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Study on The Usability of Mouse Hatched Blastocysts in Embryo Transfer

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수정란 이식시 생쥐 완전탈출 배반포기배의 유용성 검토

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요 약

본 연구는 생쥐 완전탈출 배반포기배의 체내 발달율을 조사하기 위해 실시하였다. 공시된 완전탈출 배반포기배는 체내에서 생산된 전핵기 수정란을 5일과 6일동안 체외배양하여 얻었으며, 완전탈출 배반포기배의 직경을 기준으로 small (S-HBs), medium (M-HBs), large (L-HBs)로 구분하였다. 그 결과를 요약하면 다음과 같다. 1) 체외배양 4일째에 얻어진 배반포기배를 24~48 시간동안 추가배양했던 바, 배양 5일과 6일째에 완전탈출 배반포기배의 발달율은 29.1%와 22.8%였다. 2) 또한, 완전탈출 배반포기배의 총 세포수를 조사하였던 바, S-HBs (77.7±5.3, 59.6±4.4), M-HBs (83.7±4.0, 66.8±3.5), L-HBs (100.7±2.6, 88.9±3.8)로 나타나, 완전탈출 배반포기배의 크기가 증가함에 따라 총 세포수도 증가한다는 것을 알 수 있었다. 특히, S-HBs 와 L-HBs 의 총 세포수간에는 유의적인 차이가 있었다 ($p<0.01$). 3) 분류된 완전탈출 배반포기배를 가임신 3일된 대리모의 자궁에 이식하였을 때, 배양 5일째의 임신율과 착상율이 S-HBs (28.6%, 15.7%), M-HBs (44.4%, 30.9%), L-HBs (62.5%, 49.1%)로서 완전탈출 배반포기배의 크기가 커질수록 증가하였던 반면, 배양 6일째에는 이러한 양상을 볼 수 없었다. 그러나, 전체 착상율에 대한 정상산자율을 조사하였던 바, 배양 5일째의 S-HBs (87.5%) 가 다른 군에 비하여 유의하게 높게 나타났다 ($p<0.01$). 따라서, 이러한 결과로 미루어 볼 때, 체외에서 배양된 건강한 완전탈출 배반포기배는 높은 임신율과 착상율 및 정상산자율을 얻을 수 있음을 확인하였던 바, 이는 인간포배기 배아이식시 잉여의 완전탈출 배반포기배의 유용성 검토를 위한 기초자료로서 이용될 수 있음을 시사한다고 하겠다.

(Key words : Mouse, Hatched blastocysts, Pregnancy rate)

I. INTRODUCTION

At present, it proved that embryos recoverd from the one-cell to the blastocyst stage can be transferred into the reproductive tract of a pseudopregnant recipient and derived their complete embryonic development (Brigid et al., 1994). Generally, embryo transfer in mouse has been mainly performed at from morula to the zona-intact blastocyst. However, there were only a few reports for mouse embryo transfer at

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the stage of hatched blastocysts (HBs) (Zhu et al., 1996; Kim et al., 1997). Especially, Kim et al. (1997) reported that 25.6% of HBs was implanted, while the implantation rates for embryo transfer of expanding/hatching blastocysts were appeared to 50.9% and 56.6%, respectively. It means that implantation rates are variable according to development stage of embryo. Also, it indicates that immediately zona removed HBs were probably to be sensitive in outer environment. On the other hand, in human IVF programs, *in vitro* culture techinques now allow blastocyst formation at high rates (Menezo et al., 1992; Quinn, 1994). In human blastocyst transfer, sometimes, supernumerary hatching or HBs can be produced at day 5 or 6 after IVF. Especially, there was no report on usability of HBs. The objective of this study was to obtain the fundamental data for the human blastocyst transfer program through the examination of the developmental potential of mouse HBs. In addition, the quality of HBs was investigated using total cell count.

Ⅱ. MATERIALS AND METHODS

1. The production of mouse HBs

Hybrid F_1 female mice (4~5 weeks old) from C57BL/6 × CBA/N were superovulated by intraperitoneal (i.p.) injection with 7.5 IU pregnant mare's serum gonadotrophin, followed by i.p. injection with 7.5 IU human chorionic gonadotrophin (hCG) 50 hr later. The mice were mated with adult male mice of the same strain immediately after the injection of hCG and checked for mating the following morning. Zygotes produced *in vivo* were collected at 20 hr post hCG and they were cultured *in vitro* until the formation of HBs (day 5 and day 6 after culture) in m-CR1 medium (Park et al., 1995).

2. The classification of HBs

The HBs were classified into three groups on the basis of their diameter size (Fig. 1A) ; small (S-HBs : $\theta < 130\ \mu m$), medium (M-HBs : $130 \leq \theta \leq 180\ \mu m$) and large (L-HBs : $\theta > 180\ \mu m$). The size was measured by eyepiece micrometer fitted on inverted microscope (×100).

3. Total cell count

For the count of total cell number of HBs according to size, the classified HBs were fixed with 2% formalin solution for 2~3 min and staind with bisbenzimide solution (No. 33342, 2.5 μg/ml, Sigma). Observation was carried out under ultra violet filter incorporated fluorescent microscope (Fig. 1B).

4. *In vivo* development of HBs according to size

The classified embryos were transferred surgically to one or both uterine horns (6~8 embryos/horn) of ICR mice on day 3 of pseudopregnancy. All recipients were examined on days 15 of gestation to score the total number of fetuses including the resorption sites.

5. Statistical analysis

Difference in number of cells between the classified HBs groups was compared using the Student's t-test. *In vivo* development was compared with Chi-square test using SAS institute software.

Ⅲ. RESULTS

1. *In vitro* development of mouse embryos

The rates of *in vitro* development of mouse zygotes produced *in vivo* were shown in Table 1. The cleavage rate was 97.6% and blastocysts rate at day 4 was 74.0%. Also, when the HBs at day 5 and day 6 (delayed developed) *in vitro* cul-

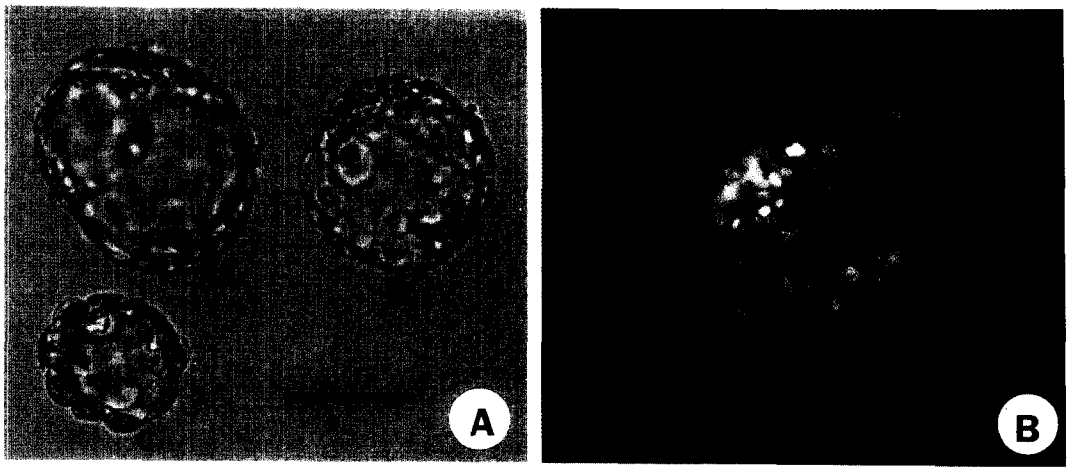

Fig. 1. A. Morphology of day 5 mouse hatched blastocysts (HBs) produced *in vitro* culture. a) large HBs (L-HBs), b) medium HBs (M-HBs), c) small HBs (S-HBs). ×300. Scale bar=100 μm. B. Fluorescence micrographs of nuclei stained with bisbenzimide. Total cell number (TCN) of mouse L-HBs produced at day 5 (TCN : 100). ×300

Table 1. *In vitro* development of mouse embryos

No. of oocytes	No. of cleaved (%)	No. of blastocyst (day 4)	No. (%) of HBs*							
			Day 5				Day 6			
			Total	S	M	L	Total	S	M	L
1,058	1,033 (97.6)	764 (74.0)	222 (29.1)	50 (22.5)	109 (49.1)	63 (28.4)	174 (22.8)	58 (33.3)	80 (46.0)	36 (20.7)

* HBs : Hatched blastocysts.

S : small (θ<130 μm), M : medium (130≤θ≤180 μm), L : large size (θ>180 μm) HBs.

ture were classified to small, medium and large size, their development rates of HBs were 22.5%, 49.1% and 28.4% on day 5 and were 33.3%, 46.0% and 20.7% on day 6, respectively.

2. Total cell number of *in vitro* cultured mouse HBs

The counts of total blastomere were presented in Table 2. Total cell numbers of classified HBs on day 5 and day 6 to small (77.3±5.3, 59.6±4.4), medium (83.7±4.0, 66.8±3.5) and large (100.7±2.6, 88.9±3.8) were increased as their size increases. Also, there were significantly different between S-HBs and L-HBs ($p<0.01$).

3. *In vivo* development of HBs according to size

When the *in vivo* developmental potential of classified HBs were examined, in Table 3, on day 5 HBs, the pregnancy and implantation rates of S-HBs (28.6%, 15.7%), M-HBs (44.4%, 30.9%) and L-HBs (62.5%, 49.1%) were increased as their size increases. Especially, the implantation rates of day 5 HBs were significantly different between S-HBs and L-HBs ($p<0.05$).

Table 2. Total cell number of mouse hatched blastocysts

Size of HBs	Day of HBs	No. of HBs	Cell number*
S-HBs	5	11	77.7±5.3 (49~ 98)[a]
	6	5	59.6±4.4 (48~ 68)[c]
M-HBs	5	12	83.7±4.0 (61~106)[a]
	6	13	66.8±3.5 (52~ 90)[c]
L-HBs	5	12	100.7±2.6 (87~115)[b]
	6	13	88.9±3.8 (62~112)[d]

* Values are means±standard errors.

[a,b/c,d] Means in the column without common superscripts are significantly different ($p<0.01$).

Table 3. *In vivo* development of mouse hatched blastocysts

Size of embryos	Day of culture	No. of		*No. of		
		Pregnant recipient (%)	Transferred embryos (PR/T)	Resorption	Live fetus	Total implantation (P, T)**
S-HBs	5	2/7 (28.6)	8/51 (15.7)	1 (12.5)	7 (87.5)[a]	8 (100, 15.7)[c]
	6	3/9 (33.3)	21/64 (32.8)	11 (73.3)	4 (26.7)	15 (71.4, 23.4)
M-HBs	5	4/9 (44.4)	34/68 (50.0)	6 (28.6)	15 (71.4)[ab]	21 (61.8, 30.9)[cd]
	6	4/8 (50.0)	32/60 (53.3)	11 (78.6)	3 (21.4)	14 (43.8, 23.3)
L-HBs	5	5/8 (62.5)	29/51 (56.9)	13 (52.0)	12 (48.0)[b]	25 (86.2, 49.1)[d]
	6	2/6 (33.3)	17/32 (53.1)	11 (91.7)	1 (8.3)	12 (70.6, 33.3)

PR : No. of transferred embryos on pregnancy recipient.

T : No. of transferred embryos to total recipient.

* : No. of implantations on day 15 of pregnancy.

** : Percentage of embryos transferred to recipients that became pregnant (P) and in total (T).

Means in the columns without common superscripts are significantly different ($p<0.01$)[ab] ($p<0.05$)[cd].

However, this pattern was not showed in day 6 HBs although their developmental potential was slightly lower than that of day 5 HBs. But, when the live fetuses formation against total implantation rates were observed, the result (87.5%) of S-HBs of day 5 was significantly higher than that of the others ($p<0.01$).

Ⅳ. DISCUSSION

Mammalian embryos have been successfully transferred from one-cell to zona intact blastocyst stage. In the human, recently, blastocyst transfer has been doing in IVF-ET program

(Menezo et al., 1992, 1996; Kaufmann et al., 1995; Heo et al., 1996; Lee et al., 1998). Especially, Menezo et al. (1997) reported that 61 % of the patients having supernumerary embryo have blastocysts frozen. Thus, the study on the characteristics of supernumerary blastocysts of different development stage was to need to wide the usability of supernumerary embryos (including hatching or hatched blastocyst) in human blastocyst transfer program. In the viewpoint, we examined the *in vivo* developmental potential of *in vitro* cultured zona-free mouse blastocysts (hatched blastocysts : HBs). For these experiments, the HBs were obtained in 120~132 hr (day 5) and 142~150 hr (day 6) *in vitro* culture and they were examined according to size. The zona pellucida has to removed before implantation to allow direct cell-cell interaction between the trophectoderm and uterine epithelial cells (Yu, 1994). But, it has known that a presence of zona pellucida plays a role of protection for change of external environment. Under *in vitro* conditions, mouse blastocysts undergo hatching following a series of alternating contraction and relaxation. Especially, hatching *in vitro* is a more prolonged process. There are conspicuous differences between hatching *in vitro* and *in vivo*. Hatching *in vitro* appears to be a more pronounced splitting of the zona pellucida than that *in vivo*. Hatched condition of blastocysts indicates the ready for implantation. However, in embryo transfer, HBs have limitation than zona-intact embryo, because they are more sensitive to culture condition and poor tolerance to external stress. Especially, the change of osmotic effect from culture medium to uterine fluid may be damage to survival of HBs.

In this experiment, 396 HBs were obtained at day 5 and day 6 from 764 blastocysts of day 4 and classified to various size (Table 1). The embryos of various size had a different total cell number (Table 2). There were significantly different between S-HBs and L-HBs ($p<0.01$). Also, when the rates of pregnancy, implantation and live fetuses of HBs produced at day 5 and day 6 were investigated (Table 3), the pregnancy rates of day 5 HBs were increased as their size increases and this pattern also was presented in implantation rates. However, significant difference was shown only between S-HBs and L-HBs of day 5 ($p<0.05$). Thus, these results denote that the developmental potential of HBs may be related with their cell number, because a total cell number of S-HBs was low and significantly different with L-HBs ($p<0.01$). But, in this experiment, interesting fact that the proportion of live fetuses in implanted embryos was not low in S-HBs indicated the live fetus formation of 87.5%. In addition, on day 6 HBs, the rates of resorption sites were higher than those of live fetuses formation. On the basis of above data, it demonstrated that the HBs can derive highly normal development independent of their size, if they were fast developed and healthy embryos. Also, as indicated in Table 2, cell number was not critically increased as their size increases.

Therefore, this study demonstrates that *in vitro* cultured healthy HBs can not only be developed normally with good pregnancy rates, implantation rates and live fetuses formation, but also served as a fundamental data for utility of supernumerary HBs in human blastocyst transfer.

V. SUMMARY

This study was carried out to investigate the *in vivo* developmental potential of mouse zona-hatched blastocysts (HBs). The HBs were cultured *in vitro* until day 5 and day 6 from zygotes produced *in vivo* and classified to small

(S-HBs), medium (M-HBs) and large (L-HBs) on the basis of embryo diameters. The results obtained in these experiments were summarized as follows ; 1) when the blastocysts at day 4 were further cultured for 24~48 hr, HBs obtained at day 5 and day 6 culture *in vitro* were 29.1% and 22. 8%, respectively. 2) Also, when the total cell number of HBs were counted, cell numbers of classified HBs on day 5 and day 6 to small (77.3±5.3, 59.6±4.4), medium (83.7±4.0, 66.8±3.5) and large (100.7±2.6, 88.9±3.8) were increased as their size increases. Especially, there were significantly different between S-HBs and L-HBs ($p<0.01$). 3) In addition, when the classified HBs were transferred into day 3 pseudopregnant recipients, the pregnancy and implantation rates of S-HBs (28.6%, 15. 7%), M-HBs (44.4%, 30.9%) and L-HBs (62. 5%, 49.1%) at day 5 were increased as their size increases. However, this pattern was not showed in embryo transfer of day 6 HBs. But, when the live fetuses formation against total implantation rates were observed, the result (87.5%) of S-HBs of day 5 was significantly higher than that of the others ($p<0.01$). Therefore, this study demonstrates that *in vitro* cultured healthy HBs can not only be developed normally with good pregnancy rates, implantation rates and live fetuses formation, but also served as a fundamental data for utility of supernumerary HBs in human blastocyst transfer.

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