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ICM - Trophectoderm Cell Numbers of Mouse IVF/IVC Blastocysts

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체외생산된 생쥐 배반포기배의 ICM과 Trophectoderm 세포수에 관한 연구

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= 국문초록 =

본 연구는 Polynucleotide-specific 형광물질을 이용한 Differential labelling기법으로 체외수정 후 배양 4일째 생산된 B6CBA F1 생쥐 배반포의 Total, ICM, Trophectoderm의 세포수를 조사함으로서 생쥐의 착상전 후기 배발달에 대한 기초 자료를 얻고자 실시하였다. 공시 배반포는 과배란처리에 의해 얻어진 난자를 1×10^6cells/ml의 정자로 수정시키고, 95시간동안 M16배양액과 37℃, 5% CO_2배양기 내에서 배양하여 배반포강의 확대와 투명대 두께의 감소를 기준으로 early, middle, expanded와 hatching으로 구분하였다.

본 연구에서 얻어진 결과는 다음과 같다.

1) 체외수정 후 95시간째 배반포 발달율은 86.7%였으며, early, middle, expanded와 hatching으로 구분하였을때의 발달율은 각각 16.3%, 18.9%, 10.5%, 40.9% 였다.

2) Bisbenzimide를 이용한 배반포의 총 세포수는 early, middle, expanded, hatching 각각 35.6±10.4, 49.4±8.6, 60.8±10.7 과 62.7±13.9를 얻었다.

3) Polynucleotide-specific형광물질을 이용한 Differential labelling으로 배반포 ICM과 Trophectoderm의 세포수를 early, middle, expanded, hatching으로 나누어 조사한 결과, ICM세포수는 각각 9.6±3.0, 13.6±3.9, 16.0±3.3, 19.5±4.6개 이었고, Trophectoderm세포수는 30.6±5.1, 39.9±5.8, 42.2±8.1, 43.7±11.1 개로 나타나 ICM과 Trophectoderm 모두 동일하게 발달의 진행정도에 따라 세포수의 증가양상을 나타내었다. 또한, Bisbenzimide와 Differential labelling에서 얻어진 총세포수의 비교에서도 동일하게 발달의 진행정도에 따라 세포수의 증가를 나타내었으며 그와 동시에 세포수도 거의 유사하였다.

이러한 결과로 미루어 볼때, Differential labelling을 이용한 빠르고도 간편한 세포수 계산법은 착상전 후기 배발달을 고찰하는데 유용하며, 배양조건에 따른 Embryo의 Quality를 반영하는 Indicator로서 이용될 수 있다는 것을 시사한다.

INTRODUCTION

Mouse blastocysts consist of two distinct cell types; an outer epithelial layer of trophectoderm(TE) and inner cell mass(ICM) attached to the inner surface of the TE. The TE is functioned as the transport of fluid for

the blastocyst formation in the early stage and as the attachment and invasion of the uterine for the implantation in the later stage and the ICM is formed to the embryonic proper in the future as well as complementary contributions to the extraembryonic tissues (Gardner et al., 1975). Allocation and differentiation to these two cell lineage occur during the fourth and fifth division (Fleming, 1987) which is affected by experimental treatment of eggs and cleavage stage embryos (Handyside et al, 1984).

Handyside and Hunter(1984) established a rapid and simple method can treat a number of mouse blastcyst. According to differential labelling of TE and ICM nuclei by using two polynucleotide-specific fluorochromes and using fluorescent microscopy with appropriate filter combination, the propidium iodide(PI)-labelled TE nuclei appeared pink or red; the bisbenzimide-labelled ICM nuclei blue or unlabelled. Therefore, study on cell number and allocation of ICM and TE cells in later preimplantation stage was already carried out in mouse (Handyside, 1978; Copp, 1978, 1979), pig(Papaioannou et al., 1988), bovine (Iwasaki et al., 1990) and human (Hardy et al., 1989). In this viewpoint, ICM and TE number have been using as an indicator of embryo quality according to culture condition variables (Iwasaki et al., 1994a,b; Ray et al., 1995; Conaghan et al., 1993).

This work has been carried out to examine the cell number of Total, ICM and TE cells of F1 mouse blastocysts at day 4 after IVF by differential labelling of the nuclei with polynucleotide-specific fluorochromes and to obtain a fundamental information of mouse preimplantation blastocyst according to development progression.

MATERIALS AND METHODS

1. Mouse IVF and blastocyst production

Oocytes were recovered from four to six week old F1 hybrid female mice(C57BL/6×CBA) which had been superovulated by intraperitoneal injections of 7.5IU pregnant mare's serum gonadotrophin(PMSG; Sigma) followed 50hr later by 7.5IU human chorionic gonadotrophin(hCG;Sigma). At 13.5h post hCG injection, the mice were killed by cervical dislocation and their oviducts were removed in M2 containing 4mg/ml BSA and then oocyte cumulus complexes(OCCs) were transferred into 50μl drop of M16 for culture medium until sperm insemination. Spermatozoa for mouse IVF were obtained from the dissected caudae epididymis of mature F1 hybrid mice(C57BL/6×CBA) from eight to ten week old age. Spermatozoa were recovered by gentle squeezing out in M16 supplemented with 4mg/ml BSA(Fraction V. Sigma) and then incubated for 2hrs 5% CO_2 in air at 37℃ to capacitate them. At 14h post hCG injection, sperm of about final conc. 1×10^6 cells/ml were introduced into the 50μl drop containing eggs and sperm and eggs were incubated for 5hrs at 37℃. And then eggs were collected and transferred into fresh culture drops. Fertilization was assessed by the presence of 2-cell cleavage on day 1 after IVF and the confirmed 2-cell groups were selected and transferred into the new culture drop and then cultured until can obtain blastocysts on day 4 after IVF.

2. Blastocysts classification

Blastocysts were classified to early, middle, expanded and hatching stage at 95hrs after IVF according to the developmental morphology; blastocoel expansion and zona thickness(Fig.1)

3. Total cell count

For the count of total blastomeres, the classified blastocysts were fixed with 2% formalin solution for 2~3min prior to bisbenzimide(No. 33342, 2.5μg/ml, Sigma) treatment. Fixed blastocysts were washed, placed on a slide glass

Table 1. Fertilization and development rates of mouse IVF (r=5)

No. of oocytes	No. of ≥2-cell(%)	Development to blastocysts at 95hrs after IVF(%)				
		>Bla.	ErB	MB	EdB	HgB
452	381(84.3)	330(86.7)	62(16.3)	72(18.9)	40(10.5)	156(40.9)

Bla.; Blastocyst, ErB; Early blastocyst, MB; Middle blastocyst, HgB; Hatching blastocyst

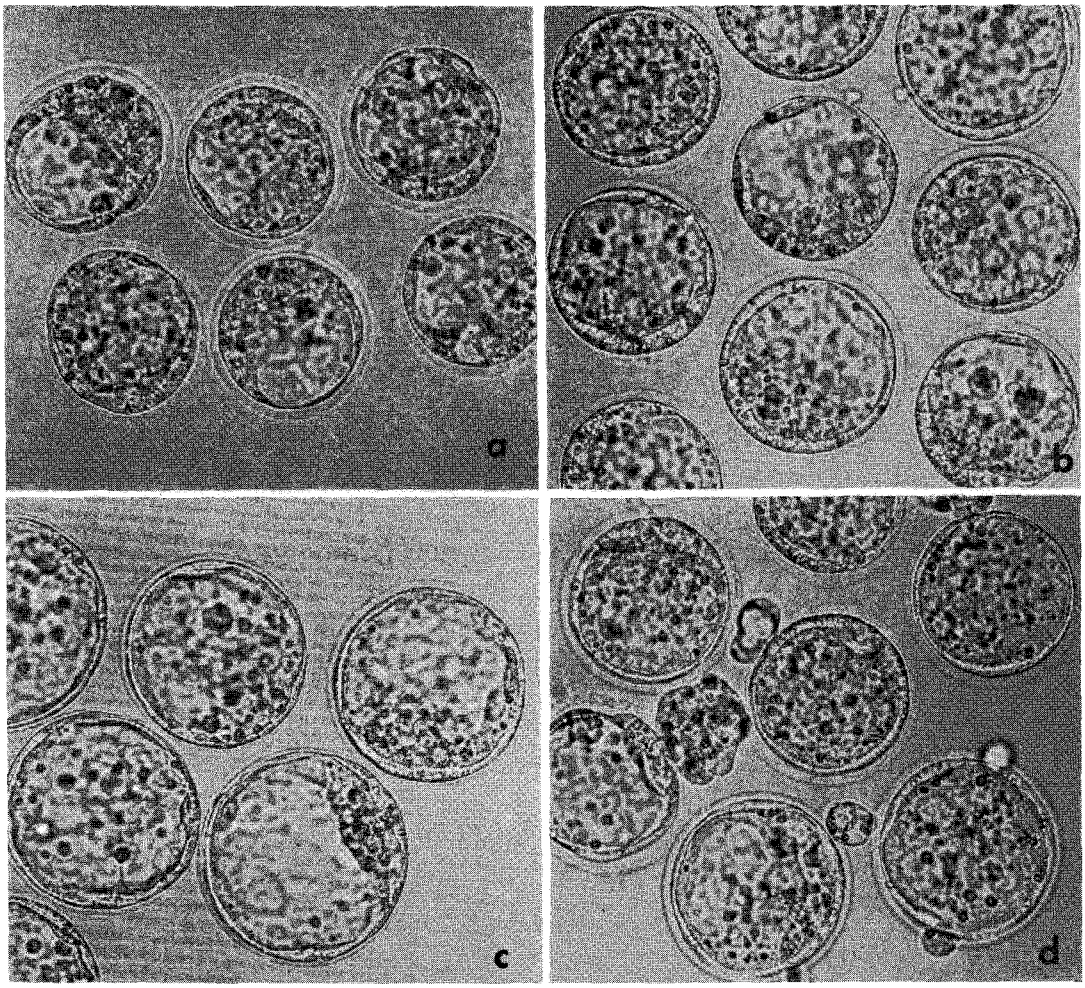

Fig. 1. Morphology of mouse IVF blastocysts; **a)** Early blastocysts(Blastocoel is smaller than 2/3 of the whole embryonic cell), **b)** Middle blastocysts(Blastocoel is larger than 2/3 of the whole embryonic cell), **c)** Expanded balstocysts(Blastocoel is larger than 2/3 of the whole embryonic cell and thinning of the zona pellucida), **d)** Hatching blastocysts.

and covered with slip and then pressed down the edges until formation of sample for the good observation. And then an appropriate bisbenzimide sol.(about 50μl) dropped beside the coverslip, filled the square under coverglass with the sol. and sealed the edges with fingernail polish. Observation was carried out under ultra violet filter incorporated fluorescent microscope on 1 day after making sample (Uhm et al., 1995)

Table 2. Total cell number of IVF mouse blastocysts by using bisbenzimide

Development stage	No. of blastocyst	Cell number
ErB	10	$35.6\pm10.4(18\sim48)^a$
MB	33	$49.4\pm8.6(29\sim67)^b$
EdB	16	$60.8\pm10.7(44\sim82)^c$
HgB	42	$62.7\pm13.9(32\sim90)^c$

*Values are Means±Standard deviation
[a,b,c] Means in the same column without common superscripts are significantly different ($P<0.01$)

4. Differential labelling of ICM and TE nuclei

Total, TE and ICM nuclei of blastocysts were differentially labelled by using a method of Hardy et al.(1989b) with some modifications. Briefly, TE nuclei are labelled first specifically with fluorochrome PI(Sigma). This fluorochrome is excluded from viable ICM cell but labelled TE cells undergoing antibody-mediated complement lysis during immunosurgery. The whole embryo is rapidly fixed and both the TE and ICM nuclei labelled with bisbenzimide. The protocol was as follows; embryo zona was removed in 0.5% pronase(Sigma) sol. and allowed to recover for 10min in TL-Hepes. Embryos were incubated on ice for 10~15min in 10mM TNBS(Sigma) containing 4mg/ml PVP(Sigma) in TL-Hepes. After washing completely, embryos were incubated in 0.1mg/ml anti-DNP-BSA(ICN Immunobiological.) in TL-Hepes for 10min at 37℃. After washing sufficiently in TL-Hepes, the embryos were incubated in 0.01mg/ml PI and 10% (v/v) guinea pig complement(Sigma) in TL-Hepes for 15~30min at 37℃. After 15min, observed them until even lysis of the outer TE cells was seen and then transferred them into 0.05mM bisbenzimide in absolute alcohol. After overnight storage at 4℃, the embryos were washed in absolute alcohol for at least 1hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under ultra violet excitation filter incorporated fluorescent microscope and counted TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

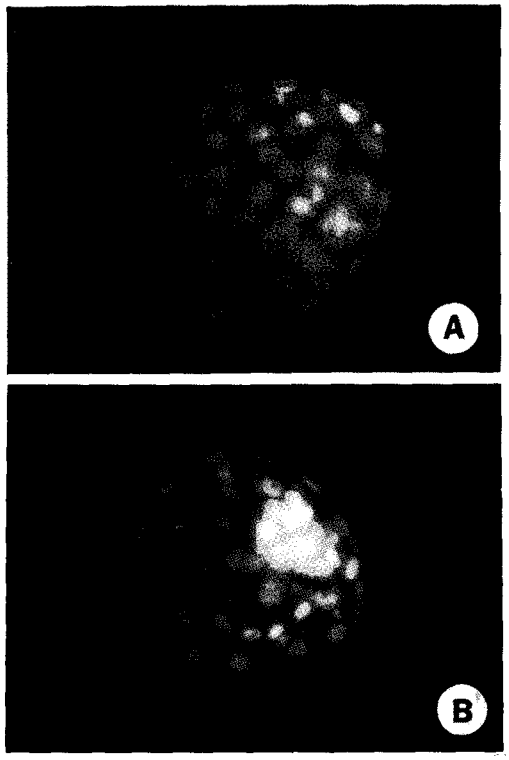

Fig. 2. Fluorescence micrographs of nuclei stained with bisbenzimide(A) or differentially stained with bisbenzimide and propidium iodide(B).
(A) Totla cell number(TCN) of *in vitro* produced mouse expanded blastocyst at 95hrs after IVF(TCN; 88). ×300. (B) ICM and TE cell numbers of *in vitro* produced mouse expanded blastocyst at 95hrs after IVF. Blue ICM nuclei and pink trophectoderm nuclei are easily distinguished(ICM; 22, TE; 62). ×300.

5. Statistical analysis

Difference in number of cells between development groups was compared using the Student's *t*-test.

RESULTS AND DISCUSSION

Table 3. Number of ICM and TE cells of IVF mouse blastocysts by using differnetial labelling

Develop. stage	No.	ICM cell number	TE cell number	Total cell number
Erb	18	9.6±3.0(4~14)[a]	30.6±5.1(20~36)[a]	40.1±7.7(24~49)[a]
MB	30	13.6±3.9(10~24)[b]	39.9±5.8(34~54)[b]	52.3±6.6(46~68)[b]
EbB	22	16.0±3.3(12~20)[b]	42.2±8.1(32~58)[b]	58.2±10.5(46~78)[b,c]
HgB	28	19.5±4.6(14~26)[c]	43.7±11.1(30~64)[b]	63.2±12.7(46~90)[c]

*Values are Means±Standard deviation
[a,b,c]Means in the same column without common superscripts are significantly different(P<0.01)

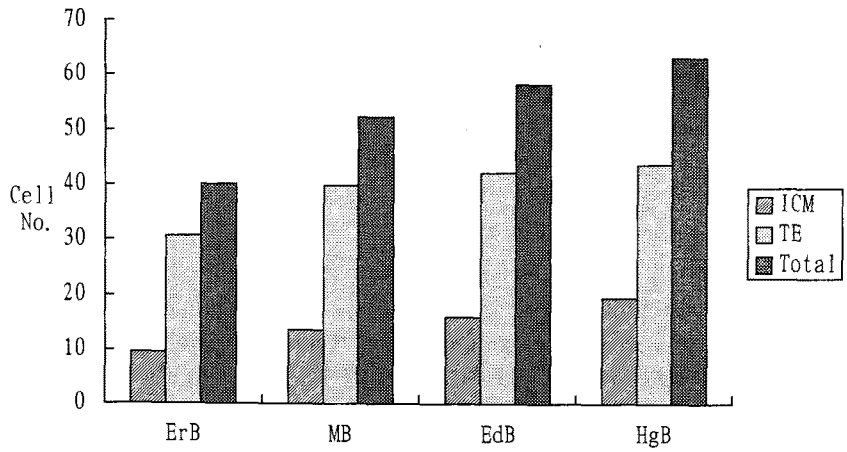

Fig. 3. Cell count of ICM & TE by using differential lablling.
ErB: Early blastocyst, MB: Middle blastocyst,
EdB: Expanded balstocyst, HgB: Hatching blastocyst

1. Development of preimplantation mouse embryos

In vitro fertilization rate of mouse eggs through the 5 replicated experimentation was 84.3% and blastocysts rate at 95hrs after IVF was 86.7% from the 2-cell embryos. Development rates of classified blastocysts to early, middle, expanded and hatching stage were 16.3%, 18.9%, 10.5% and 40.9%, respectively.

2. Total cell number of *in vitro* fertilized mouse blastocysts

The counts of total blastomere by using bisbenzimide were presented in Table 2. As shown in Table 2, total cell numbers of classified blastocysts to early, middle, expanded and hatching were 35.6±10.4(18~48), 49.4±8.6(29~67), 60.8±10.7(44~82) and 62.7±13.9(32~90), respectively. These results presented that total cell counts are increased according to devlopment level. Smith et al.(1977) reported that mouse embryos start to cavitate after completion of the 5th cleavage division, when they have ~32 cells, reaching the expanded blastocyst stage with 80~100 cells. However, data obtained in this experiment showed that the lowest cell number of blastocyst was 18 and the highest cell number was 90 under the same culture condition and culture time. This wide variation was ascertained in another domestic animal (Papaioannou, 1988; Iwasaki et al., 1990) and human (Hardy et al., 1989).

Papaioannou(1988) indicated that there is good correlation between cell number and morphological development, but cell number of each morphological category varied widely and there was considerable overlap between categories. Also, Iwasaki et al(1990) demonstrated that large variation within a category may be caused by a variation in the quality of embryos.

3. Number of ICM and TE cells *in vitro* fertilized mouse blastocysts

The count of ICM and TE cell number by using differential labelling with polynucleotide-specific fluorochromes is summarized in Table 3 and Fig. 2. As shown in Fig. 2B, the TE nuclei were labelled with PI and bisbenzimide appeared red or pink and the ICM nuclei with bisbenzimide appeared blue. In Table. 3 , ICM cell numbers of the classified blastocysts to early, middle, expanded and hatching were 9.6±3.0(4~14), 13.6±3.9(10~24), 16.0±3.3(12~20) and 19.5±4.6(14~26), respectively and TE cell numbers of the classified blastocysts to early, middle, expanded and hatching were 30.6±5.1(20~36), 39.9±5.8(34~54), 42.2±8.1(32~58) and 43.7±11.1(30~64), respectively. Especially, cell number of hatching blastocyst stage at 95hrs after IVF showed the widest variation that is originated from a part of the blastocyst has a characteristics which was hatching in the early expanding stage. However, ICM and TE cell number showed the same increase pattern according to development advance level and total cell number also showed the same pattern (Fig. 3). When compared with the results of total count were obtained between bisbenzimide only and differential labelling, both of them also had the same increase pattern according to development level and at the same time their cell numbers were almost the same according to development stage(Table 2 & 3).

Handyside and Hunter(1984) reported that ICM cell numbers of mouse blastocysts were from 10 to 25, TE cell numbers were from 17 to 37 and total cell numbers were from 27 to 60. When compared with our results, ICM cell numbers were almost the same but TE cell numbers were very high when compared with those of their results. These resuts may be caused by species difference between random and inbred mice or experimental source difference between *in vivo* blastocysts(day4 post hCG) and IVF blastocysts(day4 after IVF). Experimental time also is very important as a variable to carry output. Also, the allocation of the cells to the ICM and TE of the blastocyst is fundamental importance for later development. In the mouse, allocation to the two lineages occurs during fourth and fifth cleavage divisions at the morula stage (Fleming et al., 1987). Handyside et al(1986) reported that TE cells of mouse blastocyst increase quadruple between days 5 and 7, before reaching at a plateau coinciding with the initiation of giant cell formation, whilst the ICM cells only doubles. Also, the maximum number of TE and ICM cells in mouse blastocysts was 147 and 49, respectively. On the other hand, differential labelling method has two important advantages - that the numbers of these cell types can be determined for individual blastocysts and that spatial relationships are partially preserved so that regional interactions can be studied (Handyside et al., 1984). Therefore, differential labelling of TE and ICM nuclei *in situ* is useful in dimension of examination on later preimplantation development (mouse, Handyside et al., 1984; pig, Papaioannou et al., 1988; bovine, Iwasaki et al., 1990; human, Hardy et al., 1989). Also, differential labelling could be used as an indicator of embryo quality according to the variables of culture condition (Iwasaki et al., 1994 a,b; Conaghan et al., 1993, Ray et al., 1995).

SUMMARY

This work has been carried out to examine

the number of Total, ICM and TE cells of F1 mouse blastcysts at day 4 after IVF by differential labelling of the nuclei with polynucleotide-specific fluorochromes and to obtain a fundamental information of preimplantation mouse embryo development. Blastocysts produced by superovulated B6CBA F 1(C57BL/×CBA) eggs were inseminated with 1×10^6spermatozoa/ml and cultured in M16 medium at 37℃, 5% CO_2 incubator for 95hrs. Blastocysts were classified as early, middle, expanded and hatching stage according to the developmental morphology; blastocoel expansion and zona thickness.

The results obtained in these experiments were summarized as follows;

1) The development rate of blastocysts at 95hrs after IVF was 86.7% and classified blastocysts to early, middle, expanded and hatching were 16.3%, 18.9%, 10.5% and 40.9%, respectively.

2) The numbers of total blastomere using bisbenzimide in the classified blastocysts to early, middle, expanded and hatching were 35.6±10.4, 49.4±8.6, 60.8±10.7 and 62.7±13.9, respectively.

3) In ICM and TE cell number by using differential labelling with polynucleotide-specific fluorochrome in the classified blastocysts to early, middle, expanded and hatching; ICM numbers were 9.6±3.0, 13.6±3.9, 16.0±3.3 and 19.5±4.6, respectively and TE cell numbers were 30.6±5.1, 39.9±5.8, 42.2±8.1 and 43.7±11.1, respectively. These results showed the same increase pattern according to development advance level. Also, when compared with the results of total count were obtained between bisbenzimide only and differential labelling, both of them showed the same increase pattern according to development level and at the same time their cell numbers were almost the same.

So, rapid and simple cell count method using differential labelling can be used for the examination of later preimplantation development or as an indicator of embryo quality according to the variables of culture conditions.

REFERENCES

Conaghan J, Handyside AH, Winston RML, Leese HJ: Effects of pyruvate and glucose on the development of human preimplantation embryos *in vitro*. *J Reprod Fert* 1993, 99, 87-95.

Copp AJ: Interaction between inner cell mass and trophectoderm of the mouse blastocyst. Ⅰ. A study of cellular proliferation. *J Embryol Exp Morph* 1978, 48,109-125.

Copp AJ: Interaction between inner cell mass and trophectoderm of the mouse blastocyst. Ⅱ. The fate of the polar trophectoderm. *J Embryol Exp Morph* 1979, 51, 109-120.

Fleming TP: A quantitative analysis of cell location to trophectoderm and inner cell mass in the mouse blastocyst. *Dev Biol* 1987, 119, 520-531.

Gardner RL, Papaioannou VE: Differentiation in the trophectoderm and inner cell mass. *In* The Early Development of Mammals, 107-132, Cambridge University Press, London.

Handyside AH: Time of commitment of inside cells isolated from preimplantation mouse embryos. *J Embryol Exp Morph* 1978, 45, 37-53.

Handyside AH, Hunter S: A rapid procedure for visualizing the inner cell mass and trophectoderm nuclei of mouse blastocyst *in situ* using polynucleotide-specific fluorochromes. *J Exp Zoology* 1984, 231, 429-434.

Handyside AH, Hunter S: Cell division and death in the mouse blastocyst before implantation. Roux's Arch *Dev Biol* 1986, 195, 519-526.

Hardy K, Handyside AH, Winston RML: The human blastocyst: cell number, death and

allocation during late preimplantation development *in vitro*. *Development* 1989, 107, 597-604.

Iwasaki S, Yoshiba N, Ushijima H, Watanabe S, Nakahara T: Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *J Reprod Fert* 1990, 90. 279-284.

Iwasaki S, Mizuno J, Kobayashi K, Yoshikane Y, Hayashi T: Changes in morphology and cell number of inner cell mass of porcine blastocysts during freezing. *Theriogenology* 1994, 43, 841-848.

Iwasaki s, Yoshikane Y, Li X, Watanabe S, Nakahara T: Effects of freezing of bovine preimplantation embryos derived from oocytes fertilized *in vitro* on survival of their inner cell mass cells. *Mol Reprod Dev* 1994, 37, 272-275.

Papaioannou VE, Ebert KM: The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst *in vivo* and *in vitro*. *Development* 1988, 102, 793-803.

Ray PF, Conaghan J, Winston RML, Handyside AH: Increased numbers of cells and metabolic activity in male human preimplantation embryos following *in vitro* fertilization. *J Reprod Fert* 1995, 104, 165-171.

Smith R, Mclaren A: Factors affecting the time of formation of the mouse blastocoele. *J Embryol Exp Morph* 1977, 41, 79-92.

엄상준, 김선의, 김은영, 윤산현, 박세필, 정길생, 임진호: Epidermal Growth Factor가 돼지 미성숙난포란의 체외성숙에 미치는 영향. Ⅰ. 핵성숙에 미치는 Epidermal Growth Factor의 영향. 한국가축번식학회지 1995, 19(3), 217-226.