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## **Enhancement of NMRI Mouse Embryo Development *In vitro***

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### **ABSTRACT**

Most of the systematic studies used in the development of human embryo culture media have been done first on mouse embryos. The general use of NMRI outbred mice is a model for toxicology, teratology and pharmacology. NMRI mouse embryo exhibit the two-cell block *in vitro*. The objective of this study was to evaluate and compare the effects of four kinds of culture media on the development of zygotes (NMRI) after embryo vitrification. One-cell mouse embryos were obtained from NMRI mice after superovulation and mating with adult male NMRI mice. And then randomly divided into 4 groups for culture in four different cultures media including: M16 (A), DMEM/Ham, F-12 (B), DMEM/Ham's F-12 co-culture with Vero cells (C) and DMEM/Ham's F-12 co-culture with MEF cells (D). Afterward all of the embryos were vitrified in EFS40 solution and collected. Results of our study revealed, more blastocysts significantly were developed with co-culture with MEF cells in DMEM/Ham's F-12 medium. More research needed to understand the effect of other components of culture medium, and co-culture on NMRI embryo development.

**Keywords:** NMRI Mice, 2-cell block, Mouse Embryonic Fibroblast Cell, Blastocyst, Vero Cells

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### **INTRODUCTION**

The media that most of the researches used in the development of human embryo culture, it has been done first on mouse embryo due to similarity of the basic metabolic pathways between human and mice (Scott *et al* 1993, Quinn & Horstman 1998). In this study the development of zygotes of NMRI outbred mice were evaluated in different culture media and co-

cultures. NMRI mouse is a model for general use, including toxicology, teratology, pharmacology (especially in psychopharmacology for behavioral studies) and physiology studies ([www.criver.com/SiteCollection Documents/NMRI-MICE.pdf](http://www.criver.com/SiteCollectionDocuments/NMRI-MICE.pdf)). Most of the outbred and inbred strains of mice stop development at the 2-cell stage, a phenomenon known as the '2-cell block'. Although it occurs in particular *in vitro* culture conditions and depends on the strain of mouse (Biggers *et al* 1998 2004, Zanoni *et al* 2009). At the first time Whitten and colleagues had shown that

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two-cell mouse embryos would develop into blastocysts in vitro conditions but not zygotes, and later researchers reported that mouse zygotes would cleavage to the two-cell stage in vitro and then degenerated. They suggested that the oviduct protects the embryo over this apparently vulnerable stage (Cole & Paul 1965, Whitten *et al* 1957).

The recent studies on two-cell block are over zygotic gene activation (ZGA) that is the critical event. Zygotic gene activation governs the transition from maternal to embryonic control of development. In the mouse, a minor burst of ZGA toward the end of the one-cell stage is followed by a major burst during the two-cell stage (Schultz *et al* 2002, Kanka *et al* 2003). More research showed that reciprocal mating between blocking and non-blocking mice strains has shown that the 2-cell block is dependent upon female, but not male mice (Zanoni *et al* 2009). Somatic cell co-culture plays a major role on development of preimplantation embryos cultured in vitro conditions (Orsi *et al* 2007). Taniguchi and colleagues reported that the co-culture system with KGN cells significantly increased the rate of murine preimplantation embryos development (Taniguchi *et al* 2004). Moreover, co-culture of human embryos with primary oviductal and granulosa cells was beneficial due to the removal of embryotoxic substances from the culture medium (Tavares *et al* 2011). In this study the development of zygotes of NMRI outbred mice was evaluated in different cultures media and co-cultures.

## MATERIALS AND METHODS

Dulbecco's Phosphate Buffered (Sigma, D5773-11), M2 Media (Sigma, M7167), M16 (Sigma, M7292), DMEM high glucose (Gibco, 52100021), Ham's F-12 medium (Biowest, P0134-N1L), Serum (Gibco, 12800-082), FBS (GIBCO, 10270-106), Trypsin (Merck, 8363), Versin (EDTA) (Fluka, 03685), Pregnant Mare's Serum Gonadotrophin (Sigma), Human Chorionic Gonadotrophin (Sigma), EFS40 solution, cells for co-cultures and mice from Razi Vaccine and Serum Research Institute.

**Superovulation Induction.** All animals were kept under standard conditions; in animal laboratory department of Razi vaccine and serum research institute. Animal usage was according to the Animal Care and Use Committee of Razi Vaccine and Serum Research Institute (MED.0008.QGL). The female NMRI mice between 5 to 8 weeks old were kept under a 12 h dark/light cycle. Mice were superovulated by intraperitoneal injection of 5 IU pregnant mare's serum gonadotrophin (PMSG, Sigma) followed 48 hours later by 5 IU human chorionic gonadotrophin (hCG, Sigma). After hCG administration, they were mated with males of the same strain and sacrificed on day 1 of pregnancy.

**One-cell embryos collection.** Collection of one-cell embryos was taken place on 0.5 d.p.c (Days Post Coitum). Dissected oviduct with a small section of uterine horn was placed in three-well watch glass containing M2 media at 38°C. The cumulus cells were removed in a hyaluronidase solution of 300 µg/ml, potassium penicillin-G 0.06 g/L and streptomycin sulfate 0.05 g/L in M2 media. A pair of fine forceps was used to grasp the oviduct near the swollen ampulla where the eggs are located. The second pair of watchmaker's forceps was used to tear the oviduct to release the fertilized eggs. Flush the oviduct with ~0.1 ml of M2 media using a 26-gauge hypodermic needle and a 1 or 2ml syringe. Embryos were washed three times in M2 fresh medium. Then 360 embryos with normal morphology were transferred to EFS40 solution directly from M2 media for vitrification immediately (Clarke 2002).

**EFS40 solution Preparation.** Originally, EFS40 solution was prepared based on Kasai protocol (Kasai *et al.*, 1990). Briefly, EFS40 solution was prepared by mixing Ethylene glycol 4.0 ml, Ficoll 70,000 MW 1.8 g, Sucrose 1.026 g and M2 medium to 10 ml (without BSA or serum). The mixture was filter through a 0.45 µm filter and stored at -20 °C (Nagy *et al* 2003).

**Preparation of Cooling/Warming Container.** A thick-walled styrofoam box with a lid was precooled by at least 5 cm of Liquid nitrogen (LN2) for at least 30 minutes before use. A Polystyrene boat was floated

(with a thickness of 1 cm) on the surface of LN2. The temperature on the surface of the boat was  $>-150$  °C. The distance from the top of the boat to the edge of the container was 8 cm.

**Cooling Procedure.** One-cell embryos (n: 360) with normal morphology were selected for cryopreservation and 10 embryos were loaded into each straw (0.25 ml) with 50  $\mu$ l of EFS40 solution by using a transfer pipette. Seal the straw at both ends with wide forceps prewarmed to  $>100$  °C. The embryos had been equilibrated with the cryoprotectant for one minute at room temperature (25 °C). Gently, but rapidly, the straw was placed on Styrofoam both in the vapor phase of the liquid nitrogen for 3-5 minutes, and then plunge them into the LN2.

**Warming Procedure and Embryo Transfer.** The straw was transferred from the storage tank to the Dewar container with LN2. The straw was placed on a boat floating on the surface of LN2 inside the Styrofoam box and was left for 3-5 minutes. Rapidly but gently lift the straw from the boat and hold steady in air for 5-10 seconds. The straw was fully immersed in water-bath at 20 °C for about 5 seconds and then upper and lower seal of it were removed. Afterward, contents of the straw were expelled using mouth pipette by 0.5 M sucrose in M2 medium. Embryos were transferred to a prepared dish containing 3 ml of M2 medium for 5 minutes. Finally, Embryo with normal morphology were either selected for immediate transfer into pseudopregnant females (15 embryos into each infundibulum), or washed through several drops of M2 medium and cultured overnight (Nagy *et al* 2003).

**Culture Mouse Embryonic Fibroblasts (MEFs).** The pregnant female was sacrificed at day 13 dpc by cervical dislocation. Dissect out the uterine horns. Briefly, embryo was washed twice in Dulbecco's phosphate buffered saline containing penicillin-streptomycin. Embryo was placed into a petridish, brain and dark red organs cut away. The embryos were finely minced until they became "pipetttable". Cells/tissue were suspended in several ml of trypsin-

EDTA solution (1.5 ml per embryo), and incubated with gentle shaking at 4 °C over night. The suspension was transferred to a falcon tube and about 5 volumes of DMEM (high glucose, Gibco), was added then centrifuged at 3000 rpm for 5 minutes to develop a pellet cell. Supernatant was discarded and the cell pellet resuspended in small flask containing DMEM (high glucose, Gibco), 10% (v/v) FCS and 1/100 (v/v) pen/strep (Sigma UK). After 72 h trypsinize adherent cells and collect cells from the flask. In this experiment cells were not treated with irradiation or Mitomycin C.

**Embryo Co-Culture in vitro.** One cell embryos (n: 300) were cultured in four groups from A to D into four wells after vitrification: group A: embryos (n:75) cultured in M16 media, group B; embryos (n: 75) cultured in DMEM/Ham's F-12 media, group C; embryos (n: 75) cultured in DMEM/Ham's F-12 media co-culture with Vero cells ( $3 \times 10^4$  cells per well), group D; embryos (n: 75) cultured in DMEM/Ham's F-12 media co-culture with MEF cells ( $3 \times 10^4$  cells per well). In each well 25 embryos were cultured and each experiment was repeated three times. During in vitro culture, embryo development was evaluated every 24 hour.

## RESULTS

### Assessment of Embryo Survival after Vitrification.

Viability of embryos after one month vitrification was evaluated based on visual examination of the integrity of embryo membrane and the normality of the cytoplasm immediately after warming. The rate of embryos viability after vitrification was 92.7%.

**Assessment of Embryo Development in Four Kinds Cultured.** The development of one-cell mouse embryos in different cultures were compared. The ratio of DMEM to Ham's F-12 media was changed from one cell to eight cell 30%/70% and from eight cells to blastocyst 70%/30%, respectively. The best rates of embryo development were obtained using DMEM/Ham's F-12 medium and co-culture with MEF cell. Culture in M16 media did not allow the 2-cell block to be

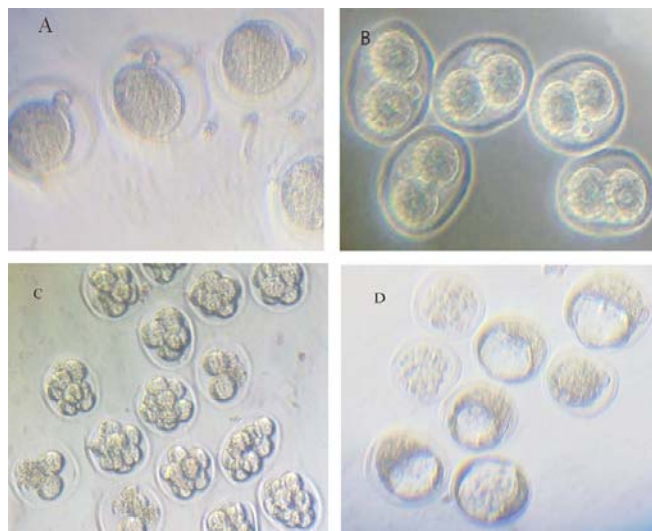
overcome (0%), very low embryos overcome the 2-cell block in DMEM/Ham's F-12 medium (10.60%) (without any development to blastocyst), co-culture with Vero cells was developed to blastocyst 38.60% and MEF cells allows high rates of blastocyst formation (70.60%). Our data showed there was a significant difference among groups Pearson chi-square= 107, 24 (P=0.000) and Fisher's exact test (P=0.006) (Figures 1 and 2) (Table 1).

## DISCUSSION

It was reported that embryos development of certain mouse strain are arrested at the 2-cell stage in cell culture ('2-cell block'), whereas those from other strains develop to the blastocyst stage under the same conditions (Neganova *et al* 1998). It has been generally accepted that preimplantation-stage embryos up to the eight-cell stage do not use glucose efficiently and require pyruvate and lactate as energy sources (Lyengar *et al* 1983, Leese 1991). Moreover, in simple culture media, glucose is responsible for retardation or developmental arrest; the replacement of glucose with glutamine in BMOC2 medium allowed zygotes of a strain blocking at the two-cell stage to develop into blastocysts (Chatot *et al* 1989). While in another research shown that one-minute exposure of 4-cell mouse embryos to glucose overcomes morula block in CZB medium (Chatot 1994, 1990).

In this experiment the amount of glucose in DMEM, Ham's F12 and M16 was 4500 mg/L, 1,802 mg/l and 1 g/l, respectively. In literature mentioned that two-cell block generally occurs in M16 medium such as one-cell mouse embryos from KM strain and B6C3F1 strain (Qiu *et al* 2003). In the present study all of embryos were stopped development at two-cells in M16 media (group A). The ratio of DMEM to Ham's F-12 media 30% to 70% respectively in groups B, C and D was changed from one-cell to eight cells and also from eight cells to blastocyst the ratio of DMEM to Ham's F-12 media was changed 70% to 30% respectively. With low concentration of glucose from one-cell to eight cells, NMRI zygotes overcome 2-cell block. After eight cell

stage the amount of glucose was increased and most of cells developed to blastocyst. Researchers compared



**Figure 1.** Development of NMRI Mice Embryos in DMEM/Ham's F-12 media co-culture with MEF cells (Group D): **A**, One-cell embryos after vitrification with EFS40 solution (the rate of embryos viability after vitrification was 92.7%). **B**, Two-cell embryo development. **C**, Embryos at morula and eight-cells development. **D**, MEF cells allows high rates of blastocyst formation (70.60%).



**Figure 2. E**, Development of NMRI Mouse Embryos in M16 media; All of embryos could not overcome 2-cell block and degraded at one-cell stage (development 0%).

**Table 1.** Development of NMRI Mice Embryos in for Groups: A, B, C and D.

Groups	A	B	C	D
Number of embryos	75	75	75	75
Development %	%0	%10.60	%38.60	%70.6

two kinds of co-culture MDBK (Madin-Darby Canine Kidney Cells) and Vero cells on mouse one cell embryo development. They showed that Madin-Darby

Bovine Kidney Cells (MDBK) allows high rates of blastocyst formation (67%) and the blastocysts obtained were viable. A Vero cell does not allow the 2-cell block to be overcome (Ouhibi et al 1990). In this experiment the best rates of embryos development were obtained using DMEM/Ham's F-12 media and co-culture with MEF cells. Culture in M16 media did not allow the 2-cell block to be overcome (0%), very low overcome the 2-cell block without complete development in DMEM/Ham's F-12 media (10.60%), co-culture with Vero cells was developed 38.60% and MEF cells allows high rates of blastocyst formation 70.60%). Data showed that there was a significant difference among groups. Regarding embryo co-culture, many studies have been reported the beneficial effects of co-cultures on embryo development in vitro in humans, domestic animals (Malekshah et al 2006, Song et al 2012). Park and colleagues compared in vitro development of DNA-injected bovine zygotes, with or without mouse embryonic fibroblasts (MEF). They demonstrated that DNA-injected embryos co-cultured with MEF (13.7%, 28/205) showed a higher developmental rate than that of the embryos cultured without MEF (6.7%, 13/193;  $P < 0.05$ ) and the pregnancy rates for two culture systems were similar (Park et al., 2000). In another study, bovine embryo co-cultured with Vero cells with 5% FCS concentration supported the embryo development to the blastocyst stage (Braz et al 2011). Further research on Vero cells demonstrated that co-culture with Vero cells produce interleukin, platelet-derived growth factor (PDGF), Leukemia inhibitory factor (LIF) and insulin-like growth factor-I (IGF-I) along with promoting an stimulatory effect during embryonic development (Duszewska et al 2000, Valojerdi et al 2002).

In this study the best rate of NMRI embryo development was obtained using co-culture with MEF cells and DMEM/Ham's F-12 media. However, in literatures is not fully elucidated why co-culture of embryos with MEF cells improves embryo development. More research needed to understand the effect of other components of culture medium, and co-

culture on NMRI embryo development because they appear to affect in vitro and subsequent in vivo mouse embryo development.

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