韓國家畜繁殖學會誌 18(4) : 257~263(1995)
Korean J. Animal Reprod.

Co-culture with Buffalo Rat Liver(BRL) Cell for IVM-IVF Bovine Embryos

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소 체외수정란의 공배양을 위한 BRL 세포의 이용

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요 약

소 체외수정란의 체외배양 체계는 현재 완전히 확립되지는 않은 상태로서 8~16 세포기 발육억제현상, 수정란의 파편화 현상, 성장지연 등 여러가지 문제점들이 현 배양체계에서 나타난다. 그러나 hepler cell들과의 공배양에 의해 이러한 문제점들은 상당히 극복되며 또한 체외발생의 촉진 및 공배양된 수정란의 이식에 의해 임신율도 향상된다. 현재 소 체외수정란의 공배양에는 난관상피세포가 가장 널리 이용되나 이러한 시스템은 몇가지 문제점이 있다. 즉, primary culture를 확보하기 위하여 신선한 조직을 주기적으로 채취해야 하며 따라서 난관채취에 시간이 소요되고, 불편하며 또한 공배양에 이용되는 세포들이 균일하지 않은바 난관상피세포에 따라 배 발생 촉진작용에 변이를 나타내기도 한다. 그러나 확립된 cell line을 이용할 수 있다면 이러한 난관상피세포의 이용에서 나타나는 문제점들이 해결될 수 있다. Buffalo Rat Liver cell은 이러한 목적에 이용될 수 있는 cell line 중의 하나로서 이들은 여러가지 성장인자(growth factor)를 분비하는 것으로 알려져 있다. 따라서 本稿에서는 소 체외수정란의 공배양을 위한 BRL(Buffalo Rat Liver)cell의 이용성, 이용방법 및 이용에 있어서 고려하여야 할 요인들에 대하여 살펴보고자 한다.

Ⅰ. INTRODUCTION

Culture requirements for *in vitro* development of preimplantation embryos from domestic animals have not been fully defined. In cattle, few embryos develop in either simple or complex media through the 8 to 16-cell stage known as *in vitro* block stage(Wright and Bondioli, 1981). Various co-culture systems using helper cells have been evaluated which appear to overcome the developmental block and facilitate embryonic development *in vitro*(Rexroad, 1989). However, the actual role of helper cells in the co-culture has not been elucidated. The proposed mechanisms include; cells provide embryonic growth stimulatory components, or remove embryo toxic substances from the culture melieu, or both(Pinyopummintr and Bavister, 1991).

Trophoblast, granulosa, endometrial and oviductal cells have been experimented for the development of bovine embryos(Kuzan and Wright, 1982; Camous et al., 1984; Heyman et al., 1987; Eyestone and First, 1989; Berg and Brem, 1990; Ellington et al., 1990; Goto et al., 1992; Xu et al., 1992; Yang et al., 1993). Results of these experiments indicate that co-culture

cells support higher embryo development to the hatched blastocyst stage *in vitro* and higher pregnancy rates following transfer of cultured embryos to appropriate recipients. Currently, the type of cell most widely used in conjunction with *in vitro* culture of bovine embryos is bovine oviductal epitherial cells(BOEC). Oviductal epithelial cells have been successfully used to nurse preimplantation embryos from one cell to blastocyst stage *in vitro* (Eyestone et al., 1987; White et al., 1989) and for the production of transferable bovine embryos from IVM/IVF oocytes (Eyestone et al., 1987). However, this co-culture system is complicated by the collection of fresh tissue for each primary BOEC culture. The preparation of a primary culture is inconvenient, time consuming and a source of variation which can be avoided by using commercially available permanent cell line. For maximum embryotrophic activity, they must be harvested from fresh tissue and held *in vitro* less than one week.

Buffalo rat liver(BRL) cells are a liver epithelial and parenchyma-like cell line(Dulak and Temin, 1973) which have been used by various laboratories in co-culture with mammalian preimplantation embryos and embryonic stem cells (Bondioli et al., 1995; Mummery et al., 1990; Slager et al., 1993; Smith and Hooper, 1987; Voelkel et al., 1992). Further investigation revealed that this cell line produces relatively high levels of insulin-like growth factor Ⅱ (IGF-Ⅱ; Marquardt et al., 1981; Nissley et al., 1977), transforming growth factor B(TGF-B; Massague et al., 1985), leukemia inhibitory factor (LIF;Smith and Hooper, 1987), and stem cell factor(SCF; Zsebo et al., 1990). The production of these growth factors may account for unsual growth characteristics and embryotrophic activity of BRL cells (Hawk and Wall, 1994; Moelkel et al., 1992; Watanabe and Ide, 1993; Wells, 1993; Zsebo et al., 1990)

This article is focused on the maintenance, use and competence of BRL cells cocultured for *in vitro* development of bovine preimplantation embryo.

Ⅱ. MANAGEMENT OF BRL CELLS

1. Preparation of BRL cell from frozen cells

1. Thaw frozen BRL cells(BRL 3A ATCC No. CRL:1442) by plunging an ampule into a water bath of 37℃ for 40~60 seconds and clean the outside of the ampule by washing with 70% EtOH.
2. Use a diamond tip pencil to score the ampul e at the neck, break open the amplule and pipette 500,000 to 1,000,000 cells into a 25cm² tissue culture flask.
3. Add 10ml of warm culture medium (TCM199 with 10% FCS land 100 units/ml penicillin and 100 μg/ml streptomycin) to the flask.
4. Replace the cap loosely on the flask, place the flask flat on its back and culture in a incubator at 39℃ under a humidified atmosphere of 5% CO_2 in air.
5. After 24~48 hrs, replace the medium with 5 ml of prewarmed fresh culture medium. When a good monolayer in evident, the flask may be used to make co-culture wells.

2. Maintenance of cells

1. Pour the old medium into a sterile beaker and add 5 ml of prewarmed fresh culture medium.
2. Replace the cap loosely on each flask and return to incubator.
3. Replace culture medium every 3 to 4 days.

3. Subculture of cells

1. Thaw one tube of frozen trypsinizing solution(0.01% collagenase, 0.1% trypsin, 1% chick serum; CTC) for a flask to be trypsinized. Warm CTC and culture medium in a water bath of 37℃.
2. Pour off the old medium from the flask into a sterile beaker or centrifuge tube.
3. Rinse the flask twice with prewarmed CTC(use about 3 ml of CTC for the first wash and 2 ml for the second wash). Discard the washes into the sterile beaker.
4. Add 1 ml of CTC into the flask, replace the cap loosely and incubate at 39℃ for 30 minutes.
5. Make staining solution for cell counting by adding 3 ml of 0.4% trypan blue stain to 4 ml of D-PBS.
6. After incubation for 30 minutes in CTC, remove flask from the incubator. Add 4 ml of prewarmed fresh culture medium.
7. Break the released cells into a single cell by gently aspirating the cell suspension with a pasteur pipet and expel them back into the flask 2 or 3 times. Transfer the cell suspension into a 15 ml sterile tube.
8. Add 80 μl of trypan blue working solution and 20 μl of cell suspension to 1.5 ml Eppendorf microcentrifuge tube. Mix thoroughly by repeatedly pipetting prior to cell counting.
9. Transfer 10 μl of the trypan blue-cell mixure into each chamber of the hemacytometer.
10. View the hemacytometer at 50 to 100 × magnification.
11. Count all viable cells in the center square and four corner squares.
12. Repeat this procedure on the second chamber.
13. Calculate cell concentration: Cells per ml=average count per square(total number or counts /10) × dilution factor of 5 × 10,000.

    From this calculation, one can determine the volume of cell suspension needed to plate the desired number of cells in a flask or a co-cultrue well. The cell numbers in the BRL co-culture system are 25,000, 50,000, 100,000 and 200,000 cells for seeding wells and 500,000 cells for flasks.
14. Determine the volume of the cell suspension containing 500,000 cells and transfer this volume into a 25 cm^2 subculture flask along with 5 ml of fresh culture medium.
15. Loosely recap the flask and return to the incubator for propagation of the cell line.

4. Freezing excess cells for storage

1. Trypsinize cells and count total cell number.
2. Transfer the cell suspension into a 15 ml centrifuge tube and centrifuge to form a pellet for 10 minutes at 600 to 800 × g.
3. Draw out the supernatant and resuspend the pellet in an appropriate volume of 1.5 molar DMSO solution in TCM199 /10% FCS to provide 2~3 × 10^6 cells per ml for suspension.
4. Transfer 1 ml aliquots into cryotubes and allow the cells to equilibrate in DMSO for 20 minutes at room temperature prior to freezing.
5. Freeze cells by placing the cryotube in a styrofoam box into a −80℃ cooler for 24 hours or by using a autofreezer which is controlled at a cooling rate of −1℃ /minute to −60℃ and plunge into LN_2.

5. Preparation fo Co-culture wells

1. Prepare and warm modified TCM199(Enriched BRC media with 0.1 gm BSA (Sigma A4503) /10 ml) as a co-culture medium.

2. Trypsinize and count the cells as described in subculture of cells. While cells are incubating in CTC solution, pipette .5 ml of modified TCM199 into co-culuter wells(Nunc 4-well plates).
   Place these plates in the incubator for storage.
3. Transfer 200,000 cells into each co-culture well.
4. Return the plates into the incubator.
5. Maintain the cell line by seeding the remaning cells into new 25 cm^2 flasks with 500, 000 cells.

6. Performing embryo Co-culture

1. Place up to 25 embryos per co-culture well.
2. Place co-culture plates containing embryos into a modular incubator chamber and purge with a gas mixture of 5% CO_2 in air for about 3 minutes. Place the chamber into a 5% CO_2 incubator and culture for 3 to 4 days at 39℃.
3. At the start of the fourth day of the co-culture, take the primary plates out of chamber and transfer all embryos to secondary co-culture wells prepared 24 hours in advance. Gas the chamber and return it to the incubator for final 4 days of culture.

7. Managing cultures for daily use

For convenient and efficient use of the culture system, maintain two flasks for trypsinizing of Tuesday and Friday, respectively, and change the media of co-coulture wells 24 hours prior to use with fresh modified TCM199 medium.

Example seeding schedule of Tuesday for managing the culture system:

1. Plate 200,000 cells in .5 ml of modified TCM199 medium for use on Wednesday.
2. Plate 100,000 cells in .5ml of modified TCM199 medium for use on Tuesday.
3. Plate 50,000 cells ljin .5ml of modified TCM199 medium for use on Friday.
4. Seed 500,000 cells in a new flask for use following Tuesday and feed the flasks with fresh TCM199/10% FCS for co-culture on Friday.

Ⅲ. DISCUSSION

Bovine oviduct epithelial cells are commonly used for the producion fo transferable bovine embryos from IVM/IVF oocytes. This co-culture system has a limitation by the complication the preparation fo primary BOEC culture. Commercial BRL cell lines have some advantages; the use of this cell line stimulates the *in vitro* development for the bovine embryo to the blastocyst stage and eliminates the variation among the primary cell cultures(Van Inzen et al., 1993). Rehman et al. (1994a) reported higher cleavage rate lin the co-culture groups using BOEC or BRL when compared to the control group. But no differences were found between BOEC and BRL treatments in cleavage and development to 8-cell stages. However, the percentage of embryos developing to blastocyst stage was higher in BRL co-culture system compared to BOEC co-culture system. The low embryo development in BOEC co-culture system may be due to the difference in cell population used among different trials.

It is known that conditions needed to successfully culture embryos through the developmental block stages are different from those required for later stage embryos. In farm animals, the block has been overcome by the use of co-culture sysem (Camous et al., 1984; Heyman et al., 1987; Rehman et al., 1994b). If embryos are transfered from BOEC to BRL cells, the transition might have effect on embryo development. This concept lead us to compare four dif-

ferent culture systems. Treatment 1 consisted of culturing embryos in BRL conditioned medium for 48hrs and then transferring them on BRL. Treatment 2 was a co-culture of embryos with BRL cells for 9 days. Teatment 3 was a co-culture of embryos with BOEC in embryo culture medium (ECM) for 72 hrs and then with BRL. Treatment 4 was made up of a co-culture with BOEC in ECM for 72 hrs and then with BOEC in TCM 199. The results showed that higher percentages of blastocyst and fully expanded blastocyst, as 14.8% and 9.1%, were obtained in treatment 3 compared to other treatments. In cattle, *in vitro* embryo developmental block occurs at the 8- to 16-cell stage, roughly 72 hrs after fertilization or at the time that the embryos pass the utero-tubulin junction. This might be related to the enhanced development by changing co-culture system from BOEC to BRL cells at 72 h post-fertilization.

Reed and White (1995) reported that BRL cells maintained and subcloned in TCM 199 lose their viability after about eight passages. This lead us to evaluate the effect of prolonged maintenance of BRL cells on their embryotrophic activity. Cells were divided into two groups, 4 to 5 passages and 13 to 14 passages. The rusult of the experiment indicated that the passage number does effect the embryotrophic activity of BRL cells. There was a tendency to be more embryotrophic to the expanded blastocyst stage for the earlier passage than the later passage with the effect most pronounced when the entire post-ferilization time was BRL cell co-culture. However, there was no marked change in morphology or viability.

Recently, cell lines that retain over many passages the ability to provide the conditions that allow development of embryos through the transition stage and on to blastulation have been investigated(Bongso et al., 1991). Buffalo rat liver clone 3A (BRL) is one of these lines. Commercially available BRL cell lines are tested for microbes and endotoxins, easy to maintain in culture and provide a reproducible distribution of cell population between replicates and experiments (Rehman et al., 1994a). The BRL cell line provide a successful co-culture system for the development of *in vitro* matured and fertilized bovine oocytes to the hatched blastocyst stage (Hernandez-Ledezma et al., 1993, 1995)

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