Effect of Various Culture Media on Mouse 2-cell Development and Their Viability *In Vitro*

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Abstract

Developing a culture system for embryos has important biotechnological implications due to the potential to produce large number of preimplantation embryos. As an initial step toward accomplishing this long-term goal, a study was conducted to determine the effects of culture media on embryo development *in vitro*. These studies were designed to examine the development of mouse embryos from the 2-cell to the hatched-blastocyst stages in the presence of various co-culture systems comparing with culture medium alone. *In vivo* fertilized 2-cell embryos were surgically collected from superovulated mice 48 hrs after mating. Embryo were randomly divided into 7 culture groups as follows: culture with TCM199+BSA, commercial 1 or commercial 2; co-culture with bovine, porcine, swamp buffalo oviduct epithelial cells or mouse granulosa cells. After 72 hrs in culture, the proportion of 2-cell embryos developed to blastocyst in commercial 2 or swamp buffalo oviductal cells were significantly higher than other culture media or co-culture. The embryos developed to hatched blastocyst were significantly higher in commercial 2 medium than swamp buffalo. Physicochemical analysis indicated that the substances in all culture media were predominantly detected at 66 kDa. These results revealed that embryos cultured in medium alone were more effective in supporting mouse embryo development than the embryos cultured in co-culture system. Therefore, the available commercial culture medium has provided a new *in vitro* model for embryo production research in numerous species of animals.

Keywords: mouse embryo, embryo development, hatched-blastocyst, co-culture

Introduction

Preimplantation embryos of most mammalian species normally do not develop into blastocysts in *in vitro* culture systems. It has been difficult to culture mouse embryos *in vitro* due to block at the two- and four-cell stage (Chatot et al., 1985). A similar developmental block *in vitro* was reported in bovine embryos at the 8- to 16-cell stage (Thibault, 1966), pig embryos at the four-cell stage (Davis and Day, 1987), hamster embryos at the two- to four-cell stage (Whittingham and Bavister, 1974), and human embryos at the two-cell stage (Tarin et al., 1994). Many studies have been undertaken to optimize culture conditions and several chemically-defined media that support development of embryos have been reported (Whittingham et al., 1974; Ellington et al., 1990). The concept of improved preimplantation development and implantation development, and implantation ability by co-culturing embryos in the presence of another cell type (feeder cells) as led to the development of the co-culture system. Most commonly used cell types include cumulus cells (Saito et al., 1994; Quinn and Margalit, 1996), oviductal epithelial cells (Neimer et al., 1993; Pavasuthipaisit et al., 1994; Minami, 1996), trophoblastic vesicles and granulosa cells (Plachot et al, 1995). Numerous reports have demonstrated the beneficial effects of co-culture on embryo development, such as improved quality of embryos and the increased rate of embryo development (Freeman et al., 1995; Weimer et al., 1998). Reproductive physiology during preimplantation development has been widely studied and the results have been utilized for establishing effective embryo culture systems. In most mammalian species, *in vivo* fertilized embryos develop through early cell cycle stages in the lumen of the oviduct (Sirard et al., 1985; Minami et al., 1988; Rieger et al., 1995). The environment within the oviduct enhances the fertilization potential of sperm and promotes the development of cleaving embryos. The suggested beneficial effects of co-cultures include the secretion of embryotrophic factors such as nutrients and substrates, growth factors, and cytokines and the removal of potentially harmful substances such as

heavy metals, ammonium, and free radical formation, detoxifying the culture medium (Weimer and Cohen, 1990). However, one difficulty with such primary cultures has been the inconsistencies between different preparations in their ability to sustain embryo development. At the same time, culture media capable of sustaining embryo development to the blastocyst stage without the use of co-culture systems were developed (Huisman et al., 1994; Krisher et al., 1999; Langendonchkt et al., 2001; Macklon et al., 2002). Increasing understanding of the dynamic nature of early embryo metabolism (Leese et al., 1986; Hardy et al., 1989; Gott et al., 1990; Mao et al., 2002) and the changing environment encountered by the preimplantation embryo *in vivo* (Gardner et al., 1996) led to the formulation of sequential culture media. These were designed to support the growth of human pronuclear embryos to the blastocyst stage (Barnes et al., 1995; Behr et al., 1999). Results achieved with culture media appeared to be the same range as those reported when embryos were co-cultured with feeder cell (Jones, 1998; Swain et al., 2001).

The aim of this study was to compare the four co-culture systems: bovine oviductal cells, swamp buffalo oviductal cells, porcine oviductal cells, or granulose cells with commercially available culture media; tissue culture medium 199 supplemented with BSA (TCM 199 + BSA), commercial culture medium company 1 and commercial culture medium company 2, in terms of developmental capacity to the blastocyst and hatching blastocyst stage. The proteins profile of each co-culture system and culture medium were also tested.

Materials and Methods

Isolation of oviductal tissue

Bovine, porcine, and swamp buffalo oviducts from post-ovulatory animals were obtained from slaughterhouses and transported to the laboratory in 157mM NaCl containing 50µg/ml of gentamicin at 4^{o}C. Following removal of connective tissue, the oviducts were rinsed with 157 mM NaCl (containing 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B) (Life Technologies, USA) and blotted on sterile gauze to remove excess fluid and blood. The oviducts were then placed in sterile 100 mm petri dishes (Nunclon, Denmark), grasped with a forceps at the isthmic end and scraped gently toward the infundibulum with a glass microslide. Mucosal tissue was extruded from the ostium abdominale and transferred to a 12 ml conical centrifuge tube with 10 ml of HEPES buffered Tyrode's medium (TALP-HEPES) supplemented with 10% heat-treated fetal calf serum (HTFCS) (Life Technologies, USA). The tissues was then washed in 5 to 7 changes of TALP-HEPES (containing 10% HTFCS, 50 µg/ml of gentamicin) (Life Technologies, USA) and then resuspended in TCM 199 to a ratio of 1:50. Five milliliters of the suspension was placed in a 60 mm Falcon dish and cultured at 39^{o}C under an atmosphere of 5% CO_2 and 95% air. The cells from 3 species were used for co-culture when they were above 80% confluency (Days 3 to 5).

Superovulation and embryo recovery

ICR mice (4-6 weeks old) were obtained from the National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. They were housed under a 12:12-h light-dark cycle and maintained at 24$\pm$1^{o}C. The protocol had been approved by the Naresuan University Ethical Committee.

Female ICR mice were induced to superovulate by intraperitoneal (i.p.) injection of 5 IU pregnant mare serum gonadotrophin (PMSG) (Sigma, USA) 5 IU human chorionic gonadotrophin (hCG) (Sigma, USA) given 48 hrs apart. They were paired with males of the same strain after the injection of hCG and inspected the following morning for the presence of vaginal plug (Day 1).

Two-cell embryos were flushed from the excised oviducts at 48 hrs after hCG injection using Dulbecco's phosphate buffered saline supplemented with 10% heat inactivated calf serum (CS) (Life Technologies, USA). After collection, morphologically normal embryos were washed twice with PBS and 10% CS medium.

In Vitro culture

Seven different culture treatments were employed in this study; TCM199 supplymented with 3 mg/ml Bovine serum albumin (BSA) (Sigma, USA), commercially available medium company 1, commercially available medium company 2, co-culture in oviductal cells collected from porcine, and swamp buffalo or bovine. TCM199+BSA was considered as control groups. Harvested 2-cell embryos were randomly cultured in 50 µl droplets of medium or co-culture with oviduct cells under paraffin oil (10 to 20 embryos/droplet). The culture was maintained in 37^{o}C, 5% CO_2 and 95% air. Embryos were cultured for 96 hrs to assess their ability to develop into blastocyst and expanded blastocyst stages. Embryos completely free of the zona pellucida were counted as having completely hatched.

SDS-PAGE and protein staining

The culture supernatants from each culture media were analyzed by 12.5% SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel eletrophoresis) according to the method of laemmli (Laemmli, 1970). The samples were mixed with Laemmli sample buffer at equal volume, boiled at 95^{o}C for 5 mins, and electrophoresis at constant current of 40 mM for 1 hr. The gel was stained with coomassie brilliant blue (CBB) (Merck, Germany) for 2 hrs followed by destaining. The molecular weight of the proteins was estimated by using the molecular weight marker.

Statistical analysis

The results were subjected to one way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. P values of < 0.05 was considered to be statistically significant.

Results

In vitro development of two-cell stage mouse embryos

Table 1 shows the proportion of two-cell mouse embryos developed to the blastocyst and the hatching blastocyst stage during 5 and 7 days of *in vitro* culture. Embryos completely free of the zona pellucida were counted as having completely hatched (Figure 1). The 2-cell mouse embryos could survive and develop to blastocyst in both medium alone or in co-culture system. The survival rate of 2-cell mouse embryos were lower in basic medium (TCM199 + BSA) ($p<0.05$) compared with other mediums. Further, development of embryos to blastocyst stage gave 81%, 27% and 19% in commercial medium company 2, commercial medium company 1 and TCM 199 + BSA, respectively. In the co-culture group, the proportion of embryos developed to blastocyst in swamp buffalo oviductal cells, porcine oviductal cells, granulosa cells, and bovine oviductal cells (83%, 47%, 45% and 38%), respectively. The significantly more embryos developed to the blastocyst stage in swamp buffalo oviductal cells and commercial medium company 2 compared with other culture media. However, there were differences in the percentage of embryos reaching the hatching blastocyst stage between co-culture in swamp buffalo oviductal cells (33%) and cultured in commercial medium company 2 medium (54%).

Table 1 The number and percentages of embryos developing to blastocyst and hatched blastocyst stages in TCM199+BSA, commercial 1, bovine oviductal cells, swamp buffalo oviducal cells, porcine oviductal cells, granulose cells and commercial 2 treatments after 5 days of culture

Experimental group	No. of 2-cell embryos	No. of survival (%)	Blastocyst (%)	Hatched blastocyst (%)
1. TCM 199 + BSA	64	15 (23)[a]	12 (19)[a]	0 (0)[a]
2. Commercial medium company 1	103	80 (78)[c]	28 (27)[b]	10 (10)[b]
3. Commercial medium company 2	99	87 (88)[d]	80 (81)[d]	53 (54)[d]
4. Granulosa cells	121	98 (81)[c]	54 (45)[c]	30 (25)[c]
5. Swamp buffalo oviductal cells	96	84 (88)[d]	80 (83)[d]	32 (33)[c]
6. Bovine oviductal cells	80	56 (70)[b]	30 (38)[c]	11 (14)[b]
7. Porcine oviductal cells	72	61 (85)[d]	34 (47)[c]	12 (17)[b]

Note: [a, b, c, d] Different superscripts within columns indicated significant differences (P<0.05)

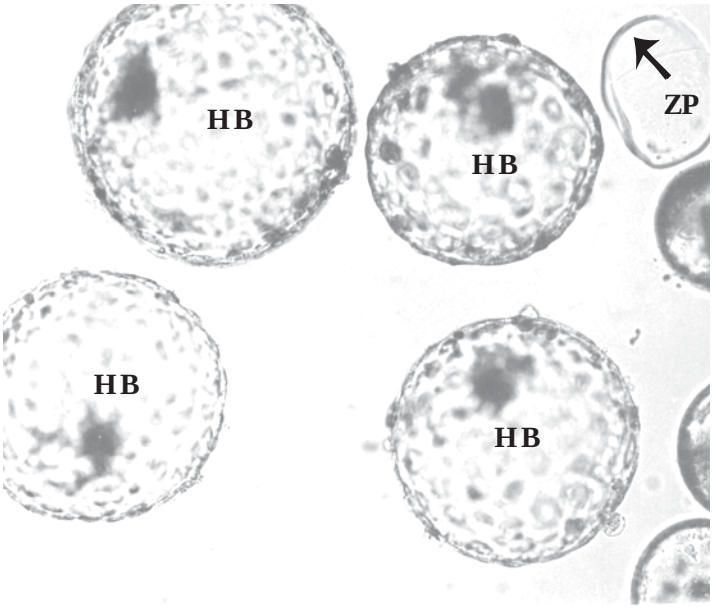

Figure 1 Representative hatched blastocyst stage of mouse embryos HB = Hatched blastocyst; Zp = Zona pellucida

Comparison of protein profile of culture media

The protein profile collected from supernatant of each culture media was analyzed by SDS-PAGE. As shown in Figure 2, protein at 66 kDa was predominantly presented in all culture media but only slight differences in intensity. The 14-45 kDa were not presented in commercial medium company 1 and commercial medium company 2 culture media but presented in all co-culture media and TCM199+BSA.

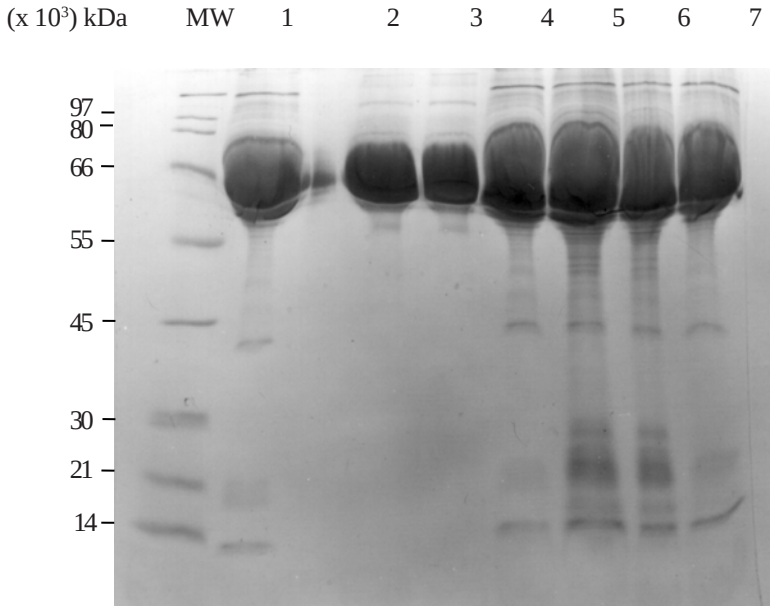

Figure 2 The profile of protein in each culture media was analyzed by SDS-PAGE. Samples were electrophoresed on 12.5% separating gels under reducing conditions and stained with Coomassie blue. Lane 1, TCM199 + BSA; Lane 2, commercial culture medium 1; lane 3, commercial culture medium 2; lane 4, granulose cell; lane 5, swamp buffalo oviductal cells; lane 6, bovine oviductal cells; lane 7, porcine oviductal cells. Molecular mass markers are also indicated to the left of the gel.

Discussion and Conclusion

The presence study was designed to compare the different approaches to enhance the proportion of embryos reaching the blastocyst and hatching blastocyst stage. Our interest in starting with two-cell embryos was to avoid the ambiguity of gauging that successful fertilization had taken place. Two-cell mouse embryos could develop to the blastocyst stage in all culture system but the rate of blastocyst formation was significantly higher in commercial medium company 2 (81%) and swamp buffalo oviductal cell (83%). Compared with other commercial complete media, co-culture system from various species provided a superior culture system for blastocyst development *in vitro.* A variety of complete media including TCM199+BSA and commercial medium company 1 have been used in attempt to develop culture system for blastocyst formation in a number of species. Moreover, in a comparison of two commercial available culture media, no difference in outcomes in the blastocyst formation was observed. The presence study has shown that oviductal cells from swamp buffalo, porcine and bovine support mouse embryo development *in vitro*.

Many co-culture studies of mammalian embryos, including human cells cultured with various other cell types, have generally shown that co-culture improves the quality of embryos, the rate of blastulation, and the rates of implantation and pregnancy following embryo transfer (Freeman et al., 1995). The result of this study confirms previous reports that the beneficial effects of oviduct on embryonic development from 2-cells to expanded and hatched blastocyst stages are not-species specific (Pavasuthipaisit et al., 1994). On the other hand, the proportion of hatching blastocyst formation in commercial medium company 2 (54%) was significantly higher than that observed in swamp buffalo oviductal cell (33%). When culture was prolonged to the hatching blastocyst stage, higher implantation rates were achieved. The results indicated that the synthetic media, commercial medium company 2 medium responded well to the need of the embryo despite the absence of a more physiological cellular environment.

The embryos cultured in swamp buffalo oviductal cells could not develop to hatched blastocyst stage, the zona pellucida hardening may occurred. The pattern of proteins was shown similar in all co-culture media. Most intensity band of protein from both co-culture media and commercial culture media were detected at 66 kDa. This protein may play an important role in embryonic development. Moreover, all co-culture media and TCM199+BSA medium contain proteins molecular weight between 14-45 kDa, but did not found in commercial media commercial medium company 2. These proteins may not necessary for embryo development. This result suggested that commercial medium company 2 may contain other embryotrophic components such as glycoproteins or growth factors that necessary for embryo development. The present results also showed that commercial medium company 2 culture medium overcame co-culture system and allowed the two-cell mouse embryos to successfully develop to the hatching blastocyst.

It may be concluded that commercial medium company 2 medium contains the nutrients required by the preimplantation embryo at the different stages of development. The applicability of our results to other laboratory settings is inevitably limited by other local factors which may have affected the study outcome. However, the results of this study indicate that mouse embryos do not necessarily have to be cultured in co-culture to fully develop into preimplantation embryo.

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