Embryonic Stem Cell Conditioned Medium Supports In Vitro Maturation of Mouse Oocytes

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Abstract

Background: This study aimed to investigate the maturation and fertilization rates of immature mouse oocytes using Embryonic Stem Cell Conditioned Medium (ESCM).

Methods: Germinal Vesicle (GV) stage oocytes were observed in 120 NMRI mice, aged 4-6 weeks. GV oocytes with or without cumulus cells were subjected to IVM in either ESCM, Embryonic Stem Cell Growth Medium (ESGM), or α-minimum essential medium (α-MEM). After recording the Metaphase II (MII) oocyte maturation rate, the oocytes were fertilized in vitro. The fertilization success rate was recorded after 24 hr. The embryos were maintained in potassium Simplex Optimization Medium (KSOM) for 96 hr and allowed to grow until the blastocyst stage. After recording developmental competence, they were transferred into the uteri of pseudopregnant mice and their birth rates were recorded.

Results: No significant difference existed between the maturation rates in α-MEM (68.18%) and ESCM (64.67%; p>0.05), whereas this rate was significantly higher for both α-MEM and ESCM compared to ESGM (32.22%; p<0.05). A significant difference in IVF success rate existed for oocytes grown in α-MEM (69.44%), ESCM (61.53%), and ESGM (0%). A significantly higher developmental competence was observed at the blastocyst stage for oocytes grown in α-MEM (51.2%) compared to ESCM (35%; p<0.05). 17 days after embryo transfer into the uteri of pseudopregnant mice, there was a nonsignificant (p>0.05), similar birth rate between α-MEM and ESCM (47 vs. 40%).

Conclusion: ESCM is an effective medium for preantral follicle growth, oocyte maturation, and subsequent embryo development.

Keywords: Assisted reproductive technologies, Embryonic stem cells, Mice, Oocytes

Introduction

In vitro culture and maturation of Germinal Vesicle (GV) oocytes is an efficient method to generate mature oocytes. This technique, as an adjuvant treatment for infertility, is of paramount importance for assisted reproductive technology. In recent years, numerous attempts have been made to grow and stimulate immature follicles with the intent to eventually treat infertility attributed to polycystic ovary syndrome, premature ovarian failure, and infertility following cancer treatments.

Although the growth of in vitro preantral follicles can result in fertilizable oocytes and successful childbirth, the technique of In Vitro Maturation (IVM) needs to be further enhanced because GV oocytes have a reasonable ability to mature and reach the Metaphase II (MII) stage. However, only 40 to 80% of the oocytes from IVM become fertilized and pass the early embryonic stage. Only 15% of these are implanted and produce viable embryos after transfer. GV oocytes are the main units of ovaries which produce a suitable environment for the growth and maturation of oocytes. Therefore, these oocytes are a great source for folliculogenesis based studies and embryo production. One of the main challenges for growth and development of in vitro follicles is the preparation of an appropriate medium that creates in vitro conditions which mostly mimic the complex in vivo environment. Embryonic stem cells are an in vitro copy of embryonic cells at
the blastocyst stage, called the inner cell mass \(^{18,19}\). These cells are pluripotent cells similar to the blastomeres that form embryos which have the capability to differentiate into all cell types in vivo \(^{20,22}\).

There are reports that discuss the biological secretions and activated proteins by embryonic stem cells which indicate their capacity to provide an environment with mitogenic factors, growth factors, cytokines, and chemokines \(^{23-26}\). These factors prevent the growth of cancer cells and cardiovascular cell apoptosis, play a role in cardiomyocyte division, and lead to angiogenesis \(^{23-26}\). The conditioned medium secreted by embryonic stem cells can regulate the ultimate fate of these cells \(^{27,28}\).

Undifferentiated mouse ES cells grown in ES cell culture medium produce a number of biologically active cytokines such as Epidermal Growth Factor (EGF), Insulin Growth Factor (IGF-1), IGF-2, Stem Cell Factor (SCF), Leukemia Inhibitor Factor (LIF), and Transforming Growth Factor-β (TGF-β) \(^{25,26}\). According to previous reports \(^{25,26}\), meiotic progress, oocyte growth, and chemokines \(^{23-26}\). These factors prevent the growth of cancer cells and cardiovascular cell apoptosis, play a role in cardiomyocyte division, and lead to angiogenesis \(^{23-26}\). The conditioned medium secreted by embryonic stem cells can regulate the ultimate fate of these cells \(^{27,28}\).

Materials and Methods

**Mouse embryonic stem cell culture and preparation of conditioned medium**

TT2 embryonic stem cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 15% Fetal Bovine Serum (FBS, Gibco), 1 mM sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), 10 ng/ml LIF (ESGRO-LIF, Gibco), 0.1 mM β-mercaptoethanol (Sigma), 100 U/ml penicillin, and 50 μg/ml streptomycin (Gibco). ESCM was prepared when cultured embryonic stem cells reached 70% to 80% confluency. The supernatant cell culture was collected from the growing embryonic stem cells, which was defined as ESCM. This supernatant was filtered through a 0.22 μm membrane. A total of 15 ml of ESCM was placed into the tubes and centrifuged at 3000 RPM for 40-60 min and immediately used.

**Animals**

A total of 20 male and 120 female NMRI mice were housed and bred in the Central Animal House of Kurdistan University of Medical Sciences. Animals were maintained on a 12 hr light/12 hr dark schedule, a temperature range of 22-24°C, and adequate food and water ad libitum.

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Figure 1. A) Immature germinal vesicle (GV) oocytes isolated from 4-6 week-old mice 48 hr after injection with 5 IU pregnant mare serum gonadotropin (PMSG), enclosed with or without compact cumulus cells (Scale bar: 50 μm). B) GV oocytes at 24 hr of culture with distinct first polar body (Scale bar: 50 μm). C) Developed 2-cell and D) Blastocyst embryos obtained from fertilized GV oocytes cultured in potassium simplex optimization medium (KSOM) (Scale bar: 50 μm).

**Collection and in vitro maturation (IVM) of immature germinal vesicle (GV) oocytes**

Each 4-6 week old female mouse received an injection of 5 IU pregnant mare serum gonadotropin (PMSG). At 48 hr after the injection, immature GV oocytes from the ovaries of these mice were obtained. The GV oocytes were released from the ovaries by puncturing the follicles with a sterile 28-gauge needle as visualized under a stereomicroscope. The preantral follicles were pooled and randomly divided for further analysis (Figure 1A).

After washing human tubal fluid medium, 1199 GV oocytes were subjected to IVM. These oocytes were randomly divided, with or without cumulus cells, into three groups. The groups were placed in various maturation media and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 24 hr. GV oocytes were subjected to IVM in either ESCM (n=603), Embryonic Stem cell Growth Medium (ESGM, n=332), or α-Minimum Essential Medium (α-MEM, n=264). The experiments were repeated for a total of seven times per group. Oocyte maturation was evaluated at 24 hr by a stereomicroscope. Only those oocytes that displayed distinct first polar bodies were classified as MII oocytes. The MII oocytes underwent fertilization and in vitro development (Figure 1A).

**In vitro fertilization (IVF) and embryo development**

The developmental potential of oocytes that had undergone Germinal Vesicle Breakdown (GVBD) or reached the MII stage via IVM were assessed by IVF. Sperm were collected from the cauda epididymis of mature NMRI males, capacitated for 1 to 1.5 hr at 37°C, and diluted in HTF to a final concentration of...
0.7-1.3x10^6 sperm/ml. MII oocytes were incubated with spermatozoa for 4-6 hr. Subsequently, the oocytes were washed to eliminate extra spermatozoa and then cultured overnight in separate dishes, in a drop of potassium Simplex Optimization Medium (KSOM). This medium is a conventional and chemically defined medium for development of mouse embryos. After insemination, the obtained 2pn embryos were cultured in KSOM under mineral oil at 37°C in an atmosphere of 5% CO_2 and air for four days until the blastocyst stage (Figure 1D). Their developmental stages were determined by morphological evaluations conducted every 24 hr under a stereomicroscope. Fertilization rate was scored as the percentage of 2-cell embryos observed 24 hr after insemination. The maturation rate, fertilization rate, and embryo development were also determined.

**Embryo transfer**

Pseudopregnant recipient mice were obtained by mating fertile female NMRI mice to vasectomized males. The following morning after mating, vaginal plugs were examined and those mice with vaginal plugs were used as pseudopregnant recipients for embryo transfer. On day 3 of pseudopregnancy (2.5 days postcoitum), the selected recipient mice were anesthetized with isoflurane in oxygen. Anesthesia was confirmed by lack of refraction in the eyes. After anesthetization, a 4 cm long skin incision was generated parallel to the dorsal midline in an attempt to expose the oviduct and uterus. An average of 10 embryos were transferred into each oviduct of the recipient mice as described by Nagy et al. 10. On day 19.5 of pregnancy, recipients which did not have natural delivery were euthanized by cervical dislocation. After dissection of the uterus, the number of live pups and resorbed fetuses were recorded. Pups that appeared pink, and could move and breathe were identified as viable. Fully formed fetuses that did not breathe or move were considered nonviable.

**Statistical analysis**

Maturation rate, IVF success, and developmental competence to the blastocyst stage in mouse oocytes were calculated for each developmental stage category and compared between the ESGM, ESCM and α-MEM groups. The data were analyzed according to the three sample test for equality of proportions with continuity correction. Birth rates for embryos matured in the study groups were analyzed by the two sample proportion test with R V.3.1.0 software. P≤0.05 was considered significant.

**Results**

**Effects of ESCM, ESGM, and α-MEM on the maturation of immature mouse oocytes**

Each 6-8 week-old mouse received an injection of PMSG. After 48 hr, the oocytes were separated from the ovaries and cultured in either α-MEM, ESGM, or ESCM. Oocytes were assessed in terms of nuclear de-

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| Group | GV oocytes (N) | PB (%) | IVF success (N) (%) | Blastocyst (N) (%) |
|-------|----------------|--------|---------------------|--------------------|
| α-MEM | 264 (68.18)    | 125 (69.44) | 64 (51.2)            |
| ESCM  | 603 (64.67)    | 240 (61.53) | 84 (35)             |
| ESGM  | 332 (32.22)    | 0 (0.0)   | -                   |

GV: Germline vesicle oocyte; PB: First polar body; Metaphase II [MII]; α-MEM: α-minimum essential medium; ESGM: Embryonic stem cell conditioned medium; ESCM: Embryonic stem cell growth medium.

**Birth rates for matured embryos according to the study groups**

| Groups | Blastocysts N | Birth rate N (%) |
|--------|---------------|------------------|
| α-MEM  | 17            | 8 (47.05)        |
| ESCM   | 30            | 12 (40.00)       |
| ESGM   | -             | -                |

α-MEM: α-minimum essential medium; ESGM: Embryonic stem cell conditioned medium; ESCM: Embryonic stem cell growth medium.

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Discussion
This study evaluated the fate of IVM MII oocytes subjected to three different culture mediums with subsequent embryo growth. Oocyte survival in α-MEM, ESGM, and ESCM were evaluated. Our results showed that 48 hr after in vivo PMSG priming, isolated GV oocytes from 4-6 week-old mice cultured in ESCM reached a similar meiotic competence compared to those cultured in α-MEM (64 vs. 68%) and a higher developmental competence compared to ESGM. Furthermore, 61% of oocytes that matured in ESCM were fertilized in HTF medium from which 35% grew and reached the blastocyst stage in KSOM medium. Blastocyst transfer into pseudopregnant mice resulted in healthy pups.

According to a number of reports, embryonic stem cells secrete a variety of cytokines and growth factors which can affect different cell types 25,26. LaFramboise et al have reported that mouse embryonic stem cells secrete numerous proteins that mediate cardiac repair via paracrine mechanisms and cell division 26. Giuffrida et al observed that human embryonic stem cells secreted factors which arrested the growth of human epithelial, prostate, and breast cancer cell lines 27. According to Guo et al, murine embryonic stem cells released factors that stimulated the growth of normal bone marrow myeloid progenitor cells and increased survival of these cells 27.

Embryonic stem cells release EGF, SCF, LIF, FGF2, TGF-β, and IGF 25,26, which are known to support IVM of oocytes. The beneficial effects of these factors on IVM have been demonstrated in several species 31,38. IGF-1 stimulates oocyte maturation 32,33. According to several studies, EGF is beneficial to IVM. Oocyte maturation 32,33 and cumulus cell proliferation 39 is stimulated by EGF. The effects of EGF are possibly attributed to mitogen activated protein kinase (MAP) activities and increased H1 kinase in oocytes, mediated by cumulus cells 40. The beneficial effects of EGF on IVM have been demonstrated in different species, including mice 32,33 and humans 32,41.

Oocyte maturation is also stimulated by TGF-β in rats 34. It has been reported that maturation of both cumulus-oocyte complexes and follicle-enclosed oocytes is accelerated by TGF-β, with an increased percentage of oocytes that exhibit GVBD 34. Matos et al have stated that cumulus expansion in human and mouse cumulus-oocyte complexes were similarly induced by LIF 42. Maturation of sheep oocytes improved the following supplementation of the medium with FGF2 43. Klinger and De Felici reported that SCF promoted oocyte growth 44. Therefore, an elevated IVM rate with the use of ESCM might be due to the combined effects of growth factors secreted by undifferentiated ES cells.

Similar to our results, Gelber et al observed that ESGM supported in vitro development of 8-cell embryos to blastocysts; however, when embryos were cultured from the 2-cell stage, such benefits were not observed 45. Recovery of immature oocytes followed by IVM and fertilization is a potential treatment for infertility. Recently, it has been shown that IVM is a practicable alternative to conventional IVF 36,47.

Conclusion
In conclusion, for the first time, the current study data showed that ESCM contained putative growth factors that could efficiently stimulate IVM of mouse oocytes, which resulted in the delivery of healthy pups following embryo transfer into recipient female mice.

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References
1. Nottola SA, Cecconi S, Bianchi S, Motta C, Rossi G, Continenza MA, et al. Ultrastructure of isolated mouse ovarian follicles cultured in vitro. Reprod Biol Endocrinol 2011;9:3.
2. Desai N, Alex A, AbdelHafez F, Calabro A, Goldfarb J, Fleischman A, et al. Three-dimensional in vitro follicle growth: overview of culture models, biomaterials, design parameters and future directions. Reprod Biol Endocrinol 2010;8:119.
3. Wang X, Catt S, Pangestu M, Temple-Smith P. Successful in vitro culture of pre-antral follicles derived from vitrified mouse ovarian tissue: oocyte maturation, fertilization, and live births. Reproduction 2011;141(2):183-191.
4. Ling B, Feng DQ, Zhou Y, Gao T, Wei HM, Tian ZG. Effect of conditioned medium of mesenchymal stem cells on the in vitro maturation and subsequent development of mouse oocyte. Braz J Med Biol Res 2008;41(11):978-985.
5. Kim DH, No JG, Park JJ, Yoo JG. Successful in vitro development of preantral follicles isolated from vitrified mouse whole ovaries. Reprod Dev Biol 2012;36(4):255-260.
6. Hasegawa A, Hamada Y, Mehandijev T, Koyama K. In vitro growth and maturation as well as fertilization of mouse preantral oocytes from vitrified ovaries. Fertil Steril 2004;81 Suppl 1:824-830.
7. Child TJ, Abdul-Jalil AK, Gulekli B, Tan SL. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. Fertil Steril 2001;76(5):936-942.
8. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil Steril 1994;62(2):353-362.
9. Spears N, Boland NI, Murray AA, Gosden RG. Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. Hum Reprod 1994;9(3):527-532.

10. Chian RC, Lim JH, Tan SL. State of the art in in-vitro oocyte maturation. Curr Opin Obstet Gynecol 2004;16(3):211-219.

11. Moor RM, Dau Y, Lee C, Fulka J Jr. Oocyte maturation and embryonic failure. Hum Reprod Update 1998;4(3):223-226.

12. Trounson A, Anderiesz C, Jones G. Maturation of human oocytes in vitro and their developmental competence. Reproduction 2001;121(1):51-75.

13. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. Fertil Steril 1991;55(1):109-113.

14. Child TJ, Phillips SJ, Abdul-Jalil AK, Gulekli B, Tan SL. A comparison of in vitro maturation and in vitro fertilization for women with polycystic ovaries. Obstet Gynecol 2002;100(4):665-670.

15. Brower PT, Schultz RM. Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev Biol 1982;90(1):144-153.

16. Picton HM, Harris SE, Muruvi W, Chambers EL. The in vitro growth and maturation of follicles. Reproduction 2008;136(6):703-715.

17. Gilchrist RB, Thompson JG. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. Theriogenology 2007;67(1):6-15.

18. Fathi F, Alvariño JM, Mowlwa SJ, Movahedin M. Transplantation of retinoic acid treated embryonic stem cells & behavioural deficit in Parkinsonian rats. Indian J Med Res 2010;131:536-544.

19. Nichols J. Introducing embryonic stem cells. Curr Biol 2001;11(13):R503-505.

20. Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. Stem Cells 2001;19(3):193-204.

21. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391):1145-1147.

22. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000;227(2):271-278.

23. Giuffrida D, Rogers IM, Nagy A, Calogero AE, Brown TJ, Casper RF. Human embryonic stem cells secrete soluble factors that inhibit cancer cell growth. Cell Prolif 2009;42(6):788-798.

24. Fatma S, Selby DE, Singla RD, Singla DK. Factors released from embryonic stem cells stimulate e-kit-FLK-1(+) progenitor cells and enhance neovascularization. Antioxid Redox Signal 2010;13(12):1857-1865.

25. Guo Y, Graham-Evans B, Broxmeyer HE. Murine embryonic stem cells secrete cytokines/growth modulators that enhance cell survival/anti-apoptosis and stimulate colony formation of murine hematopoietic progenitor cells. Stem Cells 2006;24(4):850-856.

26. LaFramboise WA, Petroso P, Krill-Burger JM, Morris DR, McCoy AR, Scalice D, et al. Proteins secreted by embryonic stem cells activate cardiomyocytes through ligand binding pathways. J Proteomics 2010;73(5):992-1003.

27. Fuchs E, Tumbar T, Guusch G. Socializing with the neighbors: stem cells and their niche. Cell 2004;116(6):769-778.

28. Naveiras O, Daley GQ. Stem cells and their niche: a matter of fate. Cell Mol Life Sci 2006;63(7-8):760-766.

29. Bendall SC, Hughes C, Campbell JL, Stewart MH, Pittock P, Liu S, et al. An enhanced mass spectrometry approach reveals human embryonic stem cell growth factors in culture. Mol Cell Proteomics 2009;8(3):421-432.

30. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the mouse embryo: A laboratory manual. 3rd ed. USA: Cold Spring Harbor Laboratory Press; 2003. 764 p.

31. Das K, Stout LE, Hensleigh HC, Tagatz GE, Phipps WR, Leung BS. Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. Fertil Steril 1991;55(5):1000-1004.

32. Gómez E, Tarín JJ, Pellicer A. Oocyte maturation in humans: the role of gonadotropins and growth factors. Fertil Steril 1993;60(1):40-46.

33. De La Fuente R, O’Brien MJ, Eppig JJ. Epidermal growth factor enhances preimplantation developmental competence of maturing mouse oocytes. Hum Reprod 1999;14(12):3060-3068.

34. Pawshe CH, Appa Rao KB, Totey SM. Effect of insulin-like growth factor-I and epidermal growth factor on the cytoplasmic maturation of mouse oocytes. J Reprod Immunol 1997;35(1):11-29.

35. Fung P, Catt KJ, Knecht M. Transforming growth factor-beta stimulates meiotic maturation of the rat oocyte. Endocrinology 1988;122(1):181-186.

36. Lorenzo PL, Illera JC, Silván G, Munro CJ, Illera MJ, Illera M. Steroid-level response to insulin-like growth factor-1 in oocytes matured in vitro. J Reprod Immunol 1991;60(1):11-29.

37. Pawse CH, Appa Rao KB, Totey SM. Effect of insulin-like growth factor-I and its interaction with gonadotropins on in vitro maturation and embryonic development, cell proliferation, and biosynthetic activity of cumulus-oocyte complexes and granulosa cells in buffalo. Mol Reprod Dev 1998;49(3):277-285.

38. Lorenzo PL, Illera MJ, Illera JC, Illera M. Influence of growth factors on the time-dependent mitotic progression of the bovine oocytes during their in vitro maturation. Rev Esp Fisiol 1995;51(2):77-83.

39. Lorenzo PL, Rebollar PG, Illera MJ, Illera JC, Illera M, Alvariño JM. Stimulatory effect of insulin-like growth factor I and epidermal growth factor on the maturation of rabbit oocytes in vitro. J Reprod Fertil 1996;107(1):109-117.

40. Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. Role of epidermal growth factor
in bovine oocyte maturation and preimplantation embryo development in vitro. Biol Reprod 1996;54(6):1420-1429.

40. Sakaguchi M, Dominko T, Yamauchi N, Leibfried-Rutledge ML, Nagai T, First NL. Possible mechanism for acceleration of meiotic progression of bovine follicular oocytes by growth factors in vitro. Reproduction 2002;123(1):135-142.

41. Goud PT, Goud AP, Qian C, Laverge H, Van der Elst J, De Sutter P, et al. In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. Hum Reprod 1998;13(6):1638-1644.

42. De Matos DG, Miller K, Scott R, Tran CA, Kagan D, Nataraja SG, et al. Leukemia inhibitory factor induces cumulus expansion in immature human and mouse oocytes and improves mouse two-cell rate and delivery rates when it is present during mouse in vitro oocyte maturation. Fertil Steril 2008;90(6):2367-2375.

43. Mondal S, Mor A, Reddy IJ, Nandi S, Parameswaragupta PS. Effect of fibroblast growth factor 2 (FGF2) and insulin transferrin selenium (ITS) on in vitro maturation, fertilization and embryo development in sheep. Braz Arch Biol Technol 2015;58(4):521-525.

44. Klinger FG, De Felici M. In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells. Dev Biol 2002;244(1):85-95.

45. Gelber K, Tamura AN, Alarcon VB, Marikawa Y. A potential use of embryonic stem cell medium for the in vitro culture of preimplantation embryos. J Assist Reprod Genet 2011;28(8):659-668.

46. Vanhoutte JL. Strategies to improve in vitro maturation of human and mouse oocytes [dissertation]. [Ghent, Belgium]: Ghent University. Faculty of Medicine and Health Sciences; 2009. 129 p.

47. Lin YH, Hwang JL, Seow KM, Huang LW, Chen HJ, Tzeng CR. Effects of growth factors and granulosa cell co-culture on in-vitro maturation of oocytes. Reprod Biomed Online 2009;19(2):165-170.