Development of Mouse Two-Cell Embryos to Hatched Blastocysts in a Medium Supplemented with Bovine Amniotic Fluid

Akira OKANO and Masashi TAKAHASHI

Department of Animal Reproduction, National Institute of
Animal Industry, Tsukuba Norin Kenkyu Danchi,
Ibaraki-ken, 305

(Received April 13, 1994)

Abstract This study was conducted to determine the effect of bovine, porcine and avian amniotic fluid (AmF) on the *in vitro* development of mouse embryos. The growth rates from 2-cell embryo through to hatched blastocyst (Hblast) were examined in whole AmF or in culture media supplemented with and without AmF for 96 h. Whole bovine, porcine and avian AmF were not effective for the development of mouse embryos cultured *in vitro*. In a BSA-free M16 medium supplemented with 10% bovine AmF, the development of 2-cell embryos was equal to that in M16 supplemented with BSA. The effective factor in bovine AmF was destroyed by heat treatment at 100°C for 5 min, and was not absorbed with Dextran-charcoal treatment. The molecular weight (MW) of this fraction was less than 10^4 . Therefore, this factor is not a steroid or a lipid but presumably a polypeptide of MW less than 10^4 .

Anim. Sci. Technol. (Jpn.) 65 (10) : 923-927, 1994

Key words : bovine, amniotic fluid, mouse embryo, hatching

Various culture media have been developed for culturing early mammalian embryo *in vitro*. However, most culture media are either supplemented with 10 to 20% bovine serum, bovine serum albumin (BSA) or serum of the same species, and the effective factors in the sera of the some species or in BSA are still unknown^{4,7)}. On the other hand, GIANAROLI *et al.*²⁾ found that human amniotic fluid (AmF) was effective for *in vitro* fertilization of human oocytes and their subsequent culture as well as *in vitro* culture of mouse embryo. DORFMANN *et al.*¹⁾ reported that human AmF was a more effective medium for the *in vitro* culture of mouse embryos than defined synthetic media. The effective factor in avian and porcine AmF on early embryos cultured *in vitro* is also un-

known. The effects of bovine AmF and allantoic fluid as culture media for mouse embryos have been studied³⁾. Others¹⁻³⁾ have proposed that the effect of AmF on culturing embryos depends on the fact that AmF originates from the developing fetus.

Our study was conducted to determine the effects of bovine, porcine and avian AmF on the development of mouse early embryos cultured *in vitro* from 2-cell embryos through to hatched blastocysts (Hblast).

Materials and Methods

Collection and preparation of amniotic fluid : Bovine and porcine AmF were directly obtained by syringe from the amniotic cavity of removed and incised uteri. Bovine AmF was

collected at the slaughterhouse from five fetal calves of approximately 70 days gestation. Fetal ages were estimated from their crown-rump length⁶⁾. Porcine AmF was collected from an experimental slaughterhouse in our Institute from five pigs of 20 to 30 days gestation. Avian AmF was directly taken from the amniotic cavity of fifty fertilized eggs at 6 days following brooding, by manually cracking the egg shell and exposing the air sac. To remove the cells and other debris, AmF from the three species was centrifuged and then inactivated by heating at 56°C for 30 min, and stored at -80°C until used in experiments. AmF from the three species was pooled and filtered through a 0.45 µm pore size filter unit (Toyo Roshi, Tokyo, Japan) to remove microorganisms.

Treatment and fractionation of bovine amniotic fluid: Bovine AmF was treated as follows: 1) heated at 100°C for 5 min (H. treat) or, 2) steroids and lipids were absorbed as follows: a 10% volume of Dextran-charcoal suspension (DC) was added to AmF in a tube, and the mixture was shaken for 15 min at room temperature. The tube was centrifuged at 1500 G for 15 min, and supernatant was removed as DC treated AmF. DC was prepared by dissolving 25 mg Dextran T70 (Pharmacia, Uppsala, Sweden) in 100 ml PBS and adding 250 mg Norit A charcoal (J.T. BAKER, Phillipsberg, NJ). Bovine AmF was fractionated based on molecular weight (MW) as follows: 3) MW less than 10^4 , 4) MW between 10^4 to 3×10^4 , 5) MW more than 3×10^4 . MW fractions of AmF were separated by centrifugation at 1500 g, over 20 min, using Ultracento-10 and -30 (Tosoh, Tokyo, Japan). Ultracento-10 and -30 cuts off over 99% of molecules whose MW are larger than 10^4 and 3×10^4 , respectively.

Collection of 2-cell mouse embryos: Adult female ICR mice were kept on a schedule of 12 h light and 12 h darkness. They were injected with 5 IU PMSG, and 24 h later, ovulation was induced by 5 IU HCG. They were mated with

ICR male mice and checked the following morning for the presence of copulatory plug. Two-cell embryos were flushed from the oviducts with PBI approximately 44 to 46 h after mating⁸⁾.

Culture and observation of mouse embryos: Two-cell embryos were placed in 50 µl drop of culture medium, and covered with mineral oil (Squibb, Princeton, NJ). The embryos were incubated at 37°C in 5% CO₂/95% air, and were observed after 96 h for Hblast formation. The control medium was M16 supplemented with BSA (4 mg/ml)⁸⁾. Growth rates of embryo culture in whole AmF from the three species were compared to those in M16. In addition, the growth rates of embryos cultured in M16 and/or BSA-free M16 (M16NBSA) supplemented with 10% (V/V) avian, porcine and bovine AmF, or the fractions of bovine AmF, were also compared with each other.

Statistical analysis: Differences in the development of 2-cell embryos through to Hblast were analyzed by χ^2 test.

Results

The results of development of 2-cell embryos through to Hblast, cultured in whole bovine porcine and avian AmF, are shown in Fig. 1. Twenty-nine of forty-eight 2-cell embryos (60.4%) cultured in M16 (control) reached Hblast level. One of forty-nine 2-cell embryos (2.0%) cultured in bovine AmF, none of forty-six 2-cell embryos (0%) cultured in porcine AmF, and four of forty-six 2-cell embryos (8.7%) cultured in avian AmF, developed into Hblasts, respectively. Almost half of the 2-cell embryos culture in M16NBSA could not develop beyond 2-cell stage. A few of the 2-cell embryos cultured in M16NBSA developed into morulae and approximately 10% of embryos developed into Hblasts.

The results of the development of 2-cell embryos into Hblasts cultured in M-16 and supplemented with 10% bovine, porcine and avian AmF are shown in Fig. 2. Thirty-eight

of fifty-four 2-cell embryos (70.4%) cultured in M16 (control) developed into Hblast. Sixteen of twenty-seven 2-cell embryos (59.3%) cultured in M16 supplemented with bovine AmF, twelve of twenty-eight 2-cell embryos (42.9%) cultured in M16 supplemented with porcine AmF, and thirty-three of fifty 2-cell embryos (66.6%) cultured in M16 supplemented with avian AmF, developed into BHlasts, respective-

Fig. 1. Development of mouse 2-cell embryos to hatched blastocysts in M16 or in whole bovine, porcine and avian amniotic fluid. M16: containing 4mg/ml BSA., M16NBSA: not containing BSA., AmF: amniotic fluid., **: $P < 0.01$

Fig. 2. Development of mouse 2-cell embryos to hatched blastocysts in M16 containing 10% bovine, porcine and avian amniotic fluid. M-16: containing 4mg/ml BSA., AmF: amniotic fluid., N.S.: not significant

ly. No significant difference in the growth rates from 2-cell embryos to HBlasts for M16 (control) and M16 supplemented with AmF from the three species could be detected. When M16NBSA was supplemented with 10% AmF from the three species, the growth rates of 2-cell embryos to HBlasts were thirty-seven of fifty-three 2-cell embryos (69.8%) (M16, control), six of forty-four 2-cell embryos (13.6%) in M16NBSA without BSA, thirty-six of forty-five 2-cell embryos (80.0%) in M16NBSA supplemented with bovine AmF, thirteen of forty-three 2-cell embryos (30.2%) supplemented with porcine AmF, and twenty-one of forty 2-cell embryos (52.5%) in M16NBSA supplemented with avian AmF, respectively (Fig. 3). The growth rates of 2-cell embryos cultured in either M16 (control) or in M16NBSA supplemented with bovine AmF were significantly higher than those for embryos in M16NBSA supplemented with porcine and avian AmF.

The growth rates of 2-cell embryos cultured in M16NBSA supplemented with treated bovine AmF are shown in Fig. 4. Twenty-nine of fifty-five 2-cell embryos (52.7%) cultured in M16 (control) developed into HBlasts.

Fig. 3. Development of mouse 2-cell embryos to hatched blastocysts in M16NBSA containing 10% bovine, porcine and avian amniotic fluid. M16: containing 4mg/ml BSA., M16NBSA: not containing BSA., AmF: amniotic fluid., *: $P < 0.05$, **: $P < 0.01$

Fig. 4. Development of mouse 2-cell embryos to hatched blastocysts in M16NBSA added 10% bovine and treated amniotic fluid.

M16: containing 4 mg/ml BSA, M16NBSA: not containing BSA, AmF: amniotic fluid, I: M16 NBSA + 10% bovine AmF, II: M16 NBSA + 10% heat treated bovine AmF, III: M16 NBSA + 10% Dextran-charcoal treated bovine AmF, IV: M16 NBSA + 10% MW $< 10^4$ fraction of bovine AmF, V: M16 NBSA + 10% MW $1-3 \times 10^4$ fraction of bovine AmF, VI: M16 NBSA + 10% MW 3×10^4 fraction of bovine AmF, *: $P < 0.05$, **: $P < 0.01$

Eleven of fifty-five 2-cell embryos (20.0%) cultured in M16NBSA and thirty-seven of seventy 2-cell embryos (52.9%) cultured in M16NBSA supplemented with non treated AmF developed into HBlasts, respectively. Five of forty-seven 2-cell embryos (10.6%) cultured in M16NBSA supplemented with H treated AmF and twenty-six of forty-seven 2-cell embryos (56.5%) cultured in M16NBSA supplemented with DC treated AmF developed into HBlasts, respectively. The growth rates of 2-cell embryos to HBlasts were thirty-nine of sixty-two 2-cell (62.9%) cultured in M16NBSA supplemented with a low MW fraction (less than 10^4) of AmF, and eighteen of fifty-seven 2-cell embryos (31.6%) in M16NBSA supplemented with both a middle MW fraction (10^4 to 3×10^4) and a high MW fraction (more than 3×10^4), respectively. Growth rates in M16NBSA supplemented with 10% AmF, DC treated AmF or a low MW fraction of AmF were higher than those in M16NBSA supplemented with other treated AmF.

Discussion

GIANAROLLI *et al.*²⁾ and DORFMANN *et al.*¹⁾ reported that human AmF is an effective culture medium for mouse embryos *in vitro*. Although JAVED *et al.*³⁾ demonstrated that 2-cell mouse embryos could be cultured through to blastocysts in 100% fresh bovine AmF, bovine AmF which had been frozen had an adverse effect on development. In our study, M16-NBSA supplemented with 10% bovine AmF which was frozen and pooled, was not detrimental to the development of 2-cell mouse embryos through to HBlasts.

The results of the present study indicate that intact bovine, porcine and avian AmF were detrimental to the development of mouse embryos cultured *in vitro*. Although AmF from the three species had a detrimental effect on mouse embryo development, the addition of 10% AmF to M16NBSA gave similar results to that of M16 containing 4 mg/ml BSA. The concentration of the detrimental factor in AmF was diluted ten times by M16NBSA, so the negative effect was eliminated by a dilution of AmF and an eventually positive effect remained. AmF generally contains a low level of total protein. Since the protein level in bovine AmF at around 70 days of pregnancy is approximately 150 $\mu\text{g/ml}$, this being approximated by 1/27 of 4 mg/ml BSA in M-16⁸⁾, eventually a final protein level of M16NBSA supplemented with 10% bovine AmF equals approximately 1/270 of M16 with BSA (4 mg/ml). Two major proteins in bovine AmF at around 70 days of pregnancy are α -fetoprotein (AFP) and BSA, and AFP is dominant over BSA⁹⁾. Ultracento-10 and -30 can cut off over 99% of molecules whose MW are larger than 10^4 and 3×10^4 , respectively. If the cut-off fractions with Ultracento contain trace amounts of AFP and/or BSA, their effect could be negligible on the development of 2-cell mouse embryos through to blastocysts.

Our findings demonstrate that bovine AmF

contains an effective factor for mouse embryo development. If bovine AmF contains an effective factor for the development of 2-cell mouse embryos through to HBlasts, it would likely be destroyed by heat treatment at 100°C for 5min, and not be absorbed with DC. This factor is not likely to be a steroid or a lipid because it was not absorbable with DC. Presumably it would be a polypeptide having low MW (less than 10^4) in consideration of its instability toward heat.

References

- 1) DORFMANN, A.D., M. BUSTILLO, S.D. BENDER, G. REED, P. ROBINSON, J.D. SCHULMAN and E.F. FUGGER, Cell-free human amniotic fluid as culture medium for mouse and human embryos. *Fert. Steril*, **51** : 671-674. 1989.
- 2) GIANARONI, L., R. SERACCHIOLI, A.P. FERRARETTI, A. TROUNSON C. FLAMIGNI and L. BOVICELLI, The successful use of human amniotic fluid for mouse embryo culture and human *in vitro* fertilization, embryo culture, and transfer. *Fert. Steril*, **46** : 907-913. 1986.
- 3) JAVED, M.H. and W. WRIGHT, Jr, Bovine amniotic and allantoic fluids for the culture of murine embryos. *Theriogenology* **34** : 445-460. 1990.
- 4) KANE, M.T., Culture media and culture of early embryos. *Theriogenology*, **27** : 49-75. 1987.
- 5) OKANO, A., Y. SHIOYA, T. OBATA and R. FUKUHARA, Specific protein in bovine allantoic fluid and amniotic fluid. *Jpn. J. Zootech. Sci.*, **49** : 276-282. 1978.
- 6) OKANO, A., Y. IZAIKE, K. SHIMADA, T. OISHI and T. MIYASHIGE, Fetal development in the Japanese Black Cattle. *Jpn. J. Anim. Reprod.*, **31** : 147-149. 1985. (in Japanese).
- 7) QUINN, P.C., C. BARROS and D.G. WHITTIGHAM, Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fert.*, **66** : 161-168. 1982.
- 8) WHITTIGHAM, D.G., Culture of mouse ova. *J. Reprod. Fert. (suppl)*, **14** : 7-21. 1971.

マウス胚の体外培養液へのウシ羊膜液添加の効果

岡野 彰・高橋昌志

農林水産省畜産試験場，茨城県筑波農林研究団地 305

マウス胚の体外培養のための培養液へ，ウシ，ブタおよびニワトリ羊膜液添加の効果を明らかにしようとした。羊膜液および羊膜液を添加した培養液中で，マウス 2 細胞期胚を培養し，96 時間後における透明帯脱出胚盤胞への発育率を検討した。3 動物種からの羊膜液とも無処理のままでは，マウス胚の体外培養液として用いた場合は全く効果がなく，むしろ胚の発育を阻害する傾向を示した。しかしウシ血清アルブミンを含まない M16 培養液にウシ羊膜液を 10% 添加して，マウス 2 細胞期胚を体外培養すると，ウシ血清アルブミンを含む M16 培養液と同程度に，透明帯脱出胚盤胞へ発育させえた。マウス 2 細胞期胚を透明帯脱出胚盤胞へ発育させるのに有効なウシ羊膜中の因子は，100°C で 5 分間の加熱処理で失活し，デキストラン-チャコールで吸収されなかった。このことより，この因子は脂質やステロイド様の物質ではなく，分子量 10^4 以下のペプチドあるいは遊離アミノ酸と推定された。

日畜会報, 65 (10) : 923-927, 1994