoid chemiluminescence, 105 μl of 0.5 M HCl was added to each scintillation vial. Lids were numbered and placed on vials. Each vial was shaken to mix the contents and the base was dipped in water (to help counteract static). Each vial was then "counted" for 20 minutes in a beta counter (Liquid Scintillation Analyser, 2000 CA, Tri-carb, Canberra Packard, Pangbourne, Berks, UK) (Protocol: 14C bio DPM for single counting).

The principle on which the pyruvate uptake methodology is based is as follows (Stryer, 1988):

1) Formation of acetyl-CoA from pyruvate

$$CH_3\text{-}\underline{\mathbf{C}}=O-COO^- + CoA + NAD^+ \rightarrow CH_3\text{-}\underline{\mathbf{C}}=O-CoA + CO_2 + NADH$$

(Pyruvate) (Acetyl-CoA)

2) Krebs cycle

$$CH_3\text{-}\underline{\mathbf{C}}=O-CoA + 3\,NAD^+ + FAD + GDP + P_I + 2\,H_2O \rightarrow$$

$$2\,\underline{\mathbf{C}}O2 + 3\,NADH + FADH_2 + GTP + 2H^+ + CoA$$

Pyruvate use by the embryo can be measured by using radiolabelled pyruvate and trapping the $^{14}CO_2$ product.

Media used for pyruvate metabolism assay:

Modified embryo culture medium, pyruvate free (mECM-p)

To 100 ml PBS add:

BSA (fraction V, cat No A-3311)	400 mg
Glucose	18 mg
Glutamine	14.6 mg
Pen Strep *	500 μl

* (50 IU/mL penicillin and 50 μg/mL streptomycin, cat No P-4333)

For "Hot" embryo culture medium ("hot" ECM)

Add 10.8 mg of "hot" pyruvate (American Radiolabelled Chemicals, cat No ARC222) to mECM-p

Specific activity: 5.5 mCi/mmol

Final concentration: 185 Bq/μl (0.005μCi/μl)

Store in freezer (-20°C) in 50 μl aliquots

Modified embryo culture medium (mECM+p)

To 100 ml PBS add:

BSA (fraction V, cat No A-3311)	400 mg
Glucose	180 mg
Sodium Pyruvate (cat No P-4562)	10.8 mg
Glutamine	14.6 mg
Pen Strep *	500 μl

*(50 IU/mL penicillin and 50 μg/mL streptomycin, cat No P-4333)

pH 7.2-7.4

Osmolality 270-280 mOsm

Filter and store in fridge. Use within 2 weeks.

II.2.G) Cell counts

Finally, blastocysts were fixed in individual wells containing 600 μl of a solution of ethanol:acetic acid (3:1, v:v) for a minimum of 24 hours. This arrested growth and dissolved the zona pellucida. Then, each blastocyst was placed on a glass slide, dried, stained with 14 μl of 10 μg/mL Hoechst stain 33342 in an aqueous solution of 5 μl Hoechst) (1 mg stain/1 ml H_2O) (cat No B-2261) in 500 μl Na Citrate (cat No S-

4641), then covered with a coverslip. Total cell number in each blastocyst was determined by counting stained nuclei with the aid of a binocular fluorescence microscope (400x). Cells were counted twice and a note was taken of the average of both cell counts. The counts were blind (without knowledge of whether the embryos were selected or unselected).

II. 3) Statistical analysis

A generalised linear model for binomial distribution was used in Genstat 5 software (version 6.2, 6th edition) for data analysis and comparisons of embryo yields and grading. Cell counts (log transformed) and zona-inclusive diameters were analysed by ANOVA. In all cases, replicate and selection status were the factors used in the model. In addition, a generalised linear model (binomial proportions) was used to evaluate data (pre-selection) for stage distribution (2-cell vs 3-4 cell vs 5-8 cell stages). This included relevant pairwise comparisons.