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Optimization of *In Vitro* Murine Embryo Culture Condition based on Commercial M16 Media

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ABSTRACT

In vitro culture of murine embryos is an important step for *in vitro* production systems including *in vitro* fertilization and generations of genetically engineered mice. M16 is widely used commercialized culture media for the murine embryos. Compared to other media such as potassium simplex optimization medium, commercial M16 (Sigma) media lacks of amino acid, glutamine and antibiotics. In the present study, we optimized M16 based embryo culture system using commercialized antibiotics-glutamine or amino acids supplements. *In vivo* derived murine zygote were M16 media were supplemented with commercial Penicillin-Streptomycin-Glutamine solution (PSG; Gibco) or MEM Non-Essential Amino Acids solution (NEAA; Gibco) as experimental design. Addition of PSG did not improved cleavage and blastocyst rates. On the other hand, cleavage rate is not different between control and NEAA treated group, however, blastocyst formation is significantly (P<0.05) improved in NEAA treated group. Developmental competence between PSG and NEAA treated groups were also compared. Between two groups, cleavage rate was similar. However, blastocyst formation rate is significantly improved in NEAA treated group. Taken together, beneficial effect of NEAA on murine embryos development was confirmed. Effect of antibiotics and glutamine addition to M16 media is still not clear in the study.

(Key words : mouse embryo, *in vitro* culture, NEAA, M16)

INTRODUCTION

In vitro culture of murine embryos is important step for *in vitro* production systems including *in vitro* fertilization, rederivation of frozen embryos and sperms and generations of genetically engineered mice (GEM). Because needs for the GEM such as knockout mice were very high in modern biomedical research, importance for optimizing *in vitro* production procedure also increase to reduce time and cost to get proper animal resource.

For *in vitro* culture of murine embryos, M16 media is widely used because it is commercially available and relatively cheap. However, our preliminary test showed that developmental competency of murine embryos cultured in M16 media is inferior compared to other murine embryo culture media such as potassium simplex optimization medium (KSOM). Composition of commercial M16 media (Sigma) is different from the KSOM and lacks of amino acid including glutamine and antibiotics.

To optimize *in vitro* culture condition based on M16 media, in the present study, we added commercially available amino acid supplements non-essential amino acid (NEAA) or penicillin-streptomycin-glutamine complex solutions to M16 media and analyze developmental competency of cultured murine embryos.

MATERIALS AND METHODS

1. Preparation of *In Vivo* Derived Murine Zygotes

Murine zygotes were obtained from C57BL/6 (Orient Bio, Korea) strain mouse by standard PMSG-hCG procedure. Briefly, female mice were IP injected with 5 IU PMSG (Sigma-Aldrich), followed by 5 IU of HCG (Sigma-Aldrich, MO, USA) after 48 h. The hormone injected mice were mated with male mice overnight. Mating was confirmed by vaginal plug check at the next day morning. Vaginal plug checked female mice were euthanized and the oviducts were surgically removed. Under microscope, zygotes were isolated from ampulla of the oviducts. The zygotes were washed three times with M2 medium (Sigma-Aldrich).

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2. *In Vitro* Culture of Murine Embryos

Murine embryos were cultured in drop of M16 media (Sigma-Aldrich) under mineral oil (Sigma-Aldrich). As experimental design, the M16 medium was supplemented with commercial Penicillin-Streptomycin-Glutamine solution (PSG; Gibco) or MEM Non-Essential Amino Acids solution (NEAA; Gibco). Environmental condition of the *in vitro* culture is 5% CO_2 and 5% O_2 at 37℃. Cleavage and blastocyst formation rates were recorded at day 2 and 4 of *in vitro* culture, respectively.

3. Statistical Analysis

All data were subjected to *t*-test using Prism software (Version 5.0, GraphPad Sofware, San Diego, CA, USA) to determine differences between experimental groups. Statistical significance was determined when the *P* value was less than 0.05.

RESULTS

1. Effect of PSG Supplementation during *In Vitro* Culture of Murine Embryos

Effect of PSG supplementation to the *in vitro* culture media, M16, was evaluated. In total, 59 and 58 embryos for control and PSG treated groups, respectively, were analyzed in the study. Embryos in PSG group showed slightly higher cleavage (86.0±7.7%) and blastocysts formation (94.7±2.7%) rates compared to control group (78.4±8.7% and 78.3±16.7% for cleavage and blastocyst rates, respectively) However, there is no statistically difference (Table 1).

2. Effect of NEAA Supplementation during *In Vitro* Culture of Murine Embryos

Effect of NEAA supplementation to the *in vitro* culture media, M16, was evaluated. In total, 92 and 93 embryos for control and NEAA treated groups, respectively, were analyzed in the study. As shown in Table 2, there is no statistically difference in cleavage rate between control and NEAA treated groups (82.0±5.5% vs. 72.7±4.3%). However, embryos in NEAA group showed significantly higher blastocysts formation rate (73.8±20.3%) compared to control group (49.1±25.4%).

Table 1. Effects of PSG supplementation on the development of murine pre-implantation embryos

	Total No.	Cleavage (%)	Blastocysts (%)
Control	59	44 (78.4±8.7)	36 (78.3±16.7)
PSG	58	48 (86.0±7.7)	45 (94.7± 2.7)

Table 2. Effects of NEAA supplementation on the development of murine pre-implantation embryos

	Total No.	Cleavage (%)	Blastocysts (%)
Control	92	75 (82.0±5.5)	36 (49.1±25.4)[a]
NEAA	93	68 (72.7±4.3)	53 (73.8±20.3)[b]

[a,b] Different superscripts represent significant differences.

3. Comparison of Developmental Competency between PSG or NEAA Supplementation Groups

Developmental competence between PSG and NEAA treated groups are also compared. In total 42 embryos for each groups were analyzed. As shown in Table 3, there is no statistically difference in cleavage rate between PSG and NEAA treated groups (61.9±11.9% vs. 66.6±4.8%). However, embryos in NEAA group showed significantly higher blastocysts formation rate (81.7±4.4%) compared to PSG treated group (51.9±9.7%).

DISCUSSION

In the present study, we found that addition of NEAA solution significantly improve culture condition of murine embryos. Amino acid is known as an essential factor for murine embryo development. Amino acids is present in the female reproductive tract fluids and known to help embryo development of embryos (Beebe *et al.*, 2009; Lane and Gardner, 1994). The amino acids helps embryo development by regulating osmolarity, stabilizing membrane, and maintaining pH (Boatman, 1997; Edwards *et al.*, 1998). Therefore, supply of amino acids is important for successful *in vitro* culture of murine embryos. Interestingly, among the various amino acids, glutamine (Gln) is very important for embryo culture. It is reported that Gln

Table 3. Comparision of PSG and NEAA supplementations on the development of murine pre-implantation embryos

	Total No.	Cleavage (%)	Blastocysts (%)
PSG	42	26 (61.9±11.9)	13 (51.9±9.7)[c]
NEAA	42	28 (66.6± 4.8)	23 (81.7±4.4)[d]

[c,d] Different superscripts represent significant differences.

is especially important for early stage of embryo development.

In the present study, we also found that addition of NEAA to M16 medium significantly improved developmental competency of *in vitro* cultured embryos. Interestingly, cleavage rate is not different between control and NEAA treated group. It means that addition of NEAA to M16 media had positive effect to reduce 2-cell block of the murine embryo development.

However, addition of PSG did not improve both cleavage rate and blastocyst formation rate in embryo culture. It might be related with presence of pyruvate and lactate in M16 media. Pyruvate/lactate and glutamine are share same energy metabolism pathway, thus, presence of pyruvate/lactate in M16 media is more than enough for early murine embryo development. On the other hand, PSG solution also containing widely used antibiotics penicillin and streptomycin. Addition of antibiotic to the media is convenient to reduce contamination of cultured embryos. However, addition of insufficient amount of antibiotics are detrimental for embryo culture. In the present study, addition of PSG to M16 is not detrimental for murine embryo development. Thus, it can be regarded as safe condition for murine embryo culture.

Between two condition, NEAA or PSG supplemented group, NEAA group showed better developmental competency as expected. Thus, we can recommend NEAA as a optimized supplement for murine embryo culture system based on M16 media. On the other hand, we added PSG to M2 media (M16 media with HEPES buffer and without lactate) for washing, handling/manipulating the embryo as our standard protocol (data not shown).

Taken together, beneficial effect of NEAA on murine embryos development was confirmed in the present study. Effect of antibiotics and glutamine is still not clear in the study. At least, addition of PSG is not detrimental for murine embryo development. For most *in vitro* culture system, addition of antibiotics is convenient for handling thus it can be recommended to include PSG for murine embryos culture medium. Further study for combination effect of PSG and NEAA is needed to further optimization of M16 base murine embryo culture.

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