The development of mouse early embryos *in vitro* in fibroblasts and cumulus cells co-cultures supplemented with retinoic acid

Hajializadeh, N.[1]; Babaei, H.[2*]; Nematollahi-Mahani, S. N.[3, 4] and Azizollahi, S.[5]

[1]Graduated from Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran; [2]Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran; [3]Department of Anatomical Sciences, Afzalipour School of Medicine, University of Medical Sciences of Kerman, Kerman, Iran; [4]Neuroscience Research Center of Kerman, Kerman, Iran; [5]DVM Student, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

[*]**Correspondence:** H. Babaei, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran. E-mail: Babaei_H@mail.uk.ac.ir

Summary

This study was designed to examine the effects of retinoic acid adding to cumulus and/or fibroblast cells monolayer on the development of mouse early embryos. One-cell mouse embryos were obtained from NMRI mice after superovulation by an intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG) followed 48 hrs later by 5 IU human chorionic gonadotrophin (hCG). Mouse embryonic fibroblasts (MEF) were obtained from mouse fetuses and cumulus cells (CC) were prepared from mouse cumulus-oocyte complexes (COCₛ). To produce monolayer of cumulus and fibroblast cells, 1.0×10^5 cells/ml were plated into culture dishes in 100 µl droplets. The collected mouse embryos were cultured randomly into six different conditions, being supplemented (experiment, Exp) or not (control, Con) with 0.28 µg/ml of retinol acetate methyl-β-cyclodextrin (RA) for 96 hrs at 37°C in 5% CO_2 in air, including: (1) culture media only (Con 1); (2) culture media plus RA (Exp 1); (3) co-culture with CC (Con 2); (4) co-culture with CC plus RA (Exp 2); (5) co-culture with MEF (Con 3) and (6) co-culture with MEF plus RA (Exp 3). The culture medium was Alpha Modification of Minimum Essential Medium Eagle (α-MEM) + 10% fetal bovine serum (FBS) with 100 IU/ml penicillin and 100 µg/ml streptomycin. The proportions of embryos passing the two-cell block were significantly higher in the MEF (Con 3) group compared to the other treatment groups (P<0.05). The percentage of the two-cell passed embryos developing to the blastocyst stage was significantly higher in the co-culture groups than that of the culture medium alone (P<0.05). After 96 hrs in culture, the rate of blastocyst stage for both groups of CC co-culture treatment (Con 2 and Exp 2) was identical but, adding RA into the MEF co-culture (Exp 3) resulted significantly lower *in vitro* development than that of the Con 3 group (29.2% vs. 57.7%, P<0.05). These results suggest that supplementation of co-culture groups with RA could not affect the embryos passing the block and developing to the blastocyst stage, although the presence of RA into the culture medium alone may improve passing the critical two-cell stage. Also, *in vitro* addition of RA to cells without receptors for retinol during long term co-culture may result early embryonic growth retardation.

Key words: Retinol acetate, Co-culture, Cumulus cell, Fibroblast

Introduction

A renaissance of embryo culture research has occurred in the last two decades largely because mammalians embryo development in culture was far from optimal (Petters, 1992). The varying conditions present in current embryo culture systems or in embryo such as *in vitro* developmental arrest may contribute to the poor *in vitro* development of preimplantation mammalian embryos and/or implantation rate after the transfer of *in vitro* culture embryos (Gordon, 1994). A number of studies have attempted to improve *in vitro* culture conditions by changing the media compositions and

supplements (Hadi *et al*., 2005; Babaei *et al*., 2006), and by using a co-culture system with helper somatic cells (Bongso *et al*., 1991; Dirnfeld *et al*., 1997; Joo *et al*., 2001; Rief *et al*., 2002).

It has been known for some time that retinoids, which include vitamin A and its active metabolite, retinoic acid (RA) play an important role in cell growth, differentiation (Blomhoff, 1994) and more importantly, for embryonic and placental development (Bavik *et al*., 1996; Livingston *et al*., 2004). Retinol administered to superovulated mice improved the potential development of blastocysts *in vitro* (Babaei *et al*., 2006). Recent reports indicate that retinoids may function as important regulators of oogenesis and oocyte survival in the mouse fetal ovary and positively impact events associated with oocytes maturation in adult farm species leading to improved embryonic development (Livera *et al*., 2000).

In the live cell, All-trans-RA binds to retinoic acid receptors (RAR; α, β and γ) and its isomer 9-cis of retinoic acid specifically binds to retinoic X receptors (RXR; α, β and γ) (Mangelsdrof *et al*., 1994; Chambon, 1996). Recently, most of these RA receptors, including RAR α and γ, RXR α and β, retinaldehyde dehydrogenase and peroxisome-proliferator activated receptor gamma (RAR γ), were detected from the oocyte to the hatched blastocyst stage *in vitro* (Mohan *et al*., 2001, 2002) and in cumulus cells (Mohan *et al*., 2003). It has been recently shown that addition of 9-cis-RA to *in vitro* oocyte maturation medium affects trophectoderm differentiation, total cell number and inner cell mass-trophoblast cell ratios, following fertilization in cattle oocytes (Gomez *et al*., 2003; Hidalgo *et al*., 2003).

On the other hand, numerous reports have demonstrated the beneficial effects of co-culture on embryo development such as improved quality of embryos and the increased rate of embryo development into blastocysts (Freeman *et al*., 1995; Wiemer *et al*., 1998). Somatic cells used in co-culture may produce unknown embryo growth promoting factors or delete embryo toxic factors from the culture medium (Eyestone and First, 1989). Tissues from the female reproductive tract and non-reproductive

sources have been used to support the development of embryos (Bongso *et al*., 1991). Embryonic fibroblast cells have been generally used for the development and maintenance of mouse (Evans and Kaufman, 1981), monkey (Suemori *et al*., 2001) and human (Thomson *et al*., 1998) embryonic stem cells. Moreover, co-culture with mouse embryonic fibroblasts (MEF) improved the development of bovine and ovine embryos (Rexroad and Powell, 1993; Park *et al*., 2000). Embryonic fibroblast cells may secrete various factors that enhance embryonic development (Park *et al*., 2000).

We hypothesized that combination of co-culture systems with RA may produce better results on embryo development. Therefore, the present study was conducted to investigate the effects of RA adding to cumulus and fibroblast cells co-culture on the development of one-cell mouse embryos.

Materials and Methods

Materials

Culture dishes and 5 ml centrifuge tubes were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). Fetal bovine serum (FBS, 10099133), and trypsin-EDTA solution (25300-054) were obtained from Gibco BRL (Grand Island, NY). All other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Animals

The embryos were obtained from 6- to 8-week-old female NMRI mice, which exhibit the two-cell block *in vitro*. The NMRI mice were obtained from the Neuroscience Research Center (Kerman, Iran), and were cared for and used in accordance with the International Guiding Principle for Biomedical Research Involving Animals at Kerman University of Medical Science. The mice were kept in light and temperature controlled conditions (12 hrs light:12 hrs dark; 22 ± 2°C), and provided with food and water *ad libitum*. The females were superovulated by an intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG, G4877) followed 48 hrs later by 5 IU human chorionic gonadotrophin (hCG, C8554). They were

then placed with males of the same strain and the following morning, were inspected for the presence of a vaginal plug and were considered pregnant. Cumulus-enclosed zygotes were collected at 21 hrs after the hCG administration and were denuded by incubation of less than 1 min with hyaloronidase (1 mg/ml, H4272) in a HEPES Modification of Minimum Essential Medium Eagle (H-MEM, M2645). The embryos were pooled and were washed three times in Alpha Modification of Minimum Essential Medium Eagle (α-MEM, M0894) and then were transferred into the culture treatments.

Preparation of mouse embryonic fibroblast (MEF)

Fetuses were collected from female mice (NMRI) at day 12 to 13 of pregnancy, and were washed thoroughly in Dulbecco's phosphate-buffer saline (PBS). The head and internal organs were removed, samples minced into small pieces and cultured in Dulbecco's Modified Eagle's Medium-F_{12} (DMEM-F_{12}, D8900), supplemented with 10% (v/v) FBS, 100 IU/ml penicillin (P7794) and 100 µg/ml streptomycin (S9137) in a humidified atmosphere of 5% CO_2 in air at 37°C. The primary fibroblasts were then frozen at -196°C in liquid nitrogen. Frozen cells were thawed in water at 37°C and re-seeded at a density of 1.0×10^5 cells/ml into a culture dishes in 100 µl droplets. The MEF were plated 1 day before use, and were used within 5 days of preparation.

Preparation of cumulus cell (CC)

Female mice were superovulated as previously mentioned and cumulus-oocyte complexes were obtained 17 hrs after hCG treatment by cutting off the ampulla of oviduct. Cumulus cells were then collected from the cumulus-oocyte complexes by a short term exposure to a medium containing hyaluronidase (1 mg/ml). The cells were suspended in 3 ml α-MEM and centrifuged at 700 × g for 5 min, and supernant were discarded. The pellet of cumulus cells were re-suspended in α-MEM containing 10% FBS. To produce monolayers of cumulus cells, 1.0×10^5 cells/ml were plated into culture dishes in 100 µl droplets. Culture

medium re-newed after 24 hrs of culture to remove debris.

Experimental design

The collected embryos were cultured randomly into six different treatment groups. Half of the treatment groups were not (control, Con) and the other half (experiment, Exp) were supplemented with 0.28 µg/ml of retinol acetate methyl-β-cyclodextrin (RA, R0635) according to Lima *et al.* (2004), including: (1) culture media only (Con 1); (2) culture media plus RA (Exp 1); (3) co-culture with CC in culture media (Con 2); (4) co-culture with CC in culture media plus RA (Exp 2); (5) co-culture with MEF in culture media (Con 3) and (6) co-culture with MEF in culture media plus RA (Exp 3). The culture medium was α-MEM containing 10% FBS with 100 IU/ml penicillin and 100 µg/ml streptomycin. The allocated embryos were cultured for 96 hrs at 37°C in 5% CO_2 in air.

Embryo survival

The survival of embryos was determined every 24 hrs by subsequent development to a higher stage including 2-cell, 4- to 8-cell, and blastocysts stages during *in vitro* culture for 96 hrs. During the first 48 hrs of culture or co-culture, the two cell embryos that did not undergo cleavage were removed and, at this moment, 30% of the culture media were replaced with fresh media (α-MEM or α-MEM supplemented with RA).

Statistical analysis

All experiments were repeated four times. The significance of individual comparison was evaluated by Pearson's chi-square (χ^2) test.

Results

The effects of RA on the proportions of the two-cell block passed embryos (Table 1)

There was no significant difference in the rate of the 2-cell stage between the 6 different treatment groups during the first 24 hrs of embryos cultivation. After 48 hrs in culture, the rate of development of embryos to 4- to 8-cell stage (2-cell block passed embryos) in the α-MEM + RA group (Exp

Table 1: The effects of retinol adding to two different co-cultures on the percentage of early mouse embryos passing two-cell block and developing to the blastocyst stage

Groups	Number of replicates	Number of embryos (%)			
		1-cell	2-cell	4 to 8-cell	Blastocyst
Con 1	4	35	32 (91.4)	6 (18.7)[a]	0[a]
Exp 1	4	38	35 (92.1)	9 (25.7)[ab]	1 (9.0)[a]
Con 2	4	44	40 (90.9)	18 (45.0)[bd]	9 (50.0)[bc]
Exp 2	4	35	33 (94.3)	14 (42.4)[bd]	7 (50.0)[bc]
Con 3	4	42	38 (90.5)	26 (68.4)[c]	15 (57.7)[b]
Exp 3	4	43	41 (95.3)	24 (58.5)[cd]	7 (29.2)[c]

Con 1, culture media only; Exp 1, culture media + RA; Con 2, co-culture with cumulus cells monolayer in culture media; Exp 2, co-culture with cumulus cells monolayer in culture media + RA; Con 3, co-culture with mouse embryonic fibroblast monolayer in culture media; Exp 3, co-culture with mouse embryonic fibroblast monolayer in culture media + RA. [a] = Different superscripts differ significantly within each column ($P<0.05$)

2) was higher than that of the culture medium alone (Con 1), (25.7% vs. 18.7%, respectively), although we did not observe any significant differences ($P>0.05$). However, the MEF co-culture group (Con 3) significantly showed higher two-cell block passed embryos compared to the other groups ($P<0.05$). Whereas, the rate of development to 4- to 8-cell stage in the MEF + RA group (Exp 3) was lower than that of the Con 3 group (58.5% vs. 68.4%, respectively), but we did not observe any significant difference ($P>0.05$). Also, supplementation of the culture medium with RA was not significantly able to affect the embryos to pass the 2-cell block in any treatment groups.

The effects of RA on *in vitro* development of the two-cell passed embryos to the blastocyst stage (Table 1)

The proportion of the 2-cell passed embryos developing to the blastocyst stage was significantly higher in the co-culture groups (Exp 2 and Exp 3) than that of the culture medium alone (Con 2 and Con 3, $P<0.05$). Although, the rate of 4- to 8-cell embryos in the cumulus cells monolayer group (Con 2) was significantly lower in comparison with the MEF group (Con 3) after 48 hrs in culture (45.0% vs. 68.4%, $P<0.05$), but until the end of the fourth day of culture (96 hrs in culture) it was not significant difference between the two mentioned groups (50.0% vs. 57.7%).

After 96 hrs in culture, the rate of blastocyst stage for the both groups of cumulus cells monolayer co-culture

treatment (Con 2 and Exp 2) was identical (50.0% vs. 50.0%), but adding RA into the mouse fibroblast cells co-culture (Exp 3) resulted significantly lower *in vitro* development of 2-cell passed embryos to the blastocyst stage than that of the MEF group (Con 3), (29.2% vs. 57.7%, $P<0.05$, respectively).

Discussion

The two-cell block defined functionally as the inability of fertilized eggs to develop *in vitro* past the two-cell stage in media that nonetheless supported both development up to the two-cell stage and development of *in vivo* produced two-cell embryos to blastocysts (Biggers, 1998). Recently, media have been developed in which blocking-type mouse embryos will develop from the fertilized egg to the blastocyst stage (Lawitts and Biggers, 1991; Hadi *et al*., 2005). The main changes in these media that led to relief of the two-cell block were a decrease in NaCl concentration and overall osmolarity, an altered pyruvate:lactate ratio, the addition of EDTA and glutamine, and optimized concentrations of the other components, including lowered glucose and phosphate (Biggers, 1998). In this regard, one of the best studies has been osmolarity and increased salt concentration (Baltz, 2001). It is now well known that the two-cell block can be alleviated by decreasing the osmolarity of the medium (Biggers *et al*., 1993; Baltz, 2001). Therefore, by looking through the literature, it can be concluded that the developmental block may be overcome by either neutralizing or

detoxification of the culture medium by the cells and/or by secretion of embryotrophic factors (Schillaci *et al*., 1994).

Numerous studies have demonstrated the beneficial effects of cellular monolayers of various somatic cell types on mammalian embryonic development *in vitro* (Rief *et al*., 2002; Moulavi *et al*., 2006). In this regard, many different somatic cell types have been used routinely for co-culture including granulosa cells, oviductal cells, uterine cells, fetal bovine endometrial fibroblasts and African green monkey kidney epithelial cells (Vero cells), (Maeda *et al*., 1996; Lee *et al*., 2001). Presently, the mode of action of co-culture systems has been explained largely by two mechanisms. One of the modes is negative conditioning role that includes the removal of deleterious components or oxidative stress from the culture medium, and the second mode is a positive conditioning role in which helper cells release embryotrophic factors into medium (Joo *et al*., 2001). It is well documented that fibroblastic cells produce and release cytokines and growth factors (Moulavi *et al*., 2006). On the other hand, *in vitro* studies showed that IGF-I and IGF-Π can promote DNA replication and cell proliferation (Giudice, 2001). Poueymirou *et al*. (1989) suggested that a co-culture system helps the activation of transcription mechanisms. A significant increase in the percentage of passing two-cell block embryos in fibroblastic cells co-culture groups, compared to granulosa cells co-culture groups, may be related to the improved nuclear and cytoplasmic maturation. Carneiro *et al*. (2001) reported that IGF-I has an important role not only in the nuclear maturation of oocytes but also in cytoplasmic maturation. In addition, Ferry *et al*. (1994) and Chen *et al*. (1994) reported that the conditioned media from granulosa cells allows the early mouse embryo to develop into blastocyst. Likewise, Maeda *et al*. (1996) suggested the presence of two embryotrophic substances, one between 10 and 30 kDa and the other greater than 30 kDa, in conditioned media from bovine granulosa cells and Vero cells.

The results of the present study indicate the efficiency of supplementation of α-MEM culture medium with retinol (not significant)

on passing the two-cell block (Con 1 vs. Exp 1). Reactive oxygen species, including superoxide anion and hydrogen peroxide, appear to influence the development of early embryos by inducing impairment in the phosphorylation of p34cdc2 (Nasr-Esfahani *et al*., 1990; Goto *et al*., 1993; Natsuyama *et al*., 1993). Retinol has been known to function as an antioxidant in lipid phases by quenching singlet oxygen and scavenging the peroxyl radical (Ikeda *et al*., 2005). Therefore, it is possible that extracellular RA in the medium incorporated into embryos protects them from reactive oxygen species mediated cytotoxicity, thereby enhancing the development competence of embryos. Although, in this study supplementation of co-culture groups with RA did not show any effect on passing the two-cell block. Fukui *et al*. (1991) suggested that helper cells can significantly reduce the oxygen metabolite levels, thereby reducing the inhibitory effects of embryo development resulting from superoxide anion radicals. Therefore, it is possible that the role of RA as an antioxidant in co-culture systems decreases markedly.

In our study, the notable finding was that the rate of the development to blastocyst after 96 hrs in fibroblastic cells co-culture group supplemented with RA was significantly lower in comparison with the other co-culture groups. One possible mechanism is production and release of inflammatory mediators secreted by fibroblastic cells and cumulus cells in long term co-culture systems (Berschneider and Powell, 1992). Oocytes (Hattori *et al*., 2000), embryos (Gouge *et al*., 1998), fibroblastic cells (Christopherson and Bredt, 1997) and cumulus-granulosa cells (Nishida *et al*., 2000) have been shown to produce nitric oxide (NO). Interestingly, some researchers found that the regulation of NO generation in culture affected embryo development after IVF in a cumulus-granulosa cell co-culture system (Lim and Hansel, 1998; Bu *et al*., 2003).

It has been suggested that RA may regulate the NO system in cumulus-granulosa cells and suppress NO synthesis (Sirsjo *et al*., 2000). RA inhibits NO expression through RARα receptors (Sirsjo *et al*., 2000). However, treating cumulus

cells with RA would permit the expression of gonadotrophin receptor, induction of midkine and suppression of cyclooxygenase synthesis within the cumulus cells. The detrimental effect of RA in fibroblastic cells co-culture during the development of passing two-cell stage embryos to blastocyst could be related to a retinoid overexposure. These detrimental effects related to retinoid imbalance have been well documented by Morris-Kay and Ward (1999). *In vitro* overexposure to RA during the preimplantation period resulted in early growth retardation and subsequent embryo death (Huang *et al*., 2001).

In conclusion, the present study showed that both of MEF and cumulus cells co-culture systems had beneficial effects on passing the two-cell block and embryo development in the mouse. However, the mouse embryonic fibroblast had more effects on the passing from critical two-cell stage compared to the cumulus cells. Addition of RA during the *in vitro* culture of one-cell mouse embryos improved the passing critical two-cell stage, however supplementation of co-culture groups with retinol could not affect the embryos passing the block and developing to the blastocyst stage. Our study provided the evidence that *in vitro* addition of RA to cells without receptors for retinol during long term co-culture may result growth retardation of early mouse embryos.

Acknowledgement

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