Effects of Various Addition and Exclusion Time of Glucose on Development of Mouse Two-Cell Embryos

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ABSTRACT

This study was conducted to investigate the effect of various addition and exclusion time of glucose (Control: no addition, A: 24~72 h, B: 24~48 h, C: 48~72 h, D: 0~72 h, E: 0~48 h, F: 0~24 h and 48~72 h, G: 0~24 h) on embryonic developmental capacity of 2-cell embryos in mice. Developed blastocysts were assessed for mean cell number by differential staining. The zona-intact blastocyst (ZiB) rates were higher ($p<0.05$) in group B than control. However, the zona-escape blastocyst (ZeB) rates were not significantly different in all groups. At 72 h, total blastocyst (ZiB + ZeB) formation rates were not significantly different in all groups. The mean cell number was not significantly different among all groups. The inner cell mass (ICM) cell number was higher ($p<0.05$) in group F than control, group A, B and G. The trophectoderm (TE) cell number was higher ($p<0.05$) in control than group A and D. The %ICM was higher ($p<0.05$) in group C, D and F than control. The ICM : TE ratio was not significantly different in all groups. Between control and glucose group, no significant difference was observed in the total blastocysts (ZiB + ZeB) formation rates. Also, no significant difference was observed in the mean cell number, ICM cell number and ICM : TE ratio. However the TE cell number was higher ($p<0.05$) in control than glucose group and %ICM was higher ($p<0.05$) in glucose group than control. In conclusion, glucose added in culture medium was not inhibitory on blastocyst formation but glucose added for 48~72 h in culture medium increases %ICM of blastocysts in mice.

(Key words : Blastocyst, Glucose, Cell numbers, Mouse 2-cell embryo, Inner cell mass)

INTRODUCTION

Success rates in human IVF-ET (*in vitro* fertilization - embryo transfer) programs still leave much to be desired; on major reason is a failure to mimic adequately the environment of the embryo *in vivo* (Park et al., 1998; 2000). A feature of the " second-generation" media used to culture preimplantation mammalian embryos is a reduction in the concentration, or the complete omission, of glucose during the early cleavage stages (Bavister, 1995; Quinn et al., 1995; Gardner and Lane, 1997). The finding during the late 1980s (Schini and Bavister, 1988; Seshagiri and Bavister, 1989) that physiological concentrations of glucose inhibit the development of the 2 cell hamster embryo *in vitro* was soon observed using embryos of other species: mouse (Chatot et al., 1989; Lawitts and Bigger 1991; Brown and Whittingham, 1992; Scott et al., 1993; Scott and Whittingham, 1996), rat (De-Hertogh et al., 1991; Reed et al., 1992; Miyoshi et al., 1994), sheep (Thompson et al., 1992), cow (Takahashi and First, 1992), and human (Conaghan et al., 1993; Hardy, 1994; Quinn et al., 1995; Coates et al., 1999).

Understanding the metabolic needs of preimplantation embryos is vital to improve the quantity and quality of embryo development. If the necessary energy substrates are not present in sufficient concentrations or at the appropriate time, the embryo will be unable to develop. One recurring pattern observed in embryo metabolism is increased glucose usage as preimplantation development progresses (Kim et al., 1993; Ledda et al., 1992; Lim et al., 1994; Matsuyama et al., 1993; Swain et al., 2002; Thompson et al., 1991).

The energy requirements of the preimplantation human embryo have yet to be fully determined. However, analyses of nutrient uptakes *in vitro* have revealed that the human embryo has an initial preference for pyruvate, with increasing glucose uptake as development

* This research was supported by the Daegu University Research Grant, 2004.

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procedures. The pattern of nutrient utilization by human embryos is very similar to that reported for the mouse. The mouse oocyte and zygote take up little glucose compared to pyruvate. Around the time of compaction, there is a decline in pyruvate utilization, with glucose becoming the preferred nutrient at the blastocyst.

This study was conducted to observed the effect of the various addition and exclusion time of glucose on the development of mouse 2 cell embryos.

MATERIALS AND METHODS

Preparation of Media

Embryos were cultured in Dulbecco's Modified Eagle Medium (MEM, no glucose, Gibco, USA) supplemented 20% human follicular fluid (hFF) with or without 0.5 mM glucose for 72 h. After 0.5% antibiotics (Streptomycin sulfate; Penicillin-G, Sigma, USA) added to culture media, the osmolarity of culture media was adjusted to 280 mOsmol/kg and sterilization by filteration through a 0.2 ㎛ filter (Millex-GV, Millipore, USA). The culture media were stored at 4℃ until used.

Preparation of hFF

The hFF was collected when oocyte retrieved from women undergoing treatment for assisted reproduction. The hFF was centrifuged at 3,500 rpm for 20, 10 m to remove cellular debris before heat inactivation at 56℃ for 35 m and sterilization by filteration through a 0.2 ㎛ filter. The hFF was pooled and stored at -20℃ until used.

Preparation of Two Cell Embryos

Female Institute Cancer Research (ICR) mice (3~4 weeks) were employed to induce ovulation by intraperitoneal injection of 5 IU of human chorionic gonadotropin (hCG, Sigma, USA) followed 5 IU of pregnant mare serum gonadotropin (PMSG, Sigma, USA) injection after 48 h. Female mice were naturally mated with male (10 week old) mice and checked for a vaginal plug 16~18 h after (hCG injection. Females were sacrificed by cervical vertebrae dislocation after approximately 48 h injection of hCG. Two cell embryos were flushed from the dissected oviducts of the sacrificed mice and put into MEM containing 20% fetal bovine serum (FBS, Gibco, USA). Recovered 2 cell embryos were rinsed three times in different experimental media and cultured.

Culture of Embryos

Recovered 2 cell embryos were randomly distributed in eight different culture groups(time of addition~time of exclusion, Control : no addition, A : 24~72 h, B : 24~48 h, C : 48~72 h, D : 0~72 h, E : 0~48 h, F : 0~24 h, 48~72 h, G : 0~24 h, Figure 1) and cultured for 72 h. Microdrops were placed in 60 mm culture dish (Falcon, USA) under mineral oil (Sigma, USA). Culture dishes were prepared on day before equilibrated at 37℃ in humidified atmosphere of 5% CO_2 in air.

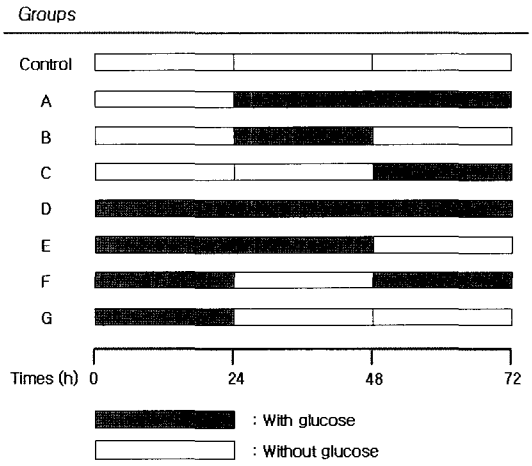

Fig. 1. The various addition and exclusion time of glucose in culture media.

Differentially Staining of Blastocysts

Mouse blastocysts were stained differentially by Park's method (Park et al., 2002b). Zona intact (ZiB, early~partially hatched blastocyst) or zona escape (ZeB, hatched blastocyst) blastocysts were first incubated (about 10 s) in 2 ㎖ solution 1 (Differential staining medium of Ham's F-10 with 1% Triton X-100 and 100 ㎍/㎕ Propidium Iodide, and immediately transferred into 1 ㎖ solution 2 (Fixative solution of 99,9% ethanol with 25 ㎍/㎕ bisbenzimide) and stored at 4℃ (≥1.5 h) for differential staining. Fixed and stained blastocysts were transferred directly from solution 2 into glycerol (Sigma, USA) without excess amount of solution 2. Blastocysts were then mounted onto slide glass in a drop of glycerol, gently flattened with a cover slide, and visualized for cell counting. Cell counting was performed on a fluorescent microscope (BX-50, Olympus, Japan) equipped with ultraviolet lamp and green filter.

Observation

The rate of embryos developed to morula, blastocyst and blastocyst (ZiB, zona intact blastocyst; ZeB, zona escape blastocyst) stage were monitored under an stereo microscope (LEICA MZ 12.5, Switzerland) at 24, 48 and 72 h, respectively (Fig. 2 A~E). At the end of the culture period, blastocysts were assessed for mean cell number, inner cell mass (ICM) cell number, trophectoderm (TE) cell number, %ICM of total cell and ICM : TE ratio of embryos by differentially staining (Fig. 2 F).

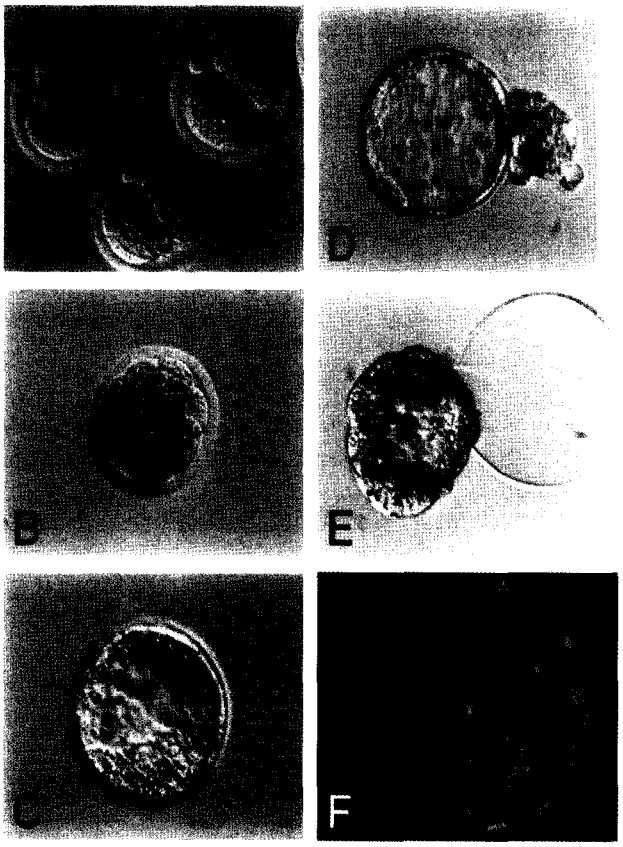

Fig. 2. Specific features of the developing and differentially stained blastocyst in mice(×200). (A). Retrieved 2 cell embryos, (B). Morula, (C). Zona intact blastocyst (ZiB), (D). Zona escape blastocyst (ZeB) : hatching blastocyst from zona pellucida (ZP), (E). Completely hatched blastocyst from ZP, (F). Differentially stained blastocyst.
Note that the intense pink color represents the chromatin in nuclei of permeabilized trophectoderm cells that are stained both red (propidium iodide) and blue (bisbenzimide). Inner cell mass nuclei remain blue only, because these cell have not been permeabilized.

Statistical Analysis

The results obtained [embryo development rates, total (mean), ICM/TE cell number, %ICM of total cells and ICM : TE ratio] were subjected to analysis of variance according to the general linear models procedure of SAS software version 8.1 (SAS Institute Inc., Cary NC, USA) and significances were compared by t-test or Duncan's Multiple Range Test. Results were considered statistically significant when p-value were less than 0.05.

Photographs

Photographs were taken with camera systems of the inverted microscope (with a Hoffman contrast- modulation system; Diaphot 300, Nikon, Japan) and fluorescent microscope [with a phase contrast and ultraviolet (UV) green filter system; BX 50, Olympus, Japan] using Kodak film (MAX, ASA 400).

RESULTS

The effects of the various addition and exclusion time of 0.5 mM glucose (Control : 0 mM, A : 24~72 h, B : 24~48 h, C : 48~72 h, D : 0~72 h, E :0~48 h, F : 0~24 h, 48~72 h, G : 0~24 h) in the culture medium on embryo development rates were summarized in Table 1.

A total of 1,352 two cell embryos was cultured in eight experimental media for 72 h. The rates of morula and blastocyst formation at 24 h and 48 h were not significantly different in all groups. The ZiB rates were higher ($p<0.05$) in group B (55.03%±23.09) than control (39.64%±24.00). However, the ZeB rates were not significantly different in all groups. At 72 h, total blastocyst (ZiB + ZeB) formation rates not significantly different in all groups.

A total of 487 blastocysts was stained differentially and cell number was counted at 72 h. The results of cell number were summarized in Table 2. The mean cell number of blastocyst was not significantly different in all groups. ICM cell number of blastocyst was($p<0.05$) in group F (13.49±8.83)than control, group A, B, and G. (10.44±6.21, 10.95±7.17, 10.71±5.94 and 10.44±7.50, respectively). TE cell number of blastocyst was higher ($p<0.05$) in control (51.35±28.57) than group A and D (42.33±27.20 and 42.78±21.71). The %ICM of total cell in blastocysts was higher ($p<0.05$) in group C, D and F (21.96%±16.14, 23.03%±16.68 and 22.42%±11.78, respectively) than control (16.9%±12.61).The ICM : TE ratio of blastocyst was not significantly different in all groups. The effects of glucose added in culture medium on formation and cell number (control vs. group) were summarized in Table 3, 4, respectively. Differences in ZiB, ZeB and total blastocysts (ZiB + ZeB) formation rates were not seen between control and glucose group. Also mean cell number, ICM cell number and ICM : TE ratio were not significantly different in two groups. But TE cell number of blastocyst was higher ($p<0.05$) in control (51.35±28.57) than glucose group (44.78±25.14) and %ICM of total cell was higher ($p<0.05$) in glucose group (20.80%±14.31) than control (16.90%±12.61).

DISCUSSION

In this study, 2 cell embryos were cultured in MEM supplemented 20% hFF with or without 0.5 mM glucose for 72 h. The ZiB (zona intact blastocyst) rates after culture for 72 h were higher ($p<0.05$) in group B (55.03%±23.09) than control (39.64%±24.00, Table 1). And, In control and glucose group, no significant difference was observed in total blastocysts (ZiB + ZeB) formation rates (68.05% vs. 70.41%, Table 3). Consequently, the glucose added in culture medium was not major inhibi-

Table 1. Effects of the various addition and exclusion time of glucose on embryo development rates

Groups	No. of examinations	No. of 2 cell embryos used	No. (%) of embryos developed for 72 h				
			Morula at 24 h*	Blastocyst at 48 h*	Blastocyst at 72 h		
					Total blastocyst*	ZiB[1)]	ZeB[2)]*
Control	18	169	51 (30.18±16.98)	69 (40.83±20.55)	115 (68.05±20.10)	67 (39.65±24.00)[b]	48 (28.41±20.81)
A	18	169	46 (27.22±14.97)	62 (36.69±18.18)	120 (71.01±12.06)	79 (46.75±18.40)[ab]	41 (24.26±15.86)
B	18	169	60 (35.50±15.75)	72 (42.60±22.34)	125 (73.96±22.04)	93 (55.03±23.09)[a]	32 (18.93±18.32)
C	18	169	49 (28.99±18.77)	64 (39.05±16.27)	118 (69.82±15.96)	76 (44.97±21.41)[ab]	42 (24.85±19.86)
D	18	169	43 (25.44±23.79)	66 (39.05±21.92)	116 (68.64±18.36)	74 (43.79±18.86)[ab]	42 (24.85±16.32)
E	18	169	54 (31.95±21.91)	68 (40.24±23.27)	119 (70.41±15.00)	72 (42.60±20.58)[ab]	47 (27.81±20.47)
F	18	169	49 (28.99±24.64)	62 (37.28±20.72)	123 (72.78±18.82)	80 (47.34±21.39)[ab]	43 (25.44±21.54)
G	18	169	50 (29.59±19.34)	62 (36.69±16.38)	112 (66.27±16.77)	72 (42.60±18.23)[ab]	40 (23.67±22.32)

• Values are means±standard deviation.
[1)] ZiB : Zona intact blastocyst.
[2)] ZeB : Zona escape blastocyst.
* No significant differences among groups.
[ab] Means with different superscript within a column were significantly different ($p<0.05$).
• Groups : Control(no addition), A(24~72 h), B(24~48 h), C(48~72 h), D(0~72 h), E(0~48 h), F(0~24 h, 48~72 h), G(0~24 h).

Table 2. Effects of the various addition and exclusion time of glucose on cell number of blastocyst

Groups	No. of examinations	No. of 2 cell embryos used	No. of blastocysts stained	No. of blastocysts stained differentially	Total cell numbers (Mean±SD)			%ICM of total cell	ICM : TE ratio*
					Total*	ICM[1)]	TE[2)]		
Control	9	88	54	54	3,337 (61.8±31.77)	564 (10.44±6.21)[b]	2,773 (51.35±28.57)[a]	16.9±12.61[b]	4.917±4.58
A	9	88	61	61	3,250 (53.28±31.30)	668 (10.95±7.17)[b]	2,582 (42.33±27.20)[b]	20.55±15.38[ab]	3.865±5.63
B	9	88	58	58	3,292 (56.76±29.84)	621 (10.71±5.94)[b]	2,671 (46.05±26.72)[ab]	18.86±11.66[ab]	4.301±5.78
C	9	88	66	66	3,716 (56.30±27.22)	816 (12.36±8.08)[ab]	2,900 (43.94±25.14)[ab]	21.96±16.14[a]	3.554±4.81
D	9	88	60	60	3,335 (55.58±25.70)	768 (12.80±9.69)[ab]	2,567 (42.78±21.71)[b]	23.03±16.68[a]	3.342±4.91
E	9	88	63	62	3,660 (59.03±26.14)	698 (11.26±7.82)[ab]	2,962 (47.77±23.67)[ab]	19.07±13.21[ab]	4.244±8.41
F	9	88	68	67	4,033 (60.19±28.64)	904 (13.49±8.83)[a]	3,129 (46.70±23.15)[ab]	22.42±11.78[a]	3.461±6.93
G	9	88	59	59	3,194 (54.14±32.55)	616 (10.44±7.50)[b]	2,578 (43.69±28.73)[ab]	19.29±14.64[ab]	4.185±7.28

• Values are means±standard deviation.
[1)] ICM : Inner cell mass.
[2)] TE : Trophectoderm.
* No significant differences among groups.
[ab] Means with different superscript within a column were significantly different ($p<0.05$).
• Groups : Control(no addition), A(24~72 h), B(24~48 h), C(48~72 h), D(0~72 h), E(0~48 h), F(0~24 h, 48~72 h), G(0~24 h).

tory effect on the blastocyst formation in mice. Coates et al. (1999) reported the outcome of a paired treatment, randomized, controlled trial using culture media with or without glucose. In view of the reported inhibitory effects of glucose on early embryo development (in humans as well as in animals), that there was no difference in clinical pregnancy rates between these two treatments. Also, They concluded that culturing pronucleate human embryos in glucose-free medium versus medium containing 5.5 mM glucose did not improve any key parameter of the clinical procedures including clinical pregnancy rates (with the exception of a slight

Table 3. Effects of glucose added in culture medium on blastocyst formation (control vs. glucose group)

	Groups*	
	Control (None glucose)	Glucose group (Added glucose)
No. of examination	18	18
No. of 2 cell embryos used	169	1,183
Development rates (mean±SD)		
Morula at 24h	51 (30.18±16.98)	351 (29.67±19.86)
Blastocyst at 48h	69 (40.83±20.55)	453 (38.29±19.68)
Total blastocyst at 72h	115 (68.05±20.10)	833 (70.42±17.04)
ZiB[1)]	67 (39.65±24.00)	546 (46.16±20.44)
ZeB[2)]	48 (28.41±20.81)	287 (24.26±19.05)

• Values are means±standard deviation.
[1)] ZiB : Zona intact blastocyst.
[2)] ZeB : Zona escape blastocyst.
*No significant differences between groups.

Table 4. Effects of glucose added in culture medium on cell numbers (control vs. glucose group)

	Groups	
	Control (None glucose)	Glucose group (Added glucose)
No. of examination	9	9
No. of 2 cell embryos used	88	616
No. of blastocysts stained	54	435
No. of blastocysts stained differentially	54	433
Cell numbers (mean±SD)		
Total	3337 (61.8 ±31.77)	24,480 (56.54±28.72)
ICM[1)]	564 (10.44± 6.21)	5,091 (11.76± 7.98)
TE[2)]	2,773 (51.35±28.57)[a]	19,389 (44.78±25.14)[b]
%ICM of total cell	16.90±12.61[b]	20.80±14.31[a]
ICM : TE ratio(1:)	4.917±4.58	3.808±6.37

• Values are means±standard deviation.
[1)] ICM : Inner cell mass.
[2)] TE : Trophectoderm.
[ab] Means with different superscript within a column were significantly different ($p<0.05$).

improvement in mean embryo quality, morphology) (Coates et al., 1999). Yet Conaghan et al. (1993) reported increased development and blastocyst cell numbers in human embryos cultured in glucose-free medium until after the 4 to 8 cell stages. About this apparent discrepancy, one possible explanation is that development of embryos, especially in the cleavage stages is optimal with a low concentration of glucose, and that both "high"(5.5 mM) and zero glucose concentration are suboptimal or inhibitory. Glucose inhibition of embryo development was first shown in hamster embryos, but low level of glucose (0.5 mM) added in culture medium increased the proportion of fetuses formed from transferred embryos (Ludwig et al., 1998). It is of interest that improvement with low-glucose medium during preimplantation embryo development was only evidenced by the timely manner in which more embryos reached the 8 cell stage (Coates et al. 1999). In addition, several studies with cow and sheep embryos reported that glucose blocks development of cleavage stages and even inhibits blastocyst formation unless its concentration in the culture medium is reduced (Bavister, 1995) However, glucose is required for optimal blastocyst development, as shown most clearly in studies by Gardner and Lane (1998). These authors have devised a "two-step"culture system wherein: [1] the medium for supporting cleavage stages contains a very low concentration of glucose together with EDTA to inhibit glycolysis; and [2] the medium for supporting morula and blastocyst development contains a higher concentration of glucose and no EDTA to facilitate glycolysis (because it is known that glucose metabolism increases considerably in these later stages).

The number of ICM and TE cells may be a valid indicator of viability and quality of mammalian blastocyst (Conaghan et al., 1993; Park et al., 2002b; Ludwig et al., 1998). Conaghan et al. 1993) reported that the significant increase in cell number of blastocyst was caused by removing glucose from the medium. Specifically, the rise in cell number was most apparent in the TE, where cell numbers was almost double in glucose-free medium (66.1±7.7 and 37.2±7.1 in 0 mM and 1.0 mM glucose, respectively). Such a significant and dramatic rise in TE cell number could improve the ability of blastocysts to implant after embryo transfer. In this study, TE cell number was higher ($p<0.05$) in control (51.35±28.57) than group D and A (42.78±21.71 and 42.33±27.20), and ICM cell number was higher ($p<0.05$) in group F (13.49±8.83) than control (10.44±6.21), group A (10.95±7.17), B (10.71±5.94) and G (10.44±7.50). The %ICM of total cell were higher ($p<0.05$) in group C (21.96±16.14), D (23.03±16.68)and F (22.42±11.78) than control (16.9±12.61). The ICM : TE ratio was not statistically different in all groups. Park et al. (2000; 2002a) reported that the number of BG (Blastocyst grade) 1 (50.8%) with high implantation capacity (15.0%) and %ICM of total cell number was higher in MEM (50.8% and 20.9%) compared with TCM (15.0% and 17.1%).

Differential staining of the mouse blastocysts was failed almost, when the blastocysts were exposed to PI morethan 16 s. However, it was found that the exposure length of bisbenzimide was not affected differential staining of mouse blastocysts(Park et al., 2002a; b). A zona pellucida did not appear to inhibit dye penetration. Triton X-100, a non-ionic detergent, was used as described in the previous studies(Ebert et al., 1985; Thouas et al., 2001). An example of differentially stained blastocyst is illustrated in Figure 2 F. The TE nuclei were labelled with Propidium Iodide(PI) and the ICM nuclei with bisbenzimide. This combination of fluorochromes

was chosen because their fluorescent spectra can be separated using appropriate combination of excitation and emission filters. PI is excited mainly by green light but is also excited to a lesser extent by UV illumination and emits in the red; bisbenzimide is excited by UV illumination only and emits in the blue region of the spectrum (Handyside and Hunter, 1984; Mishira and Seshagiri, 1998; Thouas et al., 2001; Park et al., 2002a, b).

The first report of the inhibitory effect of glucose on preimplantation development was that of Schini and Bavister (1988). The inhibitory effect was subsequently confirmed in other species. In the light of its detrimental effect on embryos when present in simple culture media, there is a growing tendency to remove glucose from media for the development of the human embryo (Quinn, 1995). However, no inhibitory effect of glucose on preimplantation development *in vitro* was found in the pig (Petter et al., 1990; Hagen et al., 1991) and mouse (Kim et al., 2000; Lawitts and Bigger, 1992).

In conclusion, glucose added in culture medium was not inhibitory effect on the blastocyst formation but glucose added for 48~72 h in culture medium increases %ICM of blastocysts in mice.

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(Received: 8 November 2004 / Accepted: 10 December 2004)