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Effect of Mouse Leukemia Inhibitory Factor on the Development of *In Vitro* Produced Pig Embryos

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돼지 체외수정란의 발달에 미치는 Mouse Leukemia Inhibitory Factor의 영향

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요 약

본 연구는 돼지 난포란으로부터 생산된 수정란의 체외발달에 미치는 아미노산, 우태아혈청 (FBS)과 Leukemia Inhibitry Factor 의 영향을 조사하였다. 돼지난포란은 도살된 돼지의 난소로부터 회수하여 39℃, 5% CO_2 배양조건하에서 1㎍/㎖ FSH-p, 1㎍/㎖ Estradiol-17β와 10% FBS가 첨가된 TCM-199 배양액내에서 42시간동안 성숙시켰다. 사출된 정자의 수정능 획득은 45와 90% Percoll density gradient법을 통한 원심분리에 의해 얻었으며, 이들 수정능획득된 정자를 성숙된 난포란이 함유된 배양액에 3×10^5/㎖의 농도로 주입하여 10 ± 1시간동안 배양함으로서 체외수정을 유도하였다. 수정된 난포란은 ; 1) 10% FBS가 함유된 TCM-199, DMEM, mKRB 또는 CR_1aa 배양액, 2) 아미노산 또는 10% FBS가 첨가된 CR_1 배양액, 3) STO 세포 또는 mLIF (1,000 unit/㎖)가 첨가된 CR_1aa (10% FBS) 배양액, 4) mLIF (1,000 unit/㎖)를 수정 직후 또는 8-세포기 이후에 첨가된 CR1aa (10% FBS)의 네가지 배양조건에서 각각 분리 배양하였다. 그결과 체외수정란의 배반포까지 발달율은 아미노산과 10% FBS가 포함된 CR_1 배양액에서 다른 배양액에서보다 양호하였고, 특히 8-세포기 이후에 mouse LIF를 첨가한 CR_1aa (10% FBS) 배양액에서는 다른 배양조건보다 현저히 높은 결과를 보였으며, 부화 배반포까지도 배발달을 유도할 수 있었다. 따라서 돼지수정란의 발달에 있어서 배양액내에 아미노산와 FBS 및 mouse LIF의 첨가는 효과가 있으며, 특히 8-세포기 이후에 있어서 mouse LIF의 첨가는 돼지의 수정란을 배반포 이후의 단계에까지 발달시킬 수 있었다.

Ⅰ. INTRODUCTION

Embryos of domestic animals may have been developed at different kinds of culture media. When bovine embryos were cultured in a medium, development to the blastocyst stages were depressed in a medium with amino acid added both phosphaste and glucose, but those were incresed in a medium with amino acid added serum (Pinyopummintr and Bavister, 1991). The additive of both lactate and pyruvate had not influence on the development of bovine morulae or blastocyst, and adding pyruvate alone decreased the percentage of morula or blastocyst (Rosenkrans et al., 1993).

Rescently, the role of growth factors in embryo culture medium has been studied. This

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kind of experiment also has been performed in many species (Heyman et al., 1987; Eyestome and First, 1989; Prather et al., 1991; Russler-Long et al., 1991; Takagi et al., 1991; Voelkel et al., 1992; Natsuyama et al., 1993; Matsuyama et al., 1993). Growth factors in embryo culture are not known to have essential effect on embryo development but these have synergistic effect. The development of bovine embryos to the blastocyst stage in media with EGF, $TGF\beta_1$, and PDGF was increased (Yang et al., 1993). Leukemiainhibitory factor (LIF) was examined to have effects on embryonic development in different species. When bovine IVM/IVF morulae were cultured in medium supplemented with mLIF, the expanded and hatched blastocyst were significantly higher in comparison with medium alone (Marquant-Le Guienne et al., 1993). Fry (1992) reported that LIF receptors were observed first at trophectoderm of expaned blastocyst, and that LIF might be involved in regulating implantation. But it is not reported that LIF has effects on development of pig embryos derived from *in vitro* matured/fertilized oocytes.

Therefore, this study was design to study the developmental ability of pig IVM/IVF embryos as follows coditions: 1) the different kinds of media, 2) the supplementation with amino acids and serum in medium, 3) co-culture with somatic cells, 4) and addition of mLIF to the culture media.

Ⅱ. MATERIALS AND METHODS

1. Recovery of immature oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline (35 to 39℃) within 1hr. The follicular oocyte-cumulus complexs were recovered by aspiration from the follicles (2~6㎜ in diameter) using a 21-gauge needle and a 10㎖ disposable syringe. The oocyte-cumulus complexes were washed three times with TL-HEPES (0.3㎎/㎖ BSA) and the maturation medium, respectively. Oocytes possessing a compact cumulus cell mass and evenly granulated ooplasm were used for this study.

2. *In vitro* Maturation (IVM)

Eight to ten oocytes were transferred into a 50μl droplet of maturation medium equibilited for 2hra in 5% CO_2, 95% O_2 incubator under warm paraffin oil in a polystyrene culture dish (60×10㎜). The maturation medium consisted of 90% (v/v) TCM-199 (with Earle's salts: Gibco, USA) supplemented with heat-treated 10% FBS (v/v: Sigma, USA), 25mM $NaHCO_3$ (Sigma, USA), 0.2mM pyruvate (Sigma, USA), 1 ㎍/㎖ FSH-p (Schering-Plough Animal Health, USA), 1 ㎍/㎖ estradiol-17β (Sigma, USA), and 25 ㎍/㎖ gentamycin (Sigma, USA), and then the oocytes for maturation were cultured at 39℃ in 5% CO_2 in air for 40~42hr.

3. Sperm capacitation and *in vitro* fertilization (IVF)

Semen were collected from a Landrace boar by hand-massage method. The sperms induced capacitation by using a Percoll density gradient. Briefly, a 90% percoll stock was prepared with a 9:1 mixture of Percoll and 10-strength of TALP Ⅱ (BSA free solution). The Percoll density gradient was prepared on a 15㎖ conical tube with 2㎖ of 90% Percoll added to the tube, which was layered with 2㎖ of 45% Percoll 1:1 mixture of 90% Percoll and single strength of TALP without BSA, semen subsequently layered onto the Percoll density gradient, and then was centrifuged at 2,000rpm for 15mim at room temperature. After centrifugation, the pellet containing capacitated sperm (95% motile)

was recovered from bottom of conical tube, and used directly for IVF.

Motile sperm cells were added into the fertilization medium containing 30~50 oocytes /mℓ to make a final concentration of 3×10^5 sperm/mℓ. Incubation conditions for IVF were 5% CO_2 in air with saturated humidity at 39℃. The fertilization medium was consisted of bovine serum albumin (BSA: 12 mg/mℓ), 0.2mM pyruvate, 2 μg/mℓ heparin, 18.2 μM pencillamine, 1.8 μM hypotaurine 9.1μM, epinephrine, and 25 μg/mℓ gentamycin. Incubation conditions for IVF were 5% CO_2 in air with saturated humidity at 39℃.

4. Preparation feeder layer

STO cells derived from mouse fibroblast were purched from ATCC Co. for co-culture of pig embryos. STO cells were cultured in 35㎠ flasks dish with DMEM containing 10% FBS until confluent under 5% CO_2 in air with saturated humidity at 37℃. Confluent monolayers in 35㎠ flasks were treated with mitomycin C (1 μg/mℓ, Sigma, USA) to prepare mitotically inactivated cells for 2~3hrs and then medium was aspirated and replaced with new DMEM (Gibco, USA) containing 10% FBS. STO cells were then treated with a mixture of 0.05% trypsin/EDTA (Sigma, USA) for 3~5min and then the agitated until detached from flask. The cells were replated onto 0.1% (w/v) gelatin-coated (swine skin) 4-well culture plate of CR_1 containing 10% FBS to promote adherence. Most cells were attached within 30min and spread within 4~5hr after plating. Mitotically inactivated cell layers were used next in the morning after replating.

5. *In vitro* embryo development

At 10±1hr post sperm insemination, the oocytes were washed 4~5 times with each culture medium. Embryos were then allocated to randomly preassigned 4-well dish with embryo culture media.

Experiment 1. To investigate the effects of culture media on pig embryos development, the culture media were prepared as follows; complex media: (1) TCM-199 + 10% FBS, (2) DMEM + 10% FBS, simple media: (3) mKRB + 10% FBS, (4) CR_1 + 10% FBS, 2% MEM amino acid (Gibco BRL, USA), 1% MEM non-essential amino acids (Gibco, USA) and 1mM L-glutamine.

Experiment 2. To examine effects of amino acids or/and FBS on pig embryo development, the embryos were randomly placed in (1) CR_1, (2) CR_1 supplemented with amino acids, (3) CR_1 supplemented with 10% FBS, (4) CR_1 supplemented with amino acids and 10% FBS.

Experiment 3. To examine the effect of mLIF or co-culture system (STO cell) on pig embryo development, the embryos were randomly placed in (1) CR_1 (containing 10% FBS and amino acids) supplemented wirh mLIF, or (2) CR_1 (containing FBS 10% and amino acids) condition by STO cells. LIF (AMRAD, ESGRO, Australia) were added to the medium at a rate of 1,000 unit/mℓ.

Experiment 4. To investigate the time when LIF functions in development of pig embryos, the embryos were randomly allocated to (1) CR_1 containing 10% FBS and amino acids (2) (1) supplemented with mLIF (1,000 unit/mℓ) at 1-cell stages, or (3) (1) supplemented with mLIF at 8-cell stages.

Embryo cultures in four kinds of experiments were performed under 5% CO_2 in air with saturated humidity at 39℃, and embryos were incubated for 6 days without disturbing.

6. Statistical analysis

All data were analyzed with Student's t-test to find the significance of differences between

two different groups.

Ⅲ. RESULTS

Experiment 1. Embryo development in different culture media

As shown in Table 1, development of pig IVM/IVF derived embryos in four different media were examined. The cleavage rates of pig embryos were no significantly different among four different media, but developmental rates to the blastocyst stage were significantly different. The highest developmental rate of pig IVM/IVF derived embryos to the blastocyst stage was found in CR_1aa media in comparision with other's medium.

Experiment 2. Effect of amino acids and FBS

Development of pig IVM/IVF derived embryo in CR_1 medium supplemented with or without amino acids or/and FBS were examined (Table 2). The cleavage rate (35.2%) of pig embryos in CR_1 alone was significantly lower than those of other's media (58.1~66.1%). Pig embryos in medium supplemented with FBS or FBS and amino acids were developed upto the blastocyst stage (5.6, 9.1%). Especially, pig embryo's development in CR_1 medium supplemented with amino acids and FBS show the highest development and only developed to the hatched blastocyst (Fig. 1).

Experiment 3. Effect of mLIF and feeder layer cells (STO) on development of pig embryo

Developments of pig IVM/IVF derived embryos were compared in two different conditions, addition of mLIF to medium and co-culture

Table 1. Development rates of porcine IVM/IVF derived embryos cultured in four different media

Media[1]	No. of examined oocytes	No. (%) of cleaved	No. (%) of blastocyst
1) TCM-199	492	264(53.7)	0(0.0)
2) DMEM	325	165(50.7)	2(1.2)
3) mKRB	241	158(65.6)	6(3.8)
4) CR_1aa	501	297(59.2)	24(8.1)

[1] Supplement with 10% FBS

Table 2. Effects of amino acids and FBS on *in vitro* development of pig IVM/IVF derived embryos in CR_1 medium

Treatment	No. of examined oocytes	No. (%) of cleaved	No. (%) of blastocyst	No. of hatching
1) CR_1	273	96(35.2)	0(0.0)	0
2) CR_1 + aa[1]	353	205(58.1)	0(0.0)	0
3) CR_1 + FBS[2]	649	429(66.1)	24(5.6)[a]	0
4) CR_1 + aa[1] +FBS[2]	646	417(64.6)	38(9.1)[b]	2

1 Containing 2% MEM amino acid, 1% MEM non-essential amino acids, 1mM L-glutamine

2 Containing 10% FBS

a, b : $p=0.022$

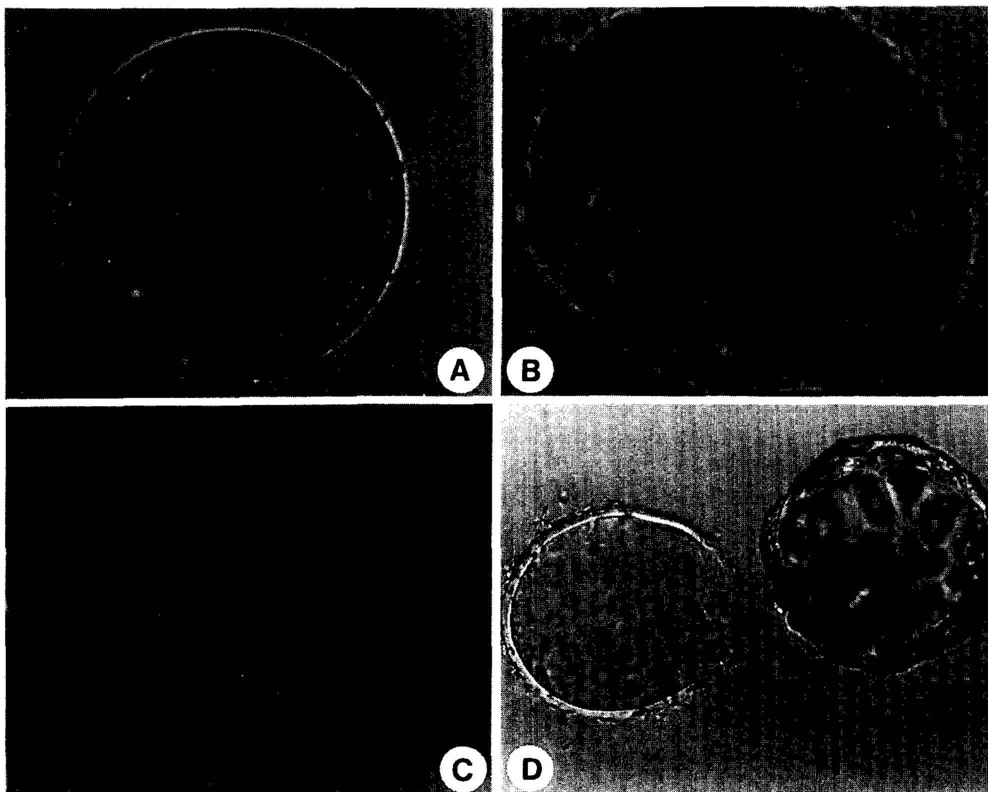

Fig. 1. ***In vitro*** **development of porcine follicular oocytes (×320)**
A. Early blastocyst **B. Late blastocyst**
C. Hatching blastocyst **D. Hatched blastocyst**

of STO cells. Development rates to the blastocyst stage in two culture conditions were not significantly different (Table 3). This finding suggests that development of pig embryo was not influenced by matrix type of LIF.

Experiment 4. Effects of mLIF on development of pig embryos

Developmental rates of pig IVM/IVF derived embryos in CR_1 medium supplement with mLIF were examined (Table 4). The cleavage rates (73.4~75.0%) of embryos developed in each medium were no significantly different. However, when mLIF was added to the embryos upto the 8-cell stage, blastocyst was significantly increased. This finding indicate that LIF actively functions beyond the 8-cell stages, but that LIF was not functions early stages below the 8-cell for embryo development.

Ⅳ. DISCUSSION

The findings obtained from this study are that pig IVM/IVF derived embryos can develop to

Table 3. Effects of pig IVM/IVF derived embryos on co-culture with STO feeder layer

Treatment	No. of examined oocytes	No. (%) of cleaved	No. (%) of blastocyst
1) CR_1 + LIF	410	243(59.3)	26(10.7)
2) CR_1 + STO feeder layer	581	327(56.3)	37(11.3)

[1] Additice 2% MEM amino acid, 1% MEM non-essential amino acids, 1mM L-glutamine and 10% FBS

Table 4. *In vitro* development of porcine IVM/IVF derived embryos in CR_1aa (10% FBS) medium supplement with mLIF

Treatment[1]	No. of examined oocytes	No. (%) of cleaved	No. (%) of blastocyst
1) CR_1	400	297(73.5)	29(9.9)[a]
2) CR_1 + mLIF	396	297(75.0)	36(12.1)[b]
3) CR_1 + mLIF[2]	414	304(73.4)	63(20.3)[c]

[1] Addition of 2% MEM amino acid, 1% MEM non-essential amino acids, 1mM L-glutamine and 10% FBS

[2] Addition of LIF (1,000 unit/mℓ) beyond 8-cell stage

[a, c] : p=0.020, b, c : p=0.036

the blastocyst stages in simple culture media containing serum or in co-culture system such as STO cell. It is previously reported that co-culture system using somatic cells overcomed *in vitro* cell block by reducing levels of inhibitory components which typically present in commercial culture medium and serum. Components such as vitamins, fatty acids, growth factor, etc, which are present in serum, were well-known to essential to late stages of embryo development (Kane et al., 1986; Kane and Bavister, 1988; Pinyopummitr and Bavister, 1991). Even though co-culture system by somatic cells overcomes the *in vitro* cell block, preparation of feeder layer for embryo culture is too much laborious.

Thus, in this study we have designed to develop new simple culture systems of pig IVM/IVF derived embryos by combination with amino acids, FBS and mLIF. Whether pig IVM/IVF embryos developed to the blastocyst stage by other investigators using conditioned media were reported or not, these data also was based on the hypothesis that addition of embryotrophic factors in simple media will be overcomed the blocks for *in vitro* embryo development.

Pig embryo development to the blastocyst stages were effected by media used. Previouse reports showed that uterine and oviduct contain at least 20 free amino acids (Fahning et al., 1967; Stanke et al., 1974). However, it is not clear how amino acids improve *in vitro* embryonic development. Development to blastocyst in medium containing amino acids and 10% FBS was significantly higher than others media as showed in Table1. Development of pig embryos to the blastocyst were found only in medium supplemented with FBS. Especially, supplementation of amino acids to CR_1 medium containing FBS showed higher development rates of embryo than those of CR_1 medium containing FBS. Furthermore, embryos cultured only in this me-

dium were developed to the hatching and hatched blastocyst stages. Therefore, this finding was comparable with previous evidences which amino acids have been reported to be essential for in vitro development of embryo (Kane and Foote, 1970; Bavister et al., 1983; Resenkrans et al., 1989; Zhang and Armstrong, 1990). Taken together, this result suggested that supplementation of amino acids promoted embryo development beyond the early blastocyst stage. It seems that supplementation of amino acids to the culture medium may also increase the pool size of endogenous amino acids, the rate of *de novo* protein synthesis, and the number of cells of blastocyst (Zhang and Armstrong, 1990 ; McLaughlin et al, 1990; Takahashi and First, 1992). Especially, it is clue that glutamine among amino acids may act as an energy source of embryo development (Rieger and Guay, 1988).

We have also investigated the effect of LIF on late development of pig embryo because mLIF receptors are expressed at the morula stage. Embryo development with mLIF only and co-culture with STO cells were examined because STO cells secreted both soluble and matrix LIF. This finding suggests that development of pig embryo was not influenced by matrix type of LIF (Table 3). Thus, the experiment was designed as follow ; 1) CR_1 plus amino acids and FBS, 2) CR_1 plus amino acids, FBS and mLIF, and 3) CR_1 plus amino acids and FBS and then supplemented with mLIF at morula stage. Interestingly, the supplementation of mLIF at 8-cell stages showed significantly higher development rate of embryo than those of two different conditions (Table 4).

Leukemia Inhibitory Factor (or differentiation inhibitory acitivity) is a 45- to 56- KDa glycoprotein that has multiple activities on various *in vitro* culture systems (Rathjen et al., 1990). The temporal and spatial expression of LIF were analyzed during embryogenesis in neonatal and adult mice. LIF is also expressed at low levels in many different tissues, but the highest level of LIF mRNA expression occurs in the endometrial glands of the uterine endometrium in mice. In addition, the expression occurs transiently on day 4 of pregnancy and reduced after implantation. This fact explains why LIF supplementation at elevated the development above 8-cell stages of pig embryos (Bhatt et al, 1991; Stewart et al., 1992). It is generally observed that LIF inhibits of embryonic stem cell differentiation *in vitro* and also suggests that it may play a role in regulating the growth and development of early mouse embryos since embryonic stem cells are derived from the inner cell mass of blastocyst. Derivation of embryonic stem cells from the inner cell mass is dependent either on the presence of a fibroblast "feeder" cell layer that secretes LIF or on the presence of the exogenous LIF added to the culture medium (Martin, 1981; Evane and Kaufman, 1981; Williams et al., 1988). Although the addition of hLIF to the culture medium had little influence on the early development of mouse embryos through the blastocyst stage, it increased the proportions of hatched blastocysts from the zona pellucida and late blastocysts. These blastocysts had a larger area of trophectoderm than that of control, but had a similar inner cell mass area. In studies on sheep embryos, the addition of hLIF to culture media increased 4-folds the number of Day-5 bovine embryos that hatched from the zona pellucida after 48hr in culture. In addition, fewer embryos degenerated when cultured in media containing hLIF (for review, Fry, 1992).

In conclusion, this study have shown that supplementation of FBS was essential for development to blastocyst *in vitro* and supplementation of amino acids increased the development rates to blastocyst. Espeically, when supplen-

mentation of LIF significantly increased the development to the blatocyst stages. We lead to onclude that LIF will be effective factor *in vitro* development for pig IVM/IVF derived embryos.

V. SUMMARY

The aim of this study was designed to investigate the effect of amino acids, fetal bovine serum (FBS) and leukemia inhibitory factor on the development of in vitro-produced embryos from pig follicular oocytes. Pig follicular oocytes were collected from the ovaries of slaughtered pigs and matured in TCM-199 containing hormones (1μg /ml of FSH-p, 1μg /ml of Estradiol-17β) and 10% FBS for 42hrs at 39℃, 5% CO_2 in air. The capacitation of pig spermatozoa was induced by centrifugation through Percoll density gradient (45 to 90%). Capacitated spermatozoa were inseminated into 1ml-well containing matured follicular oocytes and then incubated for 10 ± 1hrs. Final sperm concentration was adjusted to 3×105 per milliliter. Fertilized oocytes were transferred to the several different culture conditions, and then the effect of culture media and the additional effect of FBS, amino acids, mouse fibroblast cells and mouse LIF on *in vitro* development of pig zygotes were investigated. Development rate to the blastocyst stage of in vitro matured and *in vitro* fertilized oocytes in CR_1 containing amino acids and 10% FBS had increased rather than in others media. Especially, development of pig embryos cultured in CR_1aa supplemented with 10% FBS and mouse LIF (1000unit/ml) upto the 8-cell stage was significantly higher than those in other culture conditions. Results from this study suggest that the adding of mouse LIF to the culture medium containing amino acids and FBS may have a beneficial effect on pig embryonic development *in vitro*.

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