韓國家畜繁殖學會誌 18(2) : 87~94(1994)
Korean J. Animal Reprod.

Effects of Human Amniotic Fluid Supplemented to Whitten's Medium on Development and Outgrowth of Mouse Embryo

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Whitten 배양액내 인간양수의 첨가가 생쥐 수정란의 체외발달 및 체외신장에 미치는 영향

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요 약

본 연구는 생쥐 초기배 및 부화후 체외신장에 미치는 인간 양수의 첨가효과를 조사하고자 실시하였다. 생쥐 수정란의 체외발달에 미치는 인간 양수의 적정농도를 확인하고자 기본배양액 (Whitten's medium)에 16주령의 양수 농도를 각각 달리하여 체외배양을 실시한 결과, 20%를 첨가한 구에서 가장 높은 배반포로의 발달율과 부화율을 나타내었으며, 그 이상의 농도에서는 배발달율이 저하되었다. 포유동물 수정란 체외배양시 가장 많이 사용되는 첨가제인 fetal calf serum (FCS)과 bovine serum albumin (BSA)을 첨가한 구와의 배발달 성적을 비교한 결과, 임신중기 양수를 20% 첨가한 구의 배반포로의 발달율 (92.8%)과 부화율 (75.7%)은 기본배양액에서 배양된 것보다 배반포로의 발달율 (82.8%)과 부화율 (31.3%)은 높았으나 0.3% BSA (90.5%, 70.8%)나 10% FCS 첨가구 (94.3%, 74.3%)와는 유의한 차이가 인정되지 않았다. 임신주령에 따른 양수의 첨가효과를 조사한 결과 20%의 임신말기 양수를 첨가한 구의 배반포로의 발달율 (71.9%)과 부화율 (57.3%)은 20% 임신중기 양수를 첨가한 구보다 낮았으며, 부화후 배의 체외신장은 임신중기 양수와 FCS이 첨가된 구에서는 유기되었으나, 임신말기 양수와 BSA가 첨가된 구에서는 배의 체외신장이 유기되지 않았다. 이상의 본 연구결과를 통해 임신중기 양수 내에는 배발달 촉진인자와 배의 체외신장을 유기시키는 물질이 함유되어 있어 포유동물 배의 체외배양에 상당한 영향을 미치고 있다고 사료된다.

I. INTRODUCTION

Embryo culture systems that support a degree of development to the blastocyst and post-blastocyst stage equivalent to *in vivo* development are needed for the application of various manipulation techniques and for the study of normal development (Kane, 1987). These culture systems are usually supplemented with serum or bovine serum albumin for chelation of detrimental ions, stabilization of cell membrane, and hatching (Wright et al., 1978; Kane and Headon, 1980; Allen et al., 1982; Caro and Trounson, 1984; Shirley et al., 1985). The variability of embryo growth and occasional difficulties in

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obtaining good qualities of serum have prompted the search for serum alternatives such as peritoneal fluid, oviductal fluid, or uterine fluid (Morcos et al., 1985; Menino et al., 1988; Archibong et al., 1989; Collas et al., 1991). The addition of body fluids to culture medium or the use of body fluids alone, however, has been shown to have inconsistent effects on preimplantation development *in vitro* of embryos. An alternative for improving embryonic development *in vitro* is to use other natural body fluids that support embryonic development *in vivo*.

Mouse embryos can be cultured to the blastocyst stage in any of a number of relatively simple media of which formulations include salts, glucose, pyruvate, lactate, and bovine serum albumin. Whereas some of these blastocysts can be hatched from zona pellucida, further development will not occur unless the embryos are transferred to a richer medium which characteristically includes amino acids and serum (Gwatkin, 1966; Hsu, 1971; Spindel and Pederson, 1973). For inducing embryonic outgrowth, a number of laboratories developed *in vitro* culture systems using various culture media supplemented with serum or extracellular matrix components coated plates (Rizzino and Sherman, 1979; Hayman et al., 1985; Carson et al., 1988; Carnegie, 1991).

Amniotic fluid (AF) is an ultrafiltrate produced in utero in intimate contact with the developing fetus. Since AF is less variable in chemical composition than serum and is likely to contain the embryo (Mohamed & Noakes, 1985; Wintour et al., 1986), we considered that AF may be a more appropriate serum alternative for embryos than other alternatives currently in use. Furthermore, outgrowth of embryos *in vitro* using hAF has not been reported. In the present study, we tested hAF for its ability to support mouse 2-cell embryos culture to the blastocyst stage and to induce embryonic outgrowth.

II. MATERIALS AND METHODS

1. Preparation of human amniotic fluid

Amniotic fluid was obtained from patients undergoing amniocentesis on the mid-term (16 and 20 weeks) and full-term (38 weeks). The supernatants after centrifugation (500×g for 15 min) were heat inactivated at 56℃ for 30 min. The fluid was then filtered (0.22 μm pore size, Gelman Sci., USA) and stored at −20℃ refrigerator until required for the culture of embryos.

2. Recovery and culture of mouse embryos

Immature female ICR mice were superovulated with 5 IU of pregnant mares serum gonadotropin (PMSG : Pregnecol, Heriot, Netherland) and 5 IU of human chorionic gonadotropin (hCG : Pregnyl, Organon, USA) 48 hours after PMSG injection. Two-cell mouse embryos were flushed from the oviducts approximately 48 hours after hCG injection. Micro-drops for culture of embryos was made under paraffin oil and equilibrated for 12 hours in an atmosphere of 5% CO_2 in air at 37℃. Embryos were washed twice in the basic Whitten's medium (Whitten, 1957) before being placed into the final drop of respective treatments, and then incubated at 37℃ in an atmosphere of 5% CO_2 in air.

3. Polyacrylamide gel electrophoresis

Proteins according to gestation stages were separated on a slab gel of 8 and 12% polyacrylamide in the persence of sodium dodecyl sulfate and 2-mercaptoethanol (Laemmli, 1970). Protein bands on the gel were detected by silver staining (Silver stain kit, Sigma, USA).

4. Assessment of embryonic outgrowth

After embryo culture for 7 days, the out-

growth was examined according to morphology of inner cell mass and trophoblast with an inverted microscope.

5. Statistical analysis

Data were analyzed with a analysis of variance (ANOVA). When ANOVA revealed a significant treatment effect, each treatment was compared by student's t-test.

Ⅲ. RESULTS

1. Effects of hAF concentrations on the development of mouse embryos.

We investigated whether the beneficial effect of hAF on embryo development occurred in a dose-dependent manner. The proportion of embryos that developed into blastocyst and hatched blastocyst was significantly increased when mid-term hAF (16 weeks) was added in the embryo culture medium from 10 to 50% compared to culture medium only (Table 1). The highest development rate of blastocyst (92.8%) and hatched blastocyst stage (75.7%) was found in the culture medium containing 20% mid-term hAF. However, development rate in hAF alone was declined compared to other groups (Table 1).

2. Effects of hAF, FCS, BSA on the culture of mouse embryos

The possibility of using hAF for culture of mouse embryos in vitro was investigated by comparing the extent of development to that of em-

Table 1. Development rates of mouse embryos in Whitten's medium containing the various concentrations of mid-term human amniotic fluid (hAF) collected from 16 weeks pregnant women

Treatments	No. of 2-cell embryos cultured	No. (%) of embryos developed to	
		Blastocyst	Hatched Blastocyst
Medium only	150	121(80.7)[a]	45(30.0)[a]
10% hAF	170	155(91.2)[b]	127(74.7)[b]
20% hAF	181	168(92.8)[b]	137(75.7)[b]
30% hAF	171	150(87.7)[b]	119(69.6)[b]
50% hAF	168	150(89.3)[b]	89(53.0)[c]
100% hAF	136	19(14.0)[c]	0(0)

[abc] Values with different superscripts within columns are significantly different ($P<0.05$)

Table 2. *In vitro* development of mouse embryos cultured in Whitten's medium containing 0.3% bovine serum albumin (BSA), 10% fetal calf serum (FCS), and 20% mid-term human amniotic fluid (hAF)

Treatments	No. of 2-cell embryos cultured	No. (%) of embryos developed to		
		Blastocyst	Hatched blastocyst	Outgrowth
medium only	163	135(82.8)[a]	51(31.3)[a]	0(0)
0.3% BSA	168	152(90.5)[b]	119(70.8)[b]	0(0)
10% FCS	140	132(94.3)[b]	104(74.3)[b]	104(74.3)
20% mid-hAF	181	168(92.8)[b]	137(75.7)[b]	135(72.9)

[abc] Values with different superscripts within columns are significantly different ($P<0.05$)

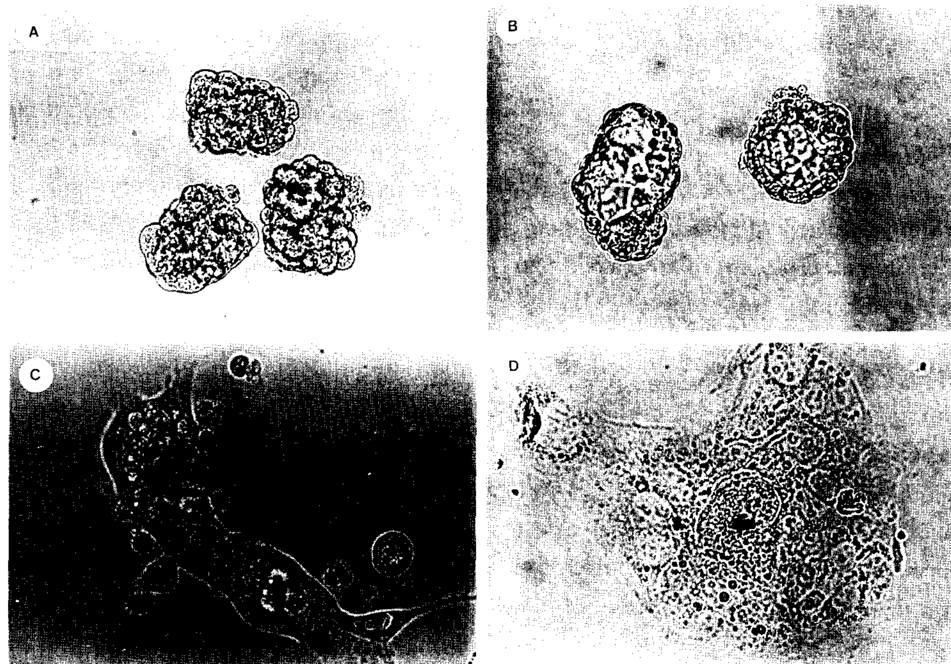

Fig. 1. Outgrowth of mouse blastocyst cultured in the presence of 20% mid-term human amniotic fluid for 5 days (A), 6 days (B), 7 days (C), and 10% fetal calf serum (D), A cluster of inner cell mass cell (arrow) can be seen lying on top of flattened, spread trophoblastic cells (A, B, C : × 320, D : ×200).

Table 3. *In vitro* development of 2-cell mouse embryos in Whitten's medium containing 20% mid- and full-term human amniotic fluid (hAF)

Treatments	No. of 2-cell embryos cultured	No. (%) of embryos developed to		
		Blastocyst	Hatched blastocyst	Outgrowth
Medium only	96	90(93.8)[a]	21(21.9)[a]	0(0)
16 weeks hAF(20%)	96	88(91.7)[a]	72(75.0)[b]	71(74.0)
20 weeks hAF(20%)	81	75(92.6)[a]	62(76.5)[b]	58(71.6)
38 weeks hAF(20%)	96	69(71.9)[b]	55(57.3)[c]	0(0)

[abc] Values with different superscripts within columns are significantly different ($P<0.05$)

bryos cultured in 10% FCS or 0.3% BSA. As shown in Table 2, no significant differences were observed among 20% mid-term hAF, 0.3% BSA, and 10% FCS treated group. Outgrowth of hatched blastocyst were observed in medium containing 20% mid-term hAF or 10% FCS but not in other groups. Embryos with FCS, however, showed a considerably greater extents of trophectodermal cell proliferation and outgrowth than those with hAF (Fig. 1, C, D).

3. Effects of hAF according to gestation

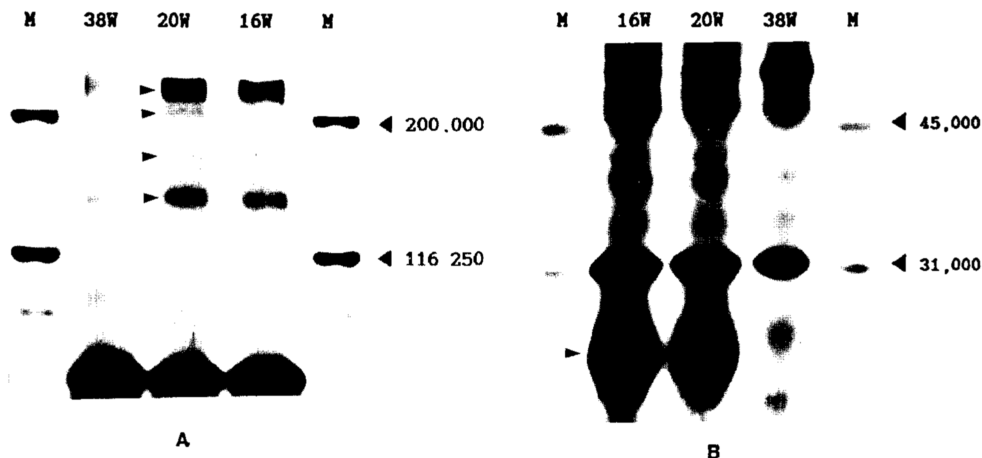

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic trends of proteins present in human amniotic fluids (hAF). M : size marker, 16W : 16 weeks hAF, 20W : 20 weeks hAF, 38W : 38 weeks hAF.

stage on development of mouse embryos.

Effects of hAF collected from different gestation periods on embryo development are depicted in Table 3. Developmental and hatched rates of 2-cell mouse embryos cultured in full-term hAF (38 weeks) were declined and their outgrowth phenomenon was not observed. But there were no significant differences between 16 and 20 weeks hAFs in development and outgrowth of embryos. Human AF samples obtained at different stages of gestation were subjected to SDS-PAGE. In the gel electrophoretic trends of proteins, mid-term hAF had more proteins in high molecular weight (>110 KD, Fig. 2, A) and low molecular weight (<30KD, Fig. 2, B) than those of full-term.

Ⅳ. DISCUSSION

At the present study, we investigated the use of hAF for the culture of mouse embryos in vitro. Development rates of mouse embryo cultured in 20% mid-term hAF was similiar to simple medium supplemented with 10% FCS or 0.3% BSA (Table 2). Serum and BSA have been shown to have detrimental effects on embryo development (Menezo et al., 1984; Shirley et al., 1985; Caro and Trounson, 1986). BSA and serum are not chemically defined and have variability from lot to lot (Kane, 1983). Purpose of using serum or BSA is that the valid effects of serum are due to cyclic adenosine monophosphate, catecholamines, vitamines, growth factors, lipids, and albumin, and those of BSA are due to amino acid source, chelation of heavy metals, and effect of trace contaminants (Kane, 1985, Gray et al., 1992). Our result (Table 2) indicates that the same effect of hAF as FCS or BSA on the culture of mouse embryo seems that mid-term hAF contain both the correct ionic and nonionic composition, and also contain the necessary growth factors to optimize the environment of embryo development (Wales, 1970; Mohamed and Noakes, 1985; Wintour et al., 1986). In full-term hAF, since concentration of urea and uric acid changes to resemble urine value (Wales, 1970), it seems that these noxious components such as uric acid or urea have detrimental

effects on embryo development (Table 3). Mid-term hAF has been sucsessfully used by itself for culture of mouse embryos (Gianaroli et al., 1986; Dorfmann et al., 1989). However, our result (Table 1) in mid-term hAF alone showed detrimental effect on the culture of mouse embryos. The reason for this discrepancy is unclear.

Human AF collected from mid-term stage showed higher rates of development and outgrowth than those from full-term stage (Table 3). Electrophoretic trends of proteins showed considerable variation in amount and composition of hAF produced between gestation periods (Fig. 2). Unfortunately, the functions of mid-term specific proteins are speculative what components of hAF are effective in development and outgrowth of embryos at this time.

Our finding (Fig. 1, C) showed that mid-term hAF-containing medium promotes their outgrowth and differentiation of trophoblast cells. However, hAF-containing medium does not support gross post-blastocyst development as typical as serum-containing medium. In particular, trophoblast outgrowths take place less extents than those in serum-containing medium. These observations lead us to suggest that cells of blastocyst are strongly disposed toward certain characteristic differentiative pathways.

In this study, our findings suggest that mid-term hAF have a beneficial effect on mouse embryo development as well as outgrowth in vitro. In conclusion, mid-term hAF may be beneficial for the development of 2-cell mouse embryos, and play a major role in inducing outgrowth of embryos.

V. ABSTRACT

The objective of this study is to evaluate the developmental ability of mouse embryo in the presence of human amniotic fluid (hAF). The highest development rate was found in the culture media supplemented with 20% mid-term hAF but this rate was concomitantly reduced with more than 20% hAF. Furthermore, mouse two-cell embryos cultured in 20% mid-term hAF were developed more consistently to the expanded and hatched blastocyst stages compared to those cultured in simple medium. However, no significant differences in the embryo development rates were observed among the supplemented effects of 20% mid-term hAF, 0. 3% bovine serum albumin (BSA), and 10% fetal calf serum (FCS). Development rates of two-cell mouse embryos cultured in 20% full-term hAF were declined compared to 20% mid-term hAF. Outgrowth of hatched blastocysts were observed when the embryos were cultured in medium containing 20% mid-term hAF or 10% FCS. But two-cell mouse embryos cultured in the presence of 20% full-term hAF or 0. 3% BSA was not observed their outgrowth. The kinetics of outgrowth processes in the presence of hAF were similar to those with 10% FCS. However, embryos with FCS showed a considerably greater extents of trophetodermal cell proliferation and outgrowth. Taken together, these data suggest that mid-term hAF may have a suitability for the mammalian embryos and induce embryonic outgrowth.

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