Optimal *in vitro* Culture Conditions for Sustainable Development of Preimplantation Mouse Embryos during Prolonged Culture

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ABSTRACT: The purpose of this study was to examine the effect of three different commercially available complex media (sequential media systems) on the sustainable development of preimplantation mouse embryos in a prolonged culture and whether the *in vitro* culture conditions could be further optimised when co-cultured with either MRC5 or cumulus cells. Hence, pronuclear embryos of ICR mice were cultured to the blastocyst stage in the sequential media systems, with and without the co-culture. Subsequently, the efficacy of the culture systems described in this paper was evaluated by the ability of the culture systems to alleviate the 2-cell block of ICR mouse embryos, and by measuring blastulation rates and nuclei number per blastocyst. The results obtained in this study suggest that specific combination between cell type and culture media is important and co-culture in sequential medium acts synergistically to improve the number and quality of blastocysts.

Keywords: blastocyst, co-culture, cumulus cells, mouse embryos, MRC5 cells, sequential culture medium

Introduction

Embryo developmental rates in mammals including human in conventional monoculture systems, either in the form of simple salts, such as Earle's balanced salt solution (EBSS) or a complex medium such as Ham's F10, have been poor (Voelkel et al. 1985; Rexroad & Powell 1988; Fissore et al. 1989; Van Blerkom 1993; Vlad et al. 1996). In vitro culture of embryos from outbred mouse strains in media such as these show developmental blocks at the 2-cell stage (Goddard & Pratt 1983). In porcine embryos, the developmental block was observed to occur at the 4-cell stage, and in bovine, ovine and caprine, between the 8- to 16-cell stage (Crosby et al., 1988; Telford et al. 1990; Jarrel et al. 1991; Prichard et al. 1992; Lequarre et al. 2003; Meirelles et al. 2004). In human a similar block occured between the 4- to 8-cell stages (Braude et al. 1988).

Numerous studies have been carried out in a variety of species using homologous and autologous cell lines as 'feeder' or 'helper' cells, which are grown together with the embryos to help overcome the cleavage arrest and to improve the embryo development. These are referred to as co-culture systems. The basic principle of the co-culture system is to mimic to some extent the *in vivo* environment of the embryo as closely as possible. Earlier studies have shown the beneficial effects of co-culture in simple and complex medium with cells of reproductive tract origin on embryo development in a variety of species such as ovine, bovine, porcine, caprine, equine, murine as well as in the human (Bongso et al. 1990; 1991; 1994; 1995). Various types of co-culture systems have been applied to improve *in vitro* culture conditions. Co-culture of human embryos with cumulus cells in either simple chemically defined media such as human tubal fluid (HTF) or complex media such as Ham's F10 have been reported to facilitate the development of good quality embryos (Mansour et al. 1994; Saito et al. 1994) and produce significantly higher blastulation rates compared to culture in simple medium (HTF) alone (Quinn & Margalit 1996). Similar observations were made with bovine embryos co-cultured with cumulus cells in TCM-199, which was a complex medium (Goto et al. 1988; Lim et al. 1996). It has been observed that a higher percentage of mouse embryos developed to blastocysts when cultured with Madin-Darby bovine kidney cells (MDBK) in both M16 (Ouhibi et al. 1990) and EBSS (Walker 1994) simple medium. Again, similar observations were made in co-culture of mouse embryos in cells originating from the human reproductive tract (Goldberg et al. 1991; Freeman et al. 1993; Desai et al. 1994) in either simple (EBSS) or complex (DMEM, Ham's F10 and DMEM/Ham'sF12) medium. Vero (African green monkey kidney) cells co-cultured in complex medium (B2-Menezo) have been reported to give similar effects for human embryos suggesting that it is not necessary for

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Published 30 June 2010

embryo co-culture to be strictly of reproductive tract origin, but rather of epithelial origin (Menezo et al. 1990; Schillaci et al. 1994).

Consequently, studies have shown improved morphology and decreased fragmentation, increased embryo development, higher implantation and pregnancy rates with co-culture of cells from reproductive tract origin such as bovine oviductal epithelial cells in HTF (Wiemer et al. 1993), foetal bovine uterine fibroblast cells in EBSS (Wiemer et al. 1989a; 1989b) and human endometrial epithelial cells in sequential culture (Simón et al. 1999). Freeman et al. (1993), observed in simple (EBSS) and complex medium (Ham's F10 and DMEM/Ham's F12) that whether or not the feeder cell types were epithelial or fibroblastic or the origins of the cells were ovarian follicles, oviductal or endometrial they all were found to exert some beneficial effects on embryo development and concluded that co-culture is not tissue or cell type specific. Conversely, Van Blerkom (1993) for human and Oakley (1997) for mouse reported no overt or statistically significant improvement in Vero cells co-cultured in EBSS compared to culture medium alone. Bongso et al. (1991) have established that co-culture systems need not be autologous to the species of the embryo. They also found that conditioned media (medium in which feeder cells are grown) gave varying degrees of beneficial effects in different species and that for some species embryo-to-cell contact is necessary for the expression of the co-culture effect. In summary, different degrees of outcomes on embryo development have been reported using various co-culture systems suggesting that the success of co-culture systems also seems to be dependent on the type of cell line used (Oakley 1997). It has also been reported that different cells in contact with different types of medium give variable responses in promoting embryo development highlighting a need in obtaining a medium that encourages optimum embryo development as well being beneficial for the feeder cells (Leppens & Sakkas 1995).

For the past three decades, co-culture was the only way to obtain high blastulation rates in domestic animals and in the human. New culture systems have been devised to develop viable blastocysts using stage-specific media or sequential complex media, which cater for the embryo's changing metabolic needs as it develops from zygote to the blastocyst stage. Sequential culture media have been formulated based on the understanding that the embryo has differing nutritional requirements at different stages of the preimplantation development. These new culture systems which employ a two-step culture protocol have resulted from an increased understanding of both the metabolism of the embryo and the environment of the oviduct and uterus (Pool 2004). Existing culture media formulations were first tested on mouse embryos and subsequently tried for human, owing to the limited usage of human embryos for metabolic research. Sequential media available and most commonly used brand names by some units around the world are Cook, MediCult and Vitrolife. Therefore, for the purpose of this study these three commercially available culture media were used. The sequential culture media consists of two different culture media to be used in sequence where the first culture medium caters for the metabolic and nutritional preferences of the preimplantation embryo from the pronuclear to the 4- or 8-cell stage, where it is then transferred to the second medium to continue culture till the blastocyst stage.

The objective of this study was to examine the effect of three different commercially available complex media (sequential media systems) on the sustainable development of preimplantation mouse embryos in a prolonged culture, and whether the *in vitro* culture conditions could be further optimised when co-cultured with either MRC5 or cumulus cells. Pronuclear embryos from ICR mouse strain were cultured to the blastocyst stage in the sequential media systems, with and without co-culture. Subsequently, the efficacy of the culture systems described in this paper was evaluated by the ability of the culture systems to alleviate the two-cell block of ICR mouse embryos, and by measuring blastulation rates and nuclei number per blastocyst.

Materials and Methods

Animals and embryo collection

The work with mice described herein was approved by, and performed in strict accordance to the guidelines of the Home Office, United Kingdom under the Animals (Scientific Procedures) Act 1986. Male and female ICR mice (Harlan, UK) used in this study were housed in light- and temperature-controlled conditions (12 h light: 12 h darkness, 21 C ± 1 C), fed with chow pellets (Harlan Teklad, UK), and received water *ad libitum*. ICR females were induced to ovulate using intraperitoneal injection with 6 IU pregnant mare's serum gonadotrophin (PMSG; Intervet, Buckinghamshire, UK) and followed 48 h later by 6 IU human chorionic gonadotrophin (hCG; Intervet, Buckinghamshire, UK). The females were mated with ICR male overnight and the following morning, successful copulation was indicated by the presence of a vaginal plug. Approximately 16-18 h post hCG, the ampulla were removed and

placed in the HEPES-buffered EBSS (Sigma) supplemented with a final concentration of 105 µg/ml human serum albumin (HSA; Sigma). Subsequently, cumulus-oocyte complexes (COCs) were denuded by a brief (less than 1 min) incubation in 1mg/ml hyaluronidase (Sigma), followed by four washes with HSA supplemented HEPES-buffered EBSS medium. Prior to transferring the embryos to the appropriate culture systems, they were washed again four times in 100 µl drops of embryo culture medium. All manipulations were carried out using a binocular dissecting microscope fitted with heated pads at 37^{o}C and under-stage illumination.

In vivo blastocyst collection

For *in vivo* embryo retrieval, blastocyst stage embryos were collected from superovulated female mice between 91-100 h post hCG (day 4). The uteri were removed, placed into a culture dish and flushed with HSA supplemented HEPES-buffered EBSS medium using a 30-gauge needle (Becton Dickinson U.K. Ltd., England) to expel the blastocysts, which were used for nuclei counts.

Preparation of monolayer for cumulus cells co-culture

The cumulus cells were difficult to detach and passage, and therefore only used for a single culture round after seeding. On the day of embryo collection, cumulus cells were either cultured upon collection or thawed from stored frozen stocks. Hence, both fresh and frozen-thawed cumulus cells were used. Cultures of cumulus cells were started by collecting the drops containing hyaluronidase (after fertilised embryos were denuded) in 10 ml of Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) supplemented with 10% foetal calf serum (FCS; Gibco BRL), 2 mM L-glutamine, and 50 IU penicillin: 376 IU streptomycin sulphate per ml, centrifuged at 200 × g for 5 min and resuspended in 1 ml of fresh RPMI 1640, 10% FCS and cultured for 72 h prior to the *in vitro* culture of embryos. Approximately 10^5 cells/ml were added to the culture well containing 0.5 ml of pre-warmed RPMI 1640, 10% FCS medium and incubated at 37 C, 5% CO_2 in air. After 72 h of culture, the cells had established a semi-confluent monolayer, covering the bottom of the well. For freezing, cumulus cells were collected in 10 ml of RPMI 1640, 10% FCS, centrifuged at 200 × g for 5 min, and resuspended in 1 ml of freezing medium, which consisted of 95% FCS and 5% dimethyl sulphoxide (DMSO). This freezing medium, devised in previous experiments improves the viability of cumulus cells during defrosting. The pellet was dispersed gently and thoroughly with the pipette. The cell suspension was divided into 0.5 ml per vial, and cryopreserved.

Preparation of monolayer for MRC5 cells co-culture

MRC5 cells were either thawed from stored frozen stocks or obtained from fresh confluent cultures by detachment with 0.05% trypsin (Sigma) in phosphate-buffered saline (PBS) at room temperature. When all cells were detached, the trypsin was inactivated by the addition of RPMI 1640, 10% FCS and collected by centrifugation at 200 × g for 5 min. The cell pellet was resuspended in 1 ml of freezing medium, which consisted of 50% FCS, 40% RPMI 1640 and 10% DMSO at room temperature then similarly cryopreserved and cultured as previously mentioned.

Embryo culture medium

EBSS used as monoculture medium for *in vitro* embryo culture was prepared 'in house'. Commercially available sequential culture media used in this study were – Cook (which consists of Sydney IVF Cleavage medium and Sydney IVF Blastocyst medium; Cook Ireland Ltd, Limerick, Ireland), MediCult (which consists of ISM1 medium and ISM2 medium; MediCult (UK) Ltd., Surrey, UK) and Vitrolife (which consists of G-1 version 3 medium and G-2 version 3 medium; Research Instruments UK, Cornwall). The sequential culture media were used according to the manufacturer's specifications.

In vitro culture of embryos

In vitro embryo culture was carried out in 4-well plastic dishes (Nunclon, Roskilde, Denmark) containing 0.5 ml of either culture medium only (control) or co-culture (cumulus cells or MRC5 cells) growing in 0.5 ml of the same medium as the control. For co-culture systems, 24 h before embryo culture, the cell monolayers used for co-culture were washed three times in an appropriate pre-warmed culture medium from the same batch as the control wells containing culture medium only, before finally 0.5 ml of pre-warmed fresh culture medium was added. All culture wells, including controls (culture medium only), were layered with paraffin oil pre-saturated with EBSS to prevent evaporation and left to equilibrate overnight at 37^{o}C, 5% CO_2 in air. Pro-nuclear embryos collected from 4-8 females were pooled, allocated randomly to different culture systems, in equal numbers where possible. The embryos were incubated at 37^{o}C, 5% CO_2 in air and the number of embryos reaching different stages of development was recorded daily for 5 days. Observation was carried out by phase-contrast

microscopy using an Olympus inverted microscope (10 × eye-piece; 10 ×, 20 × or 40 × objective lens) fitted with a heated stage at 37 C. As the embryos were sensitive to transient cooling and UV rays, the time out of the incubator was kept to an absolute minimum.

Nuclei count

In vivo developing blastocysts and *in vitro* cultured blastocysts obtained were fixed and spread on microscope slides for the counting of nuclei. Blastocysts were incubated at 37°C for 5 min in a solution of 0.5% sodium citrate (BDH AnalaR, Poole, England), and individually placed on an ethanol-cleaned microscope slide, in as small a volume as possible. A drop of ice-cold, freshly prepared Clake fixative (methanol and acetic acid, 3:1, v:v) was placed directly over the blastocyst and left to air dry. The total cell number in the blastocyst was determined after nuclei staining with Giemsa (Sigma) at room temperature for 5 min.

Data analysis

Different culture conditions were assessed by comparing blastocyst formation rates and nuclei numbers on day 5. The nuclei numbers of *in vitro* and *in vivo* developing blastocysts were also compared. Although embryo culture was carried out from the pronuclear stage nevertheless to compensate for the variable rates of fertilization the blastulation rates were calculated based on the number of embryos that developed to 2-cells at 24 h after collection. The combined data from the replicate experiments (mean ± SEM) were analysed for significance using the parametric unpaired t test, Welch corrected or non-parametric Mann-Whitney test, where appropriate. Data analysis was performed using GraphPad Instat version 3.06 for Windows (GraphPad Software Inc., San Diego, CA, USA). All tests were two-tailed and a probability of $P<0.05$ was considered to indicate statistical significance.

Results

The blastulation rates and nuclei numbers of mouse pronuclear embryos of ICR mice cultured *in vitro* are shown in **TABLE 1**. ICR pronuclear mouse embryos do not normally develop beyond the 2-cell stage in EBSS, however it was observed that out of 560 pronuclear embryos cultured 7 developed to the blastocyst stage (**TABLE 1**), which was a cumulative result of four experiments. Cook and Vitrolife without co-culture released the 2-cell block to some extent, with a blastocyst formation rate of 14.5% and 9.8% respectively (**TABLE 1**). MediCult sequential medium showed a blastulation rate of 0.9%. Subsequently, co-culture with MRC5 increased the blastulation rate for MediCult to 12.0% and for Vitrolife to 10.2%. Nevertheless, the blastulation rate for co-culture with Cook-MRC5 was not significantly different from Cook without co-culture. The highest rates of embryo development were observed in Cook-Cumulus cells. Co-culture with cumulus cells did not show a significant improvement when used in combination with MediCult or Vitrolife media, but it doubled the blastulation rate to 30.5% when used with Cook medium (**TABLE 1**).

The nuclei numbers obtained from *in vitro* culture were compared to those obtained from *in vivo* developing embryos. Only two blastocysts were obtained from co-culture in EBSS-MRC5. These two blastocysts were unfortunately lost during the spreading of the blastocysts onto the slides for staining; therefore no data was available for the nuclei count. Correlating with the highest blastulation rates, the highest mean nuclei number was obtained for Cook-Cumulus cells. The nuclei number per blastocyst for Cook-Cumulus cells and MediCult-MRC5 co-cultures was comparable to that of *in vivo* developing blastocysts. All other culture conditions produced lower nuclei number per blastocyst compared to those developing *in vivo* (**TABLE 1**). The *in vivo* blastocysts were collected and counted between 91-100 h post hCG and *in vitro* blastocysts between 98-129 h post hCG, as they were not fully expanded until then.

It was also observed that the co-cultured ICR embryos tend to cleave slightly faster than non-co-cultured embryos and reach the 8-cell stage (day 3) earlier when cultured in the Cook-Cumulus cells (unpublished observations). The blastulation rates were also significantly different when compared to culture in the Cook medium only (Cook, 14.5% versus Cook-Cc, 30.5%). Blastocyst formation was observed from 98 hours (day 4) for culture in the Cook-Cumulus cells. It was evident that morphologically the blastocysts obtained from co-culture were fully expanded blastocysts and visibly larger in diameter than those obtained from without co-culture. The results obtained here show that co-culture coupled with a suitable sequential medium has the ability to sustain embryo development to the blastocyst stage.

TABLE 1- The effect of different culture conditions, with and without co-culture of cumulus and MRC5 cells on the development of ICR mouse embryos

Culture conditions	PN	2-cell	No. of expanded blastocysts (%)	Mean no. of nuclei per blastocyst* (n, range)
In vivo	-	-	-	52.9 ± 18.1[e] n = 103, (23-112)
EBSS	560	429	7 (1.6)[a]	31.6 ± 10.2 n = 7, (18-49)
EBSS-Cc	376	233	0 (0.0)[a]	NA
EBSS-MRC5	365	250	2 (0.8)[a]	NA
Cook	452	345	50 (14.5)[c]	37.7 ± 15.1 n = 50, (11-71)
Cook-Cc	271	226	69 (30.5)[d]	53.4 ± 21.5[e] n = 64, (17-121)
Cook-MRC5	196	147	20 (13.6)[c]	40.4 ± 11.5 n = 18, (18-54)
MediCult	422	350	3 (0.9)[a]	26 ± 4.2 n = 2, (23, 29)
MediCult-Cc	214	175	7 (4.0)[ab]	28.7 ± 10.2 n = 6, (19-43)
MediCult-MRC5	214	192	23 (12.0)[bc]	53 ± 27.8[e] n = 16, (26-118)
Vitrolife	317	214	21 (9.8)[bc]	33.7 ± 10.4 n = 20, (16-53)
Vitrolife-Cc	145	89	4 (4.5)[ab]	37 ± 22.7 n = 4, (24-71)
Vitrolife-MRC5	176	118	12 (10.2)[bc]	39 ± 9.9 n = 10, (26-59)

NA = not available
n = number of blastocysts for which nuclei count was determined, range in parentheses
[a-e]Values with different superscripts within a column are significantly different (P<0.05)
Cc = Cumulus cells
MRC5 = human foetal lung fibroblast cells

Discussion

This study examined the effect of three different commercially available complex media (sequential media systems) and whether the *in vitro* culture conditions could be further optimised when co-cultured with either MRC5 or cumulus cells. The effects of the three commercially available complex media, Cook, MediCult and Vitrolife were also compared to a simple medium, EBSS with respect to blastulation rates and nuclei numbers of pronuclear mouse embryos obtained from the ICR strain. ICR mouse embryos were used in this study as they were more sensitive to suboptimal culture conditions. The rational for this was to impose a strict quality control that might detect any changes in culture conditions that helped overcome the characteristic mouse 2-cell block, and in human *in vitro* fertilization (IVF) technology might support the development of human embryos from the pronuclear stage, through the 4- to 8-cell block to expanded and hatching blastocysts.

Sequential culture media has been formulated based on the different physiological requirements of the preimplantation embryo at the cleavage stage and from morulae to blastocyst stage (Gardner & Lane 1997). In the present study, all three sequential culture media were seen to release the 2-cell block and to some extent support normal embryonic development to the blastocyst stage. The highest blastulation rate was obtained for culture in the Cook media (14.5%) followed by Vitrolife (9.8%) and MediCult (0.9%). Cook media showed the most encouraging results. Consequently, it has also been observed (F.N. Omar Farouk and M. Vlad, unpublished observations) that culturing of embryos in the Cook media decreases the amount of fragmentation occurring when compared to the other culture media (this has also been reported to be true for human embryos – Agerholm (2005) - personal communications). The mouse embryo assay (MEA) is the most common animal model for detecting toxic factors within an embryo culture system and is used by Cook to culture embryos from hybrid mice (C57BL6♀ mated to CBA♂) from the pronuclear stage to blastocysts. MediCult also used embryos from hybrid mice (B6C3F1♀ mated to B6D2F1♂), culturing them from the 2-cell stage to blastocysts. Similarly Vitrolife like Cook, claimed to conduct the MEA from the pronuclear stage as opposed to the 2-cell stage but the strain of mice was not mentioned. In this study, using ICR embryos and culturing them from the pronuclear stage gave a much more stringent quality control for determining the most

effective culture media as it is more sensitive to mild changes. Subsequently, an aberrant outcome was observed with culture in the EBSS where development to blastocyst was observed on four separate occasions although at very low rates (1.6%). This outcome is unusual as ICR embryos do not normally develop beyond the 2-cell stage in simple salt media such as EBSS. There is a likelihood that the culture media may have been contaminated causing the 2-cell block to be alleviated in this instance or (highly unlikely) that those embryos did not possess a strict 2-cell block and went on to form blastocysts. In terms of nuclei number per blastocyst, embryos cultured in sequential media only have lower numbers compared to the *in vivo* developing embryos.

There is an evidence to support the hypothesis that the results obtained with the sequential media could be further improved when coupled with co-culture. In this study, the effects of co-culturing mouse pronuclear embryos with cumulus cells and MRC5 cells in sequential culture media were assessed by observing whether the blastulation rates and nuclei number per blastocysts could be further improved. The embryo development in co-culture was compared to culture in the same medium without the presence of feeder cells. The mean nuclei number of blastocysts obtained in all culture conditions was compared to that shown by the *in vivo* developing embryos. Various embryo species that have shown to benefit from the co-culture methods are bovine (Eyestone & First 1989; Lim et al. 1996), ovine (Rexroad 1989), caprine (Prichard et al. 1992; Yadav et al. 1998) and murine (Lai et al. 1992; Freeman et al. 1993; Walker 1994; Vlad et al. 1996; Oakley 1997). Numerous studies have shown that *in vitro* development of human embryos could be improved by co-culture with a monolayer of various cell lines such as human ampullary cells (Bongso et al. 1989; Vlad et al. 1996; Hwu et al. 1998), oviductal (Yeung et al. 1992) and endometrial cells (Simón et al. 1999; Mercader et al. 2003), bovine oviductal cells (Wiemer et al. 1993), autologous granulosa cells (Freeman et al. 1995; Feng et al. 1996), autologous cumulus cells (Mansour et al. 1994; Saito et al. 1994; Quinn 1994; Quinn & Margalit 1996) and Vero cells (Menezo et al. 1990; Schillaci et al. 1994; Guerin & Nicollet 1997). As for the choice of co-cultures, one of the concerns of using heterologous or animal reproductive or non-reproductive cells for co-culture particularly in human IVF is the cross-contamination of diseases from donor cells to the patient's embryo. Therefore, for obvious ethical reasons, the use of feeder cells of autologous origin for co-culture is an attractive option. The cell types chosen for co-culture in this study were mouse cumulus cells and MRC5 cells with potential clinical applications in mind. Cumulus cells originate from the ovary of the female reproductive tract and are a primary cell line. They were chosen because, in mice like in humans, they can be easily obtained from cumulus-oocyte complexes, therefore being safe and ideal for the human in vitro system. In the intra-cytoplasmic sperm injection (ICSI) procedure human oocytes are stripped off of their cumulus cells before sperm injection. These can be used for the culture of pronuclear embryos the following day for the same patient, eliminating the need for further testing for possible viral infection when cells from different patients or species are used. The MRC5, a diploid (2n=46) cell line derived from human foetal lung fibroblast cells, was chosen because it is available commercially, is relatively easy to handle and has shown good results in previous experiments with B6CBF1 mouse and human embryos (Walker 1994; Oakley 1997). The benefit of using commercially available cell lines like MRC5 for co-culture is that they are quality-control tested for microbes and other pathogens, and the labour involved in establishing primary oviductal cell lines can be circumvented.

For co-culture in the Cook-Cumulus cells, the blastulation rate and nuclei number were significantly higher compared to all other culture conditions with the exception of MediCult-MRC5 which despite having a much lower blastulation rate (12.0%) compared to the Cook-Cumulus cells (30.5%), gave a mean nuclei number that was similar to the Cook-Cumulus cells. Results observed for MRC5 indicate that for the co-culture to be effective it does not necessarily have to be reproductive tract or epithelial cell specific. On the contrary, reduced rates of embryo development were observed for co-culture in the Cook-MRC5 and Vitrolife-Cumulus cells compared to medium alone. This may suggest that the proportion of embryos developing to blastocysts was influenced by the combination of culture media and cell line. One of the concerns of co-culture systems is finding a culture medium which is suitable for both the cell monolayer and embryo. In this study, it has been demonstrated that both cell types MRC5 and cumulus cells show differing effects on blastulation rates in different types of media. For ICR mouse embryos, it seems that the best combination for an improved embryo development is to co-culture in Cook-Cumulus cells where Cook media seems to be able to strike a balance between the needs of the feeder cell and of the embryo.

Cumulus cells normally detach from the embryo after fertilisation. A characteristic of most somatic cells in culture is to utilize glucose and produce lactate (Gardner et al. 1996). The cumulus cells used in this study were collected after hyaluronidase treatment and either seeded immediately or frozen for later use. It was observed that some cumulus cells that were frozen tend to lose viability. Autologous cumulus cells are useful for co-culture and easily obtained as

all oocytes are surrounded by cumulus cells when retrieved from the ovary. The cumulus cells can be seeded and used for culture on the same day as the embryo retrieval, and as such they are an attractive option for busy assisted reproductive units. For patients undergoing ICSI it is a normal procedure for the cumulus cells to be removed with hyaluronidase prior to sperm injection. The cumulus cells could be collected, seeded into a drop of fresh medium and left to adhere to the culture dish while the sperm injection procedure is carried out. The same day or the following day, the injected oocytes or pronuclear embryos can be placed into a drop of culture medium with the cumulus cells. The cumulus cells that are not attached by the following day can easily be removed by pipetting. After attachment to the culture dish, it proved to be difficult to detach cumulus cells for further passaging using trypsin/EDTA. Therefore, all the co-culture with cumulus cells in this study were carried out with a monolayer obtained by seeding either fresh or frozen cumulus cells. A recent study reported improved embryo quality and blastocyst formation of ICSI derived embryos when they were co-cultured with autologous cumulus cells, suggesting the potential of using this culture system (Carrell et al. 2000). A similar study of this nature, found good pregnancy (44% per transfer) and implantation rate (46%) through this mode of co-culture for ICSI derived embryos (Wilson et al. 1998). This indicates that using cumulus cells for co-culture offers as potential clinical application particularly for ICSI patients. Furthermore, recently published studies have reported significant improvement in the morphology and blastulation rate of human embryos after cultured with attached cumulus cells left over through incomplete denudation of oocytes prior to ICSI (Ebner et al. 2006; Parikh et al. 2006) or after fertilisation for IVF oocytes (Carrell et al. 1999; 2000). Our findings confirm these observations on the benefits of cumulus cell co-culture.

In summary, the present study shows that the results obtained with sequential media can be further improved when used in co-culture. Nevertheless, the combination between the type of cell and the culture medium seems to be important. Here, embryos co-cultured in the Cook-Cumulus cells showed good morphology and significantly higher blastulation rates and nuclei numbers than all other culture conditions. Consequently, the advantage of blastocysts co-cultured in the Cook with autologous cumulus cells is generating more blastocysts with nuclei numbers that are directly comparable to the *in vivo* developing embryos. In the human clinical setting, this will allow for more blastocysts with a higher implantation potential to be transferred. Hence, Cook seems to be the most favourable culture media to be used in combination with cumulus cells for a sustainable development of preimplantation embryos during prolonged culture.

Acknowledgements

The authors would like to thank members of the Biomedical Services Unit at the University of Warwick for their technical support. This work was funded by the Council of Trust for the Indigenous People (MARA), Malaysia. The work of Dr. Farha Naomi Omar Farouk was also supported in part by the Incentive Grant (Ref. no. T115670 MEMO 25.5.09) from Universiti Sains Malaysia.

References

1. Agerholm, I. (2005). New approaches for non-invasive embryo quality assessment. *ESHRE Campus 2005: Copenhagen (Denmark), April 30th* – personal communications.
2. Bongso, A., Ng, S.C., Sathananthan, H., Ng, P.L., Rauff, M. and Ratnam, S. (1989). Improved quality of human embryos when co-cultured with human ampullary cells. *Hum. Reprod.,* 4: 706-713.
3. Bongso, A., Ng, S.C. and Ratnam, S. (1990). Co-cultures: their relevance to assisted reproduction. *Hum. Reprod.,* 5: 893-900.
4. Bongso, A., Ng, S.C., Fong, C.Y. and Ratnam, S. 1991. Cocultures: a new lead in embryo quality improvement for assisted reproduction. *Fertil. Steril.,* 56: 179-191.
5. Bongso, T.A., Fong, C.Y., Ng S.C. and Ratnam, S.S. (1994). Blastocyst transfer in human *in vitro* fertilization: the use of embryo co-culture. *Cell Biol. Intl.,* 18: 1181-1189.
6. Bongso, A., Fong, C.Y., Ng, S.C. and Ratnam, S.S. (1995). Coculture techniques for blastocyst transfer and embryonic stem cell production. *Assist. Reprod. Reviews,* 5: 106-114.
7. Braude, P., Bolton, V., and Moore, S. (1988). Human gene expression first occurs between the four and eight-cell stages of preimplantation development. *Nature,* 332: 459-461.
8. Carrell, D.T., Peterson, C.M., Jones, K.P., Hatasaka, H.H., Udoff, L.C., Cornwell, C.E., Thorp, C., Kuneck, P., Erickson, L. and Campbell, B. (1999). A simplified coculture system using homologous, attached cumulus tissue results in improved human embryo morphology and pregnancy rates during *in vitro* fertilization. *J. Assist. Reprod. Genet.,* 16: 344-349.
9. Carrell, D.T., Jones, K.P., Hatasaka, H.H., Udoff, L.C. and Peterson, C.M. (2000). Autologous cumulus cells coculture improves embryo quality and blastocyst growth of ICSI-derived

human embryos. *Fertil. Steril.,* 73: S15-S16 (abstract).

10. Crosby, I.M., Gandolfi, F. and Moor, R.M. (1988). Control of protein synthesis during early cleavage of sheep embryos. *J. Reprod. Fertil.,* 82: 769-775.
11. Desai, N.N., Kennard, E.A., Kniss, D.A. and Friedman, C.I. (1994). Novel human endometrial cell line promotes blastocyst development. *Fertil. Steril.,* 61: 760-766.
12. Ebner, T., Moser, M., Sommergruber, M., Shebl, O. and Tews, G. (2006). Incomplete denudation of oocytes prior to ICSI enhances embryo quality and blastocyst development. *Hum. Reprod.,* 21: 2972-2977.
13. Eyestone, W.H. and First, N.L. (1989). Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. *J. Reprod. Fertil.,* 85: 715-720.
14. Feng, H.L., Wen, X.H., Amet, T. and Presser, S.C. (1996). Effect of different co-culture systems in early human embryo development. *Hum. Reprod.,* 11: 1525-1528.
15. Fissore, R.A., Jackson, K.V. and Kiessling, A.A. (1989). Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. *Biol. Reprod.,* 41: 835-841.
16. Freeman, M.R., Bastias, M.C., Hill, G.A. and Osteen, K.G. (1993). Coculture of mouse embryos with cells isolated from the human ovarian follicle, oviduct and uterine endometrium. *Fertil. Steril.,* 59: 138-142.
17. Freeman, M.R., Whitworth, C.M. and Hill, G.A. (1995). Granulosa cell co-culture enhances human embryo development and pregnancy rate following *in vitro* fertilization. *Hum. Reprod.,* 10: 408-414.
18. Gardner, D.K. and Lane, M. (1997). Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum. Reprod. Update,* 3: 367-382.
19. Gardner, D.K., Lane, M., Calderon, I. and Leeton, J. (1996). Environment of the preimplantation human embryo *in vivo*: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil. Steril.,* 65: 349-353.
20. Goddard, M.J. and Pratt, H.P.M. (1983). Control of events during early cleavage of the mouse embryo: an analysis of the '2-cell block'. *J. Embryol. Exp. Morph.,* 73: 111-133.
21. Goldberg, J.M., Khalifa, E.A.M., Friedman, C.I. and Kim, M.H. (1991). Improvement of *in vitro* fertilization and early embryo development in mice by coculture with human fallopian tube epithelium. *Am. J. Obstet. Gynecol.,* 165: 1802-1805.
22. Goto, K., Kajihara, Y., Kosaka, S., Koba, M., Nakanishi, Y. and Ogawa, K. (1988). Pregnancies after co-culture of cumulus cells with bovine embryos derived from *in vitro* fertilization of *in vitro* matured follicular oocytes. *J. Reprod. Fertil.,* 83: 753-758.
23. 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.
24. Hwu, Y.M., Lee, R.K.K., Chen, C.P., Su, J.T., Chen, Y.W. and Lin, S.P. (1998). Development of hatching blastocysts from immature human oocytes following *in vitro* maturation and fertilization using a co-culture system. *Hum. Reprod.,* 13: 1916-1921.
25. Jarrell, V.L., Day, B.N. and Prather, R.S. (1991). The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, *Sus scrofa*: quantitative and qualitative aspects of protein synthesis. *Biol. Reprod.,* 44: 62-68.
26. Lai, Y.M., Stein, D.E., Soong, Y.K., Tang, Y.X., Grifo, J., Malter, H.E., Talansky, B.E., Cohen, J., Liu, H.C. and Rosenwaks, Z. (1992). Evaluation of Vero cell co-culture system for mouse embryos in various media. *Hum. Reprod.,* 7: 276-280.
27. Leppens, G. and Sakkas, D. (1995). Differential effect of epithelial cell-conditioned medium fractions on preimplantation mouse embryo development. *Hum. Reprod.,* 10: 1178-1183.
28. Lequarre, A.S., Marchandise, J., Moreau, B., Massip, A. and Donnay, I. (2003). Cell cycle duration at the time of maternal zygotic transition for *in vitro* produced bovine embryos: Effect of oxygen tension and transcription inhibition. *Biol. Reprod.,* 69: 1707-1713.
29. Lim, J.M., Rocha, A. and Hansel, W. (1996). A serum-free medium for use in a cumulus cell co-culture system for bovine embryos derived from *in vitro* maturation and *in vitro* fertilization. *Theriogenology,* 45: 1081-1089.
30. Mansour, R.T., Aboulghar, M.A., Serour, G.I. and Abbass, A.M. (1994). Co-culture of human pronucleate oocytes with their cumulus cells. *Hum. Reprod.,* 9: 1727-1729.
31. Meirelles, F.V., Caetano, A.R., Watanabe, Y.F., Ripamonte, P., Carambula, S.F., Merighe, G.K. and Garcia, S.M. (2004). Genome activation and developmental block in bovine embryos. *Anim. Reprod. Sci.,* 82-83: 13-20.
32. Menezo, Y.J.R., Guerin, J.F., and Czyba, J.C. (1990). Improvement of human early embryo development *in vitro* by coculture on monolayers of Vero cells. *Biol. Reprod.,* 42: 301-306.
33. Mercader, A., Garcia-Velasco, J.A., Escudero,E., Remohi, J., Pellicer, A. and Simón, C. (2003). Clinical experience and perinatal outcome of blastocyst transfer after coculture of human embryos with human endometrial epithelial cells:

a 5-year follow-up study. *Fertil. Steril.,* 80: 1162-1168.

34. Oakley, W.E. (1997). *Pre-implantation embryo development in vitro: IVF and coculture*. MD thesis. Department of Biological Sciences, University of Warwick.
35. Ouhibi, N., Hamidi, J., Guillaud, J. and Menezo, Y. (1990). Co-culture of 1-cell mouse embryos on different cell supports. *Hum. Reprod.,* 5: 737-743.
36. Parikh, F.R., Nadkarni, S.G., Naik, N.J., Naik, D.J. and Uttamchandani, S.A. (2006). Cumulus coculture and cumulus-aided embryo transfer increases pregnancy rates in patients undergoing *in vitro* fertilization. *Fertil. Steril.,* 86: 839-847.
37. Pool, T.B. (2004). Development of culture media for human assisted reproductive technology. *Fertil. Steril.,* 81: 287-289.
38. Prichard, J.F., Thibodeaux, J.K., Pool, S.H., Blakewood, E.G., Menezo, Y. and Godke, R.A. (1992). *In vitro* co-culture of early stage caprine embryos with oviduct and uterine epithelial cells. *Hum. Reprod.,* 7: 553-557.
39. Quinn, P. (1994). Clinical assisted reproduction. Use of coculture with cumulus cells in insemination medium in human in vitro fertilization (IVF). *J. Assist. Reprod. Genet.,* 11: 270-277.
40. Quinn, P. and Margalit, R. (1996). Beneficial effects of coculture with cumulus cells on blastocyst formation in a prospective trial with supernumerary human embryos. *J. Assist. Reprod. Genet.,* 13: 9-14.
41. Rexroad, C.E., Jr. (1989). Co-culture of domestic animal embryos. *Theriogenology,* 31: 105-114.
42. Rexroad, C.E., Jr. and Powell, A.M. (1988). Co-culture of ovine eggs with oviductal cells and trophoblastic vesicles. *Theriogenology,* 29: 387-397.
43. Saito, H., Hirayama, T., Koike, K., Saito, T., Nohara, M. and Hiroi, M. (1994). Cumulus mass maintains embryo quality. *Fertil. Steril.,* 62: 555-558.
44. Schillaci, R., Ciriminna, R. and Cefalu, E. (1994). Vero cell effect on in-vitro human blastocyst development: preliminary results. *Hum. Reprod.,* 9: 1131-1135.
45. Simón, C., Mercader, A., Garcia-Velasco, J., Nikas, G., Moreno, C., Remohi, J. and Pellicer, A. (1999). Coculture of human embryos with autologous human endometrial epithelial cells in patients with implantation failure. *J. Clin. Endocrinol. Metab.,* 84: 2638-2646.
46. Telford, N.A., Watson, A.J. and Schultz, G.A. (1990). Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol. Reprod. Dev.,* 26: 90-100.
47. Van Blerkom, J. (1993). Development of human embryos to the hatched blastocyst stage in the presence or absence of a monolayer of Vero cells. *Hum. Reprod.,* 8: 1525-1539.
48. Vlad, M., Walker, D. and Kennedy, R.C. (1996). Nuclei number in human embryos co-cultured with human ampullary cells. *Hum. Reprod.,* 11: 1678-1686.
49. Voelkel, S.A., Amborski, G.F., Hill, K.G. and Godke, R.A. (1985). Use of a uterine-cell monolayer culture system for micromanipulated bovine embryos. *Theriogenology,* 24: 271-281.
50. Walker, D.J. (1994). *"Human IVF and co-culture systems*". MD thesis. Department of Biological Sciences, University of Warwick.
51. Wiemer, K.E., Cohen, J., Wiker, S.R., Malter, H.E., Wright, G. and Godke, R.A. (1989*a)*. Coculture of human zygotes on fetal bovine uterine fibroblasts: embryonic morphology and implantation. *Fertil. Steril.,* 52: 503-508.
52. Wiemer, K.E., Cohen, J., Amborski, G.F., Wright, G., Wiker, S., Munyakazi, L. and Godke, R.A. (1989*b)*. *In vitro* development and implantation of human embryos following culture on fetal bovine uterine fibroblast cells. *Hum. Reprod.,* 4: 595-600.
53. Wiemer, K.E., Hoffman, D.I., Maxson, W.S., Eager, S., Muhlberger, B., Fiore, I. and Cuervo, M. (1993). Embryonic morphology and rate of implantation of human embryos following co-culture on bovine oviductal cells. *Hum. Reprod.,* 8: 97-101.
54. Wilson, M., Marek, D., Killoran, M.A., Ward, D., Goodrich, S., Kirkpatrick, S., Martin, L. and Madden, J. (1998). Autologous somatic cell co-culture of human intracytoplasmic sperm injection (ICSI) derived embryos: effect on pregnancy rate, implantation rate and blastocyst development. *Theriogenology,* 49: 369 (abstract).
55. Yadav, P.S., Saini, A., Kumar, A. and Jain, G.C. (1998). Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Anim. Reprod. Sci.,* 51: 301-306.
56. Yeung, W.S.B., Ho, P.C., Lau, E.Y.L. and Chan, S.T.H. (1992). Improved development of human embryos *in vitro* by a human oviductal cell co-culture system. *Hum. Reprod.,* 7: 1144-1149.