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Effect of LH Treated Ovine Oviductal Epithelial Cell Co-Culture System on Murine Pre-Embryo Development

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Abstract

Background: This study was designed to develop a new co-culture system, assess the effect of luteinizing hormone (LH) using sequential media to promote development and increase the quality of 2-cell murine embryos through the 8-16 cell stage to morula and blastocyst stages.
Materials and Methods: Monolayers for co-culture were prepared from ovine oviduct epithelial cells (OOEC) in DMEM/F12 medium and *in-vivo*-fertilized 2-cell embryos were collected by flushing from superovulated mice. Co-culture media was treated with 10ng/ml LH. For the control groups, embryos were cultured solely in G1/G2™Ver.5 drops and containing LH; and on OOEC monolayers in G1/G2™Ver.5 drops alone and containing LH as the experimental groups. Development and quality rates were determined for all embryos daily and statistically compared. At the end of the cultivation period, differentially stained trophectoderm (TE) and inner cell mass (ICM) of expanded blastocysts from each group were examined microscopically.
Results: The embryos cultured on an OOEC monolayer in G1/G2™Ver.5 drops treated with LH had a significantly higher developmental rate than those of the group without LH and the control groups ($p \leq 0.05$). The blastocysts from the LH treated co-culture, in comparison with the group without LH and the control groups, also had a significantly higher mean cell number ($p \leq 0.05$).
Conclusion: These findings suggest that elevated periovulatory LH levels may promote preimplantation embryo development. These results have important implications for assisted reproductive technologies in which co-cultures are used to improve pregnancy rates. OOEC cell co-culture system treated by LH could improve *in vitro* preimplantation embryo development both in terms of quality (increasing blastocyst cellularity) and developmental rate.

Keywords: Co-Culture, Ovine Oviduct, Epithelial Cells, Luteinizing Hormone, Embryo Development

Introduction

The reproductive tract creates a dynamic environment for the development of mammalian embryos *in vivo*. The cyclic morphologic and physiologic changes in the oviduct and uterus suggest that these organs offer different microenvironments for the preimplantation embryo at various stages of development. The nutrient requirement of the embryo also changes as it grows, as reflected by the alteration in its energy metabolism (1) and its differential requirement for amino acids (2). The development of embryos cultured in a single chemically defined medium is inferior to that of their counterparts *in vivo* in terms of developmental rate, blastomere number and implantation potential (3, 4).
Over the past 10 years, a number of studies have attempted to improve *in vitro* culture conditions by changing the media compositions and supplements (5) and by using a co-culture system with helper somatic cells (6). G series medium is one of the commercially available sequential culture systems for human embryos. G1 and G2 medium contain different concentrations of energy substrates which mimics the change in energy substrate environment that the embryos encounter when they are traveling from the oviduct to the uterus. However the search for a better embryo culture system is ongoing. One approach is to mimic the oviductal conditions since oviductal cells produce a number of embryotrophic factors that enhance embryo development via different mechanisms.

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Oviducts are targets of estradiol and progesterone produced in response to FSH and LH stimulation of ovaries (7). These steroid hormones are delivered to oviducts, perhaps by local and systemic routes (8). LH, a hormone released from the anterior pituitary gland in response to GnRH stimulation from the hypothalamus, is among the circulatory hormones that can regulate oviductal functions (9-13). LH regulates indirectly by increasing ovarian synthesis of steroid hormones and directly by activating the LH receptors, which have been demonstrated in oviducts of several species (7, 14-16). LH receptor activation results in upregulation of cyclooxygenases 1 and 2, 5-lipoxygenase, oviductal glycoprotein (OGP), endothelin 1, and endothelin receptor types A and B, which play important roles in different oviductal functions (10-12). Bovine, ovine and murine oviducts contain LH receptors (14-16), whose activation results in an increased synthesis of OGP (11), which binds to embryos to increase their development (17-21).

Therefore, new investigations have been focused on the role of different macromolecules in embryo culture media.

Relying on these findings and considering that very little study has been done to assess the development and growth of embryos in a co-culture system using sequential media and oviductal epithelial cells (OECs), we hypothesize that LH treatment of co-cultures with oviductal epithelial cells in sequential media would further increase embryonic development into blastocysts.

Materials and Methods

The experiments of this study were reviewed and approved by the regional committee of Royan Institute.

Oviductal epithelial cells (OEC)

Ovine reproductive tracts were obtained from a local slaughterhouse, transported back to the laboratory in PBS Buffer (Gibco, Invitrogen, USA) containing 0.05 mg/ml streptomycin (Sigma, USA) and 0.06 mg/ml penicillin (Sigma). The epithelial tissues were mechanically isolated as follows: tissues were trimmed of all attached fat and connective tissue, and were washed several times in PBS. The oviducts were then slit open longitudinally and clean, pure samples of epithelial cells with minimal blood contamination were collected by scraping with a blade in the DMEM/F12 (Sigma) media and centrifuged at 1200g for 5 minutes. After two washes by centrifugation, the resuspended cells were seeded into 25 cm^2 plastic flasks (TPP, Switzerland) containing 5 ml of DMEM/F12 supplemented with 10% FBS (Gibco).

Embryos

The embryos were obtained from 6 to 8-week old inbred female NMRI (Naval Medical Research Institute) mice obtained from Pasteur Institute (Tehran, Iran) and were maintained on a light-dark cycle that consisted of lights on at 5:00 a.m. and off at 7:00 p.m. The females were superovulated by an intraperitoneal injection of pregnant mare's serum gonadotrophin (PMSG; Intervet Folligon 5000 IU; Holland) followed 48 hours later by hCG (Intervet Chorulon 1500 IU; Holland). Immediately following the second injection, the females were placed overnight with NMRI males of proven fertility. Mating was confirmed by the presence of a vaginal plug the following morning. The 2-cell embryos were collected at 48 hours after the hCG injection, pooled and the cumulus cells surrounding embryos were removed by pipetting and washing in T6 medium containing 0.4% bovine serum albumin (BSA; Sigma).

Embryo Culture and Evaluation

Sequential media culture: The embryos were then cultured sequentially in drops of G-1™ver 5 (G1.5; Vitrolife, Sweden) and 30µL G-2™ver 5 (G2.5; Vitrolife) media containing 10% recombinant serum albumin (rHA; Vitrolife, Sweden) alone or containing 10 ng/ml LH hormone (Sioux Biochemical Inc., U.S.A) as control groups 1 and 2 respectively for 6 days in a humified incubator at 37°C and 5% CO_2.

Co-culture: Fourth passage cells were harvested with 0.05% (w/v) trypsin/0.53mM EDTA solution. At 24 hours before the introduction of the embryos, the viable oviductal epithelial cells were plated into DMEM/F12 drops (25 µL) under mineral oil and incubated until the cells formed a confluent monolayer on the base of the drop. An hour before adding the embryos, the DMEM/F12 media was replaced with fresh G1.5 medium containing 10% rHA and incubated at 37°C and 5% CO_2. For experimental group 2, 10 ng/ml LH was added to the G1.5 medium before placing the culture drops. The flushed two cell mouse embryos were cultured in the 25 µL microdrops under mineral oil in a humidified atmosphere of 5% CO_2 oviductal cell monolayers.

Development and Morphology Assessment

The age of the embryos was timed with reference to

the time after hCG administration. Embryos were evaluated and embryo development was assessed and recorded at 48, 72, 96 and 120 post-hCG using an inverted microscope (Olympus, Tokyo, Japan). Embryos were classified according to the following: 2-cell stage, 4-cell stage, 8-cell stage, morula, early blastocyst and expanded blastocyst. After reaching the blastocyst stage, embryo morphology was assessed by Hoechst 33342 differential staining and viewed under an epifluorescence microscope (Olympus BX51) (22) to determine the embryo cell number.

Number of blastomeres in blastocysts

The expanded blastocysts were first incubated in 500 µl of solution 1: BSA-free human tubal fluid (HTF) medium with 1% Triton X-100 (Sigma) and 100 µg/ml propidium iodide (Sigma), for up to 30 seconds. Blastocysts were then immediately transferred into 500 µl of solution 2: fixative solution of 100% ethanol with 25 µg/ml Bisbenzamide (Hoechst 33258, Calbiochem, San Diego, USA), and stored at 4°C overnight. Fixed and stained blastocysts were then transferred from solution 2 directly into glycerol (Sigma), taking care to avoid carryover of excessive amounts of solution 2. Blastocysts were then mounted onto a glass microscope slide in a drop of glycerol, gently flattened with a cover slip, and visualized for cell counting. Cell counting was performed on a fluorescence microscope (Olympus BX51) fitted with an ultraviolet lamp and excitation filters (380 and 420 nm for blue and red fluorescence, respectively) (23, 24). As a result, inner cell mass (ICM) and trophoectoderm (TE) stained blue and red, respectively.

Statistical analysis

Data from both control and experimental groups were analyzed statistically. Differences between the percentages of various stages of embryo development and blastocysts quality were compared by χ^2 analysis. The differences between blastomeres were also compared using one-way analysis variance (Tukey). A P value of <0.05 was considered significant. All analyses were carried out using the SPSS software program (Version 11, SPSS Inc., Chicago, IL, USA).

Results

Experimental Design

Four culture systems were used to examine embryonic developmental competence that resulted from the G1.5, G2.5 sequential media in this study:

Experimental Group 1: Embryos co-cultured with OOEC's in G1.5, G2.5 sequential culture medium;

Experimental Group 2: Embryos co-cultured with OOEC's in G1.5, G2.5 sequential culture medium supplemented with LH;

Control group 1: Embryos were cultured in the G1.5, G2.5 sequential culture medium without co-culture cells;

Control group 2: Embryos were cultured in the G1.5, G2.5 sequential culture medium without co-culture cells supplemented with LH.

Development and blastocyst cellularity

Embryos

A total of 365, 2-cell embryos were randomly allocated to the experimental groups; 190 embryos in group 1 (OOEC+G1/G2 co-culture) and 175 embryos in group 2 (OOEC+G1/G2+LH co-culture). A total of 397, 2-cell embryos were randomly allocated to the control groups; 219 embryos in control group 1 (G1/G2 sequential media alone) and 178 embryos in control group 2 (G1/G2 sequential media + LH). The rate of development in the experimental and control groups has been shown in Table 1.

During the cultivation period, embryo degeneration in the feeder co-culture groups was lower (with and without statistical difference) than that observed in control groups (Data not shown in Table 1).

At the end of the cultivation period, both feeder groups significantly enhanced embryonic development as compared to control groups; as a result, more embryos reached the blastocyst stage in the co-culture groups in comparison with those in the control groups. However there was also a significant difference between the experimental groups. Group 2 (the OOEC co-culture group supplemented with LH) had a significantly higher rate of embryonic development than group 1 (the co-culture group lacking LH).

ICM and ICM/TE

The allocation of ICM and TE of blastocysts at 120 hours after various treatments has been shown in Figure 1. In experimental group 2, the total cell number (TCN) as well as the cell number in the ICM and TE of each blastocyst increased along with the duration of co-culture.

The mean ICM index and total cell number of the blastocysts developed on the feeder layers was significantly higher than that in the control groups ($p<0.05$).

For this parameter there was also a statistically significant difference between the experimen-

Table 1: Pre-implantation development of mouse embryos when cultured in sequential media alone, sequential media with LH or co-cultured with no treated or LH treated Oovine oviductal epithelial cell monolayers.

Groups	Total number of embryos	Developmental stage	Time from culturing			
			48h	72h	96h	120h
OOEC	190	2-cell	51.35±0.05	17.60±0.08 [a, d]	10.73±0.05 [c]	10.73±0.05[c]
		4-cell	48.65±0.05	11.96±0.05	8.07±0.03 [d]	8.07±0.03
		8-cell		7.04±0.03	3.62±0.04	3.17±0.03
		Morula		19.26±0.05	4.14±0.04	2.29±0.04 [f]
		Early blastocyst		44.15±0.10 [g]	5.52±0.06	2.33±0.02
		Expanded blastocyst			67.92±0.10 [h, k, l]	73.42±0.09 [g, j]
OOEC+LH	175	2-cell	49.97±0.09	7.18±0.05 [a, b, c]	3.93±0.03 [a, b]	3.93±0.03 [a, b]
		4-cell	50.03±0.09	4.50±0.03 [e, f]	2.96±0.03 [d, e, f]	2.96±0.03 [d]
		8-cell		7.45±0.07	1.80±0.04	0±0.00 [e]
		Morula		22.24±0.07	1.76±0.03	2.77±0.03
		Early blastocyst		58.63±0.10 [g, h, i]	2.75±0.0 [g]	0.36±0.01
		Expanded blastocyst			86.80±0.07 [h, i, j]	89.98±0.04 [g, h, i]
Control 1	219	2-cell	55.84±0.07	29.47±0.08 [b, d]	23.46±0.08 [a, c]	23.46±0.08 [a, c]
		4-cell	44.16±0.07	15.62±0.07 [e]	10.11±0.05 [e]	8.53±0.06 [d]
		8-cell		10.50±0.03	5.19±0.03	5.19±0.03 [e]
		Morula		12.34±0.07	6.14±0.05	3.75±0.05
		Early blastocyst		32.08±0.08 [h]	5.26±0.04	3.39±0.02
		Expanded blastocyst			49.84±0.10 [h, j]	55.69±0.10% [h, j]
Control 2	178	2-cell	55.07±0.09	23.50±0.04 [c]	17.16±0.04 [b]	17.16±0.04 [b]
		4-cell	44.93±0.09	13.71±0.04 [f]	8.81±0.03 [f]	6.48±0.04
		8-cell		11.32±0.06	4.25±0.04	1.34±0.03
		Morula		17.60±0.03	5.04±0.03	8.35±0.02 [f]
		Early blastocyst		33.88±0.05 [i]	9.22±0.04 [g]	3.62±0.03
		Expanded blastocyst			55.53±0.04 [j, l]	63.05±0.06 [i]

Notes: The culture time is the period from collection of two2-cell stage embryos and data are shown as Mean±SD percentage. OOEC: co-culture in sequential media with Oovine oviductal epithelial cells; OOEC+LH: co-culture in sequential media with Oovine oviductal epithelial cells and LH; Control 1: culture in sequential media alone (G1.5 followed by G2.5 media); Control 2: culture in sequential media alone (G1.5 followed by G2.5 media) with LH. Values within columns with Ssame superscripts are significantly different (ANOVA, p<0.05).

Table 2: Cell proliferation and differentiation in mouse blastocysts produced by in vitro in vitro culture in sequential media alone, sequential media with LH or co-cultured with no treated or LH treated Oovine oviductal epithelial cell monolayers..

Groups	Total Blastocysts	ICM index (Mean%±SD)	ICM (Mean±SD)	TE (Mean±SD)	Total Cell Number (Mean±SD)
OOEC	30	31±0.04 [a, b, c]	15.83±2.41 [a, b, c]	36.13±5.32 [a, b, c]	51.97±5.80 [a, b, c]
OOEC+LH	30	34±0.06 [a, d, e]	21.57±4.32 [a, d, e]	42.20±6.65 [a, d, e]	63.77±8.23 [a, d, e]
Control 1	30	27±0.06 [b, d]	11.67±2.76 [b, d]	32.00±5.86 [b, d]	43.67±6.77 [b, d]
Control 2	30	27±0.04 [c, e]	12.20±2.52 [c, e]	32.53±4.55 [c, e]	44.73±5.99 [c, e]

Notes: OOEC: co-culture in sequential media with Oovine oviductal epithelial cells; OOEC+LH: co-culture in sequential media with Oovine oviductal epithelial cells and LH; Control 1: culture in sequential media alone (G1.5 followed by G2.5 media); Control 2: culture in sequential media alone (G1.5 followed by G2.5 media) with LH. ICM: inner cell mass; TE: trophectoderm. Values within columns with Ssame superscripts are significantly different (Anova, p<0.05).

tal groups. The co-culture group containing LH had a higher mean total cell and ICM index number than the co-culture group lacking LH (Table 2).

Comparison by time and developmental stage

After 48 hours, the embryos of all groups reached the 4-cell stage with no significant difference between the number of 2-cell and 4-cell stage embryos at this time.

After 72 hours, the embryos reached the early blastocyst stage. where at this time experimental group 2 had a significantly lower number of 2-cell embryos than experimental group 1 and both control groups ($p<0.05$). Additionally, experimental group 2 had a significantly lower number of 4-cell embryos than both control groups ($p<0.05$).

There was no significant difference in the number of embryos reaching the 8-cell and morula stage after 72 hours.

Experimental group 2 had a significantly higher number of embryos reaching the early blastocyst stage than experimental group 1 and the control groups ($p<0.05$), whereas there was no significant difference between experimental group 1 and the control groups.

After 96 hours, the embryos reached the expanded blastocyst stage. At this time, experimental group 2 had a significantly lower number of 4-cell embryos than experimental group 1 and both control groups ($p<0.05$).

There was no significant difference between the number of 8-cell and morula stage embryos in experimental and control groups.

Experimental group 2 had a significantly lower number of early blastocyst stage embryos than control group 2 ($p<0.05$), however no significant difference between experimental group 2 and control group 1 was seen. There was no significant difference between experimental group 2 and the control groups.

Both experimental groups had a significantly higher number of expanded blastocysts than the control groups. The number of expanded blastocysts in experimental Group 2 was also significantly higher than experimental group 1 ($p<0.05$). There was no significant difference between the control groups.

After 120 hours, no significant difference between the number of early blastocysts in experimental and control groups was seen.

Experimental group 2 had a significantly higher number of expanded blastocysts than both control groups and experimental group 1 had a significantly higher number of expanded blastocysts than control group 1 ($p<0.05$). In this term experimental group 2 was also significantly higher than experimental group 1, however there was no significant difference between the control groups.

Discussion

The results of this study show that the culture of mouse 2-cell embryos in the G1.5, G2.5 sequential medium containing LH over OOECs leads to a significantly higher number of blastocysts in comparison to the culture of embryos in the sequential medium alone.

Under *in vivo* conditions, early embryos develop in the maternal reproductive tract. The key to successful *in vitro* embryo culture is to mimic these *in vivo* micro-environments (25).

The mammalian oviduct plays a critical role in establishment of a successful pregnancy even though the gametes and embryos stay within the oviduct briefly (26). In order to mimic *in vivo* conditions, some commercial IVP systems of mammalian embryos use co-culture systems. These systems include oviductal epithelial cells, cumulus cells, granulose cells, and established cell lines. Several reports have shown that the use of co-culture results in better embryonic development, higher cleavage rate, blastulation, and increased pregnancy rate in some animals and humans (27).

Bongso, et al. (6) have suggested two possible mechanisms for action of co-culture. One is the secretion of embryotrophic factors by feeder cells into medium (positive conditioning role). The other is the removal of deleterious compounds from culture medium by the activated metabolism of the helper cells (negative conditioning role) (28).

Several other studies have also found that the somatic helper cells secrete specific glycoproteins as well as a variety of growth factors such as transforming growth factor b (TGF-b) and leukemia inhibitory factor (LIF) (6). Early preimplantation embryos have receptors for a variety of cellular growth factors (29).

In this study the beneficial effect of ovine oviductal cells on mouse blastocysts is also manifested by increases in the number of cells in the TE, ICM and TCN which confirm previous studies that oviductal cells improve embryo development with respect to blastocyst formation and hatching (30-40).

It has previously been reported that the number of ICM cells in blastocysts that develop in co-culture systems are less than those observed for blastocysts that are developed *in vivo* (41, 42). However, in a study by Lane, et al. the number of ICM cells in the expanded blastocysts developed in sequen-

tial media is significantly higher than that observed for buffalo rat liver cells (BRL) co-culture. Lanea, et al. concluded that the blastocysts derived from sequential media system better supports differentiation of the ICM compared to co-culture systems (43). The difference in the results of these studies are the use of different somatic cells and culture media for co-culture. Embryos may respond differently when co-cultured with different cell types (44). Our study supports the suggestion by XU, et al. (45), that human oviductal cells co-cultured in G1.2/G2.2 media are superior to the G1.2/G2.2 sequential system alone in producing blastocysts of better quality.

Oviducts are regulated by a wide variety of agents, including locally synthesized molecules working in an additive, synergistic or antagonistic manner to regulate different oviductal functions. LH is among the circulating hormones that can potentially regulate oviductal function. The presence of oviductal LH receptor (LHR) and/or activation by LH has been demonstrated in several species. The LHR activation resulted in up-regulation of prostaglandin H synthase-1 & -2 (10, 13), synthesis of prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2 α) (10, 12, 13, 46), 5-lipoxygenase (10), oviductal glycoprotein (11, 47) and endothelin-1 (12, 46), which have been shown to have an important role in various oviductal functions. Activation of the bovine oviductal LHR results in an increased synthesis of oviductal glycoprotein (11, 47), which binds embryos to increase their development (21). The LH treatment of oviductal epithelial cells co-cultured with 2-cell embryos results in increased embryonic development to the blastocyst stage (48). Furthermore, hCG has been shown to bind directly to the bovine oocyte, embryo and blastocyst. The effect of this binding is unknown, but a common theme in LH action outside the gonads across all species is the up-regulation of PG synthesis (14). The organization of the LHR protein is similar in all species so far studied (16). Although the mechanism of the LH effect upon the oviduct is not clear, it is possible that it can act via locally released factors, such as prostaglandins or nitric oxide (NO). Cultured cells of human oviductal mucosa respond to hCG stimulation with increased cyclooxygenase-2 (COX-2) mRNA expression, protein levels and with increased prostaglandin (PG) E2 production (13).

Results of this study also show that the culture of mouse 2-cell embryos in the G1.5, G2.5 sequential medium containing LH over OOECs lead to a significantly higher number of blastocysts in comparison to the culture of embryos in the G1.5, G2.5 sequential medium alone and the co-culture group without LH. The dependence of LH effect on the presence of oviductal epithelial cells suggests collaboration between this hormone and oviductal embryotrophic factors to further enhance embryo development.

The significant increases in the number of cells in the TE, ICM and TCN of the embryos in the G1.5,G2.5 sequential medium containing LH over OOECs compared to the sequential medium alone and the co-culture group without LH can also be related to the effect of the embryotrophic factors secreted by the oviduct as a result of the cross-talk (communication?) between embryos and the OECs (49) and also the effect of LH on the oviduct helper cells.

For somatic cells used in co-culture, it has been demonstrated that these tissues are sometimes contaminated by various pathogens. Therefore, additional risks may result in the use of co-culture cells obtained from ovaries or oviducts collected in the slaughterhouse with an unknown health status. Oviductal cells from slaughterhouse material can be contaminated with BVDV or BHV-1 (50), so that the co-culture system may induce a contamination to initially healthy oocytes or embryos.

When systems of co-culture are used, the best way to control sanitary risks is to use established cell lines, which can be tested before use for the presence of pathogens (51).

This study also supports the hypothesis previously suggested by Mishra, et al. (47), that the LH receptors on embryos and blastocysts probably play little or no role in the beneficial effect of LH in co-cultures. This hypothesis is based on the finding that LH addition to embryos cultured in medium alone has no effect on their growth and development.

Conclusion

The present findings have important physiological and clinical implications. The physiological implication is that elevated periovulatory serum LH levels may promote preimplantation embryo development in the oviduct. The clinical implication is that LH treatment of co-cultures with reproductive tract epithelial cells, which contain LH/hCG receptors, may further increase pregnancy rates achieved using assisted reproductive technologies.

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