大韓不妊學會雜誌: 第 23 卷 第 3 號 1996
Kor. J. Fertil. Steril., Vol. 23, No. 3, 1996, 12

Effects of Energy Substrates on *In Vitro* Fertilization of the Mouse Oocytes with Cumulus Mass and their Developments

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생쥐 체외수정과 배아 발달에 미치는 에너지원의 영향

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김충현 · 장은주 · 정경순 · 박소현 · 황도영 · 김기철 · 민응기

= 국문초록 =

난구세포는 lactate와 pyruvate를 쉽게 생성하고, 이로 인해 배양액내 에너지원의 농도를 변화시켜 난자의 수정과 배양에 영향을 주는 것으로 알려져 있다. Glucose, lactate 및 pyruvate의 농도가 상이한 M16, MTF 및 CZB배양액에서 난구세포를 포함한 생쥐난자의 체외 수정과 발달을 관찰하여, 이들 기질의 영향에 대하여 살펴보고 배양액의 유용성을 재검토하고자 하였다.

Glucose를 제거한 배양액 (CZ2 배양액)에서 수정율과 배반포 형성율은 다른 배양액에 비해 유의하게 감소되었으나 ($p<0.05$), 생쥐 난관액과 동일한 기질 농도로 조성된 MT1 (난관액이 난구세포를 포함하고 있을 때) 및 MT2배양액 (난관액이 난구세포를 포함하고 있지 않을 때)과 glucose를 포함한 modified CZB배양액에서는 영향이 없었다.

이와 같은 결과로 기질의 농도를 생리적 수준으로 조정한 배양액의 이용은 난구세포를 포함한 생쥐 난자의 체외수정과 그 발달을 향상시키지 못하고, glucose의 제거는 악영향을 나타내는 것으로 사료된다.

Key Words: Glucose, Lactate, Pyruvate, Mouse oviductal fluid, Cumulus cell.

ABSTRACT

Cumulus cells have possibly influence on fertilization of mouse oocytes and their subsequent development *in vitro*, because they readily produce lactate and pyruvate and can modify the concentration of substrates in the medium. *In vitro* fertilization of mouse oocytes with cumulus mass and their developments in five media which were differently composed in concentrations of glucose, lactate and pyruvate were observed.

In the absence of glucose (CZ2 medium) decreased ($p<0.01$) the percentage of fertilization and embryos reaching the blastocyst stage. But, in the same concentration of glucose, lactate and pyruvate as mouse oviductal fluid with (MT1 medium) and without (MT2 medium) cumulus mass and modified CZB medium containing glucose (CZ1 medium) had no effects ($p>0.05$). These studies indicate that the adjustments of energy substrates concentration to the physiological level did not improve the fertilization of mouse oocytes with cumulus mass and their development *in vitro*, and the dele-

tion of glucose showed adverse effects.

INTRODUCTION

Although considerable improvement has been made in culturing preimplantation mammalian embryos, complete development from the zygote to blastocyst is achieved in only a few species. Even then, embryo development *in vitro* is retarded compared with *in vivo* (Bowman & McLaren 1970; Nonogaki *et al.*, 1992). The reasons of retardation are not clear. Many investigators have tried to overcome these phenomena by alteration of media composition (Chatot *et al.*, 1989; Holst *et al.*, 1990; Gardner & Sakkas, 1993) and culture system (Nonogaki *et al.*, 1992; Bongso *et al.*, 1992; Ali *et al.*, 1993; Plachot *et al.*, 1993; Mansour *et al.*, 1994). Fertilization and development of early preimplantation embryo take place in the oviduct. So, they have maden media based on oviductal fluid (Quinn *et al.*, 1985; Gardner & Leese, 1990) and cocultured oviductal epithelium (Bongso *et al.*, 1992; Yeung *et al.*, 1992; Wiemer *et al.*, 1993) as an analogy to oviductal environment.

There are some differences in concentration of glucose, pyruvate and lactate between oviductal fluid and several media (Table 1). Although these differences were adjusted in HTF (Quinn *et al.*, 1985) and MTF (Gardner & Leese, 1990) media, they did not improve embryo development. The reason why they did not improve embryo development may be that cumulus cells were excluded in those studies. Cumulus cells connect with oocytes, metabolize actively and change concentrations of energy substrates in oviduct fluid (Gardner & Leese, 1990). Therefore, they can influence *milieu* of oocytes and zygotes (Leese & Barton, 1985; Gardner & Leese, 1990). In order to simulate natural conditions, the early preimplantation embryo culture system must include cumulus cells. And optimal concentration of substrates in media have need to be reexamined. MTF (Gardner & Leese, 1990) and CZB (Chatot *et al.*, 1989) were known as the good embryo culture media for mouse. So, we modified the substrates concentration of these media and observed fertilization rates of the mouse oocytes with cumulus cells and their development.

MATERIALS AND METHODS

Fertilization and Embryo Culture Media

The fertilization and embryo culture media used in these studies were M16 (Whittingham, 1971), MTF and CZB, three previously published, simple, embryo culture media. We modified the compositions of MTF and CZB media. They were formulated using the ionic composition of M16 except for the concentrations of sodium chloride. The variations in sodium chloride concentration were due to the adjustment of osmolarity. The concentration of glucose, lactate and pyruvate in MT1 and MT2 media were the same as mouse oviduct fluid with and without cumulus mass, respectively. Glutamine and EDTA were om-

Table 1. Concentrations(mM) of glucose, lactate and pyruvate in several media and oviduct fluid of human and mouse

	HTF*	M16*	CZB*	Human†		Mouse††	
				follicular§	midcycle‖	+Cumulus¶	-Cumulus※
Glucose	2.78	5.56	(5.56)	3.11	0.50	3.40	5.19
Lactate	21.4	23.3	31.3	4.78	10.5	4.79	4.26
Pyruvate	0.33	0.33	0.27	0.25	0.32	0.27	0.14

*: Gardner & Lane(1993a), †: Gardner *et al*(1996), ††: Gardner & Leese(1990), §: follicular phase, ‖: ovulation(days 12 to 16), ¶: oviduct fluid at presence of cumulus, ※: oviduct fluid fluid at absence of cumulus.

itted in modified CZB media (CZ1 and CZ2 medium). Glucose was present in the CZ1 medium, not in the CZ2 medium. The formulations of media used in these studies are shown in Table 2.

Collection of oocytes

Oocytes were collected from F1 (CBA×C 57BL/6) hybrid female mice. Virgin female (5~6 weeks of age) were superovulated with 7.5IU PMSG (Sigma) administered i.p. at 18:00h, followed 48 hours later by 5IU hCG (Sigma). At 14~15 hours after hCG, the oviducts were dissected free of other tissues, and rinsed in D-PBS (Gibco) contained 0.4% BSA (Sigma). The mass of oocyte cumulus complex (OCC) was teased from oviduct into 0.4% BSA in D-PBS. OCC from different females were pooled and transferred to M16 medium contained 0.4% BSA and then incubated at 37℃, in 5% CO_2. After 10 minutes OCC were dispersed and separated from each other. The oocytes were assigned randomly to each of the study media.

In vitro fertilization & embryo culture

Sperm were collected from F1 hybrid male mice (9~10 weeks of age). Before oocytes collection, one epididymis from each of two mice were dissected free of other tissues, and rinsed in D-PBS contained 0.4% BSA. The mass of sperm were teased from the epididymis into 2 ml of M16 without BSA, and then incubated at 37℃, in 5% CO_2. After 10 minutes, 0.2 ~ 0.4 ml of sperm solution were transferred to 2ml of each media contained 0.4% BSA and then incubated at 37℃, in 5% CO_2 until oocytes were ready. At insemination, 0.2ml of each prepared sperm solutions were used and counts of sperm were adjusted to 10×10^4 motile sperm/ml. After 24 hours, fertilization rates were estimated by numbers of two-cell stage embryos. Further developments were observed at 48 and 96 hours postinsemination.

Statistical analysis

Fertilization rates and the proportions of embryos reaching the four-cell and blastocyst stages were subjected to an arc-sine transformation, and the transformed values were assigned for ANOVA. When ANOVA revealed a significant treatment effect, the treatments were compared by Duncan's Multiple Range test.

RESULTS

Fertilization rates in each media are shown in Table 3. In the CZ2 medium the fer-

Table 2. Compositions(mM) of IVF and embryo culture media

	M16	MT1	MT2	CZ1	CZ2
NaCl	94.66	114.19	114.06	86.70	86.70
KCl	4.78	4.78	4.78	4.78	4.78
KH_2PO_4	1.19	1.19	1.19	1.19	1.19
$MgSO_4 \cdot 7H_2O$	1.19	1.19	1.19	1.19	1.19
$NaHCO_3$	25.00	25.00	25.00	25.00	25.00
$CaCl_2 \cdot 2H_2O$	1.71	1.71	1.71	1.71	1.71
D-glucose	5.56	3.40	5.19	5.56	-
Na-lactate	23.28	4.79	4.26	31.30	31.30
Na-pyruvate	0.33	0.37	0.14	0.27	0.27

BSA was contained in all media at 4.0mg/ml, Penicillin was contained in all media at 100IU/ml, Streptomycine was contained in all media at 50μg/ml.

tilization rate (45.6%) decreased significantly ($p<0.05$). Differences between the other media were not significant.

Developments of the mouse embryo in each media are shown in Table 4. There were no significant differences in the percentage of embryos reaching four-cell stage at 48 hours after insemination. Like the fertilization rate results, only in CZ2 medium the percentage of embryos reaching the blastocyst stage at 96 hours after insemination (22.6%) decreased significantly ($p<0.05$).

DISCUSSION

The mammalian oviduct allows fertilization of the ovum to occur and maintains the embryo in its initial stages of development (Borland *et al.*, 1980). The most commonly investigated substrates in oviductal fluid are glucose, lactate and pyruvate because of their importance to gamete and embryo metabolism (Cross & Brinster, 1973; Leese & Barton, 1984; Seshagiri & Bavister, 1989; Gardner & Leese, 1990; Nichol *et al.*, 1992; Rosenkrans CF Jr. *et al.*, 1993; Quinn *et al.*, 1995). The concentrations of these substrates in most media are different from those of the substrates in the oviductal fluid (Gardner & Leese, 1990; Gardner & Lane, 1993a). Also, the concentrations change during the oestrus cycle (Gardner & Leese, 1990; Nichol *et al.*, 1992; Gardner *et al.*, 1996). The media having the same substrate concentrations as oviductal fluid were expected to improve embryo development, but they did not (Quinn *et al.*, 1985; Gardner & Leese, 1990). These unexpected results were possibly due to exclusion of cumulus cells surrounding the oocyte. We studied the *in vitro* fertilization of mouse oocytes in the presence of cumulus mass and the subsequent development to blastocyst in five media composed of different concentrations of glucose, lactate and pyruvate. Because in the absence of cumulus mass the optimal composition of the medium may be different in the presence of cumulus mass, we did not make comparisons between presence and absence of cumulus mass.

In the CZ2 medium the fertilization rate (45.6

Table 3. Effects of several media on fertilization rate in mouse

Medium	No. of ova* inseminated	No. of ova "fertilized"†	Fertilization rate (%)
M16	121	82	67.8
MT1	138	96	69.6
MT2	138	104	75.4
CZ1	123	78	63.4
CZ2	136	62	45.6††

*: Ova collected 14~15 hours after hCG, †: Ova developed to two-cell stage 24 hours after insemination, ††: $p<0.05$.

Table 4. Effects of several media on embryo development *in vitro*

Medium	No. of embryo	Percentage of 4-cell at 48hr psi*†	Percentage of blastocyst at 96hr psi†
M16	82	70.7(58)	53.7(44)
MT1	96	71.9(69)	42.7(41)
MT2	104	87.5(91)	56.7(59)
CZ1	78	78.2(61)	46.2(36)
CZ2	62	48.4(30)	22.6(14)††

*: psi means postinsemination, †: Values in parentheses are number of embryo reaching those cell stage, ††: $p<0.05$.

%) decreased significantly ($p<0.05$). In the other four media, the differences are not significant (Table 3). Development of embryos were observed at 48 and 96 hours after insemination (Table 4). At 48 hours after insemination, there were no significant differences in the percentage of embryos reaching the four-cell stage ($p>0.05$). In the CZ2 medium the percentage of embryos reaching the blastocyst stage at 96 hours after insemination (22.6%) decreased significantly ($p<0.05$).

The lowest fertilization rate and percentage of embryos reaching blastocyst in the CZ2 medium may be the result of the absence of glucose. Glucose is utilized as the major substrate by embryos when the GLUT2 gene is expressed (Leese, 1988; Gardner & Leese, 1988; Hogan *et al.*, 1991) and cumulus cells. In the CZB medium, the absence of glucose were compensated by glutamine and cumulus cells were not included (Chatot *et al.*, 1989). But in the CZ2 medium glutamine as well as glucose was omitted. The requirement of glucose was evident by comparison of CZ1 and CZ2 media. The CZ1 medium with glucose showed a significantly higher fertilization rate and the percentage of embryos reaching the blastocyst stage than the CZ2 medium without glucose.

The concentrations of pyruvate and lactate have been shown to be important for optimal development of mouse embryos. They are the main energy substrates for one or two-cell stage mouse embryos. Also, optimal lactate/pyruvate ratio in the medium is essential for the balancing of oxidation-reduction potential in the embryos, because the early preimplantation embryo does not have the mechanism for alleviating intracellular acid loads (Balts *et al.*, 1991; Balts *et al.*, 1993). It is known that a lactate/pyruvate ratio in medium approaching 120 is beneficial for embryo development (Cross & Brinster, 1973; Chatot *et al.*, 1989). The lactate/pyruvate ratio in MT2 medium was about 30, a lower level than the other media except MT1. Although MT2 medium did not significantly improve the fertilization rate or the percentage of embryos reaching the four-cell stage, it showed the best results among the five media (Table 3 & 4). This may be due to a modification of microenvironments in the vicinity of eggs and embryos by mouse cumulus cells. Eggs and embryos may be exposed to the much higher lactate and pyruvate concentration in the presence of cumulus cells than in the absence of cumulus cells, because cumulus cells can readily produce lactate and pyruvate. Also, the rate of lactate production by cumulus cells is about 40 times greater than pyruvate (Leese & Barton, 1985). For these reasons, high lactate/pyruvate ratio in medium is possibly adverse to the fertilization of mouse oocyte surrounded with cumulus mass and their subsequent development *in vitro*.

But, the percentage of blastocyst formation in MT2 medium did not differ from other media (Table 4). Embryos move along the female reproductive tract during preimplantation stage, so their environments are not constant. At late preimplantation stage, embryos are in the uterus rather than oviducts and the cumulus mass are dispersed. In human uterine fluid, the concentrations of glucose, lactate and pyruvate are different from those of tubal fluid (Gardner *et al.*, 1996). Also, metabolic behaviors of embryo and controls of intracellular redox potential balancing are transient. In the early cleavage stage, the preimplantation embryos metabolize lactate and pyruvate predominantly. Utilization of glucose is increased since morula stage and it is predominant at the blastocyst stage (Leese & Barton, 1984; Gardner & Leese, 1988; Hogan *et al.*, 1991; Gardner & Lane, 1993a). And the controls of intracellular redox potential balancing are transient from lactate-pyruvate system to others (Gardner & Sakkas, 1993). The culture condition in the MT2 medium seems not to be suitable for late preimplantation stage, because it was adjusted to only oviductal conditions. In these studies, we used simply media. The

biological fluids have many kinds of materials which are helpful or harmful for embryo developments. Some deficiency of these helpful natural materials such as amino acid, vitamine and proteins may cause unimprovement of culture condition. Also, filtration of toxicant by the oviduct wall and exact salt concentration of reproductive tract fluid may affect embryo developments.

In conclusion, the conditions similar to environment of oviduct did not significantly improve fertilization and embryo development. And it is suggested that although these conditions might be helpful to development of early preimplantation embryo, they could not support development of late preimplantation embryo.

Use of a new culture medium in which substrates concentration are similar to uterine fluid at late preimplantation stages, the adequate salt composition and the addition of amino acid and EDTA may improve fertilization and embryo developments in mouse (Borland *et al.*, 1977; Leese *et al.*, 1979; Metha & Kiessling, 1990; Gardner & Lane, 1993b).

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