



In Vitro Maturation of Human Oocytes using Conditioned Medium of Mesenchymal Stem Cells and Formation of Embryo by Use of ICSI

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Abstract

Problems: In vitro maturation (IVM) of oocytes is a promising technique to reduce the costs and avert the side-effects of gonadotrophin stimulation in in-vitro fertilization (IVF). On the other hand there are a lot of synthetic IVM mediums with low efficiency. Current research aims to develop an innovative approach to increase fertility rate. We rely on molecules secreted in mesenchymal stem cells (MSCs) proliferation medium which include different kinds of cytokines and growth factors. These secreted bioactive factors can cause in vitro oocyte maturation by resumption of meiotic division. The present study demonstrates use of conditioned medium (CM) of MSCs can provide the suitable medium for in vitro maturation whereas matured oocytes had the capability of reaching to blastocyst stage after intracytoplasmic sperm injection (ICSI).

Experimental approach: Denuded oocytes in germinal vesicle stage were placed into two IVM medium: commercial medium and conditioned medium of MSCs which was composed of DMEM supplemented with 10% FBS. Then after 48 h of placing them in IVM mediums, they were observed by Invert microscope to identify exclusion of polar body as the symbol of oocyte maturation. We used Chi Square test to compare the obtained results of IVM mediums.

Findings: the rate of successful maturation and embryo formation in CM and commercial IVM medium were 50, 26.42, 32.1 and 23.3% respectively. Regarding these data and by use of chi square test in the level of maturing GV oocytes the difference between these two used medium was not significant but about embryo formation, they differed significantly ($P < 0.05$).

Conclusion: Obtained results have shown that CM in comparison with commercial IVM medium is a more effective medium for maturation of oocyte and didn't disturb subsequent embryonic development.

This study was approved by jihad daneshgahi (33/50/227).

Key words: In vitro maturation, Mesenchymal stem cells, Cell culture, Human oocyte, Embryo Formation

Introduction

Recently the extensive use of drugs for ovarian stimulation has been questioned (1, 2). During menstrual cycle a large number of germinal vesicles are released monthly. If we can find a way to mature these numbers of GVs which are not usable clinically, we can reduce the cost and side effects of drugs in in-vitro fertilization (IVF) method. In the other word in vitro maturation (IVM) of oocytes is a promising technique to reduce the costs and avert the side-effects of gonadotrophin stimulation in in-vitro fertilization (IVF). Beside developing a functional method for in vitro maturation of human oocyte can provide an important instrument for studying the mechanisms governing oocyte development as well as supporting clinical applications.

It is known that oocytes need to undergo cytoplasmic maturation as well as nuclear maturation to be able to support suitable fertilization and embryo development (3,4) and Culture system can provide oocyte growth and maturation conditions.

Human follicular fluid (hFF), in a close contact with maturing oocyte, has an essential role in completion of oocyte maturation. During ovarian follicular development several growth factors and cytokines are secreted by granulosa cells in to the hFF that are necessary for oocyte maturation in vivo.

On the other hand, Mesenchymal stem cells (MSCs) comprise a heterogeneous population of cells with multilineage differentiation potential. The MSCs not only have the capability of responding to the stimuli and differentiation but also they can be consider as a secretory source secreting a large spectrum of growth factors and cytokines such as MCP-1 (Monocyte Chemotactic Protein-1), VEGF-A (Vascular Endothelial Growth Factor A), EGF (Epidermal Growth Factor), FGF-2 (Fibroblast Growth Factor 2), IL-6 (Interleukin 6), LIF (Leukemia Inhibitory Factor), TGF- β (Transforming Growth Factor beta) (5,6) . In fact these cells have two distinct functions: 1) they are able to replace the expired cells of mesenchymal tissue 2) they have differential effects on cells in their vicinity to induce their differentiation.

The effect of bioactive molecules secreted by MSCs can be direct, indirect or both. In the direct way they use intracellular signaling and in the indirect way they cause secretion of functionally active agent by the cells in their vicinity.

During menstrual cycle a large number of germinal vesicles are released monthly. If we can find

a way to mature these numbers of GVs which are not usable clinically, we can reduce the cost and side effects of drugs in in-vitro fertilization (IVF) method. As a result by expanding the way to in vitro development and maturation of human oocyte we can help young couples to keep their fertility.

During recent years there are a lot of applied methods with different synthetic commercial IVM medium for human oocyte which are different in using variant molecules, type and hormonal content. Unfortunately Most of them have a relatively low success rate.

In this study, conditioned medium of MSCs and a commercial IVM medium are used (CM) to stimulate cell condition and in fact we rely on the second function of MCSs in inducing cells to differentiation.

We decided to determine the nuclear and cytoplasmic maturation of the cultured oocytes by their capability to undergo germinal vesicle breakdown (GVBD), polar body extrusion, fertilization and early cleavage after ICSI.

Material and method

This study was done in the highly specialized infertility treatment of jihad daneshgahi (Qom branch). All the patients had filled a testimonial before using their oocytes or adipose tissues. This study was approved by jihad daneshgahi (33/50/227)

1. Isolation and culture of adipose tissues-derived mesenchymal stem cells:

Adipose tissues was harvested from 24 patients (aged 24~55 years) undergoing elective liposuction surgeries. Briefly adipose tissues were rinsed with phosphate buffered saline containing 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco) and digested with collagenase I (Sigma), then adipose tissue-derived mesenchymal stem cells cultured in DMEM medium (DMEM, Sigma) supplemented with 10% FBS (Gibco), 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco) at 37°C under 5% Co₂ and 95% humidity. The medium was changed every 4 days. After reaching to complete cell confluency, they were trypsinized and seeded at a density of 350×10³. After medium changing and collecting of supernatant, it was filtered through a 0.2µm membrane for immediate use.

2. Germinal vesicle treatment:

human germinal vesicle (hGV) were obtained from stimulated ovaries of women undergoing infertility treatment during an IVF/ICSI cycle. These GVs are usually discarded in clinical methods but in this procedure we incubated them at 37°C, 5% Co₂ and 95% humidity. We used two kinds of IVM mediums: 1) conditioned medium of MSCs 2) commercial IVM medium (Vitco). After 24-48 hours the oocytes were observed by invert microscope to determine their development in to the MII (metaphase II) stage.

As it has been proved, every ones genetic and environmental conditions differ from the others and these can also affect their oocytes, so different results can be seen.

3. Intracytoplasmic sperm injection (ICSI):

We used discontinuous density gradient centrifugation method to prepare spermatozoa for ICSI procedure. The spermatozoa were injected to the matured oocytes (After releasing of polar body as a symbol of maturation) either in the conditioned medium (CM) or commercial IVM medium the matured oocytes were transferred to the HEPES medium where the spermatozoa are placed in the Polyvinylpyrrolidone (pvp, life global) droplet. After cutting of spermatozoa's tails by injection pipet, we inject the immobilized spermatozoa into the cytoplasm of matured oocytes.

Results and Discussion

IVM of oocytes with CM and commercial IVM medium

In this research, we used 260 human oocytes in germinal vesicle stage from patients undergoing infertility treatment in “Highly Specialized Jihad Daneshgahi Infertility Center” during 2 years. (Figure 1-1a)

We found that some immature oocyte can maintain for up to 48 hrs (two days) at 37°C, 5% Co2 and 90% humidity (in a Co2-incubator). After three days, some of them stopped at germinal vesicle stage and some in metaphase I.

The oocytes maturation was determined by precise microscopic assessment of its morphology and their stages were classified as 1) germinal vesicle (GV), 2) metaphase I (MI) and 3) metaphase II (MII) or polar body extrusion (Figure 1-1 b, c). We observed in conditioned medium 35.7% of GV oocytes got to MI, 50% exhibited polar body extrusion and 14.3% didn't show any changes. These numbers for commercial IVM medium are respectively 21.4, 32.1 and 32.1%

(Table 1)

a oocytes	Frequency	Percent
MI	50	35.7
MII	70	50.0
Unchanged	20	14.3
Total	140	100.0

b oocytes	Frequency	Percent
MI	30	21.4
MII	45	32.1
Unchanged	45	32.1
Total	120	100.0

Table 1(a, b) : a) the percentage of MI, MII and unchanged oocytes with conditioned medium of MSCs. b) the percentage of MI, MII and un changed oocytes treated with commercial IVM medium

Developmental competence of injected oocytes (matured in conditioned and commercial IVM mediums)

a embryos	Frequency	Percent
Two-cells	16	22.9
Four-cells	9	12.9
Blastocyst	12	17.1
Unchanged	33	47.1
Total	70	100.0

The developmental competence of in vitro matured oocytes was observed by in vitro fertilization (IVF) and subsequent culture to the blastocyst stage.

Embryo formation of injected oocytes was assessed 2, 3 and 5 days after ICSI. In conditioned medium group, percentage of 2 cells, 4 cells and blastocyst formation respectively was 22.9, 12.9, 17.1. About commercial IVM medium the

percentages were respectively: 33.3, 15.6, 13.3 (Table 2 a, b). we should mention that there were some unchanged oocyte after ICSI in both groups.

b embryos	Frequency	Percent
Two-cells	15	33.3
Four-cells	7	15.6
Blastocyst	6	13.3
unchanged	17	37.8
Total	45	100.0

Table 2(a, b): a) the percentage of 2-cells, 4-cells, blastocyst and unchanged embryos after ICSI (conditioned medium of MSCs). b) the percentage of 2-cells, 4-cells, blastocyst and unchanged embryos after ICSI (commercial IVM medium)

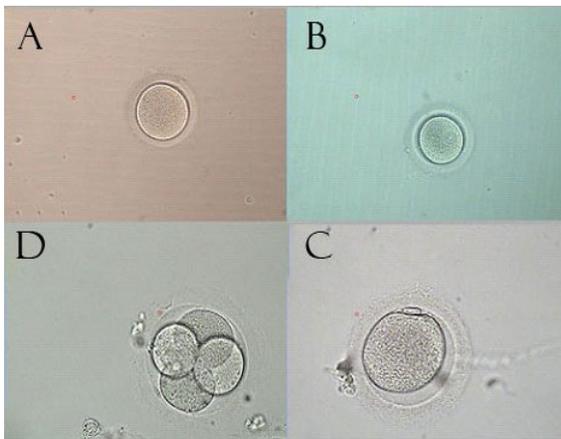


Figure1:(A, B, C & D): A) GV oocyte , B) MI oocyte , C) MII oocyte & D) 4-cells embryo



Figure2: blastocyst embryos

Oocyte maturation in vivo is a highly orchestrated, “induced” process, whereby the gonadotrophin/EGF cascade overrides the natural meiotic-inhibiting environment of the follicle (7, 8). It is shown that complete maturation of oocyte is essential for the developmental competence of embryos. Loss of developmental competence is associated with the absence of specific proteins in oocytes cultured to metaphase II in vitro (9). Kim BK and his coworkers (10) have shown that human GV oocytes from stimulated ovaries can be matured, fertilized and developed in vitro. Beside, in vitro maturation of immature oocytes together with ISCI can result in normal fertilization, embryo development, pregnancy and the delivery of healthy child (11, 12).

The EGF present in CM might resume meiosis of oocyte in vitro, similar to that which occurs in vivo and prevents spontaneous nuclear maturation of IVM oocyte caused by removing the COC from the meiotic-inhibiting effects of the follicle. Certainly, synchronous maturation of nuclear and cytoplasm in-vitro led to a competent oocyte, which was confirmed by changes of cell growth (CG) distribution during IVM and subsequent embryonic development after IVF. In this study the ability of CM of MSCs was investigated to support IVM of GV oocytes and subsequent embryo development.

It is shown that use of CM as a maturation medium will cause high maturation rate of oocytes with or without comolus and CM is an effective medium for preantral follicle growth, oocyte maturation and sequential embryo development [13]. In another study, Regardless the presence of cumulus cells, excellent meiotic and developmental competence of GV oocytes was achieved, when CM was used as maturation culture. On the other hand, when maturation in CM was compared with in-vivo maturation there was no significant difference in improvement of developmental components (13).Our research confirmed supportive effect of using CM on the oocyte maturation, but the difference between CM and commercial IVM medium was not significant (Figure 3).

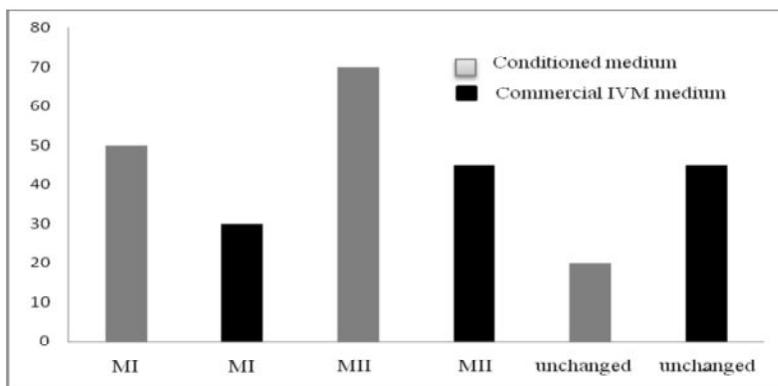


Figure 3.Comparison of MI, MII and unchanged oocytes in two conditioned and commercial IVM mediums.

Some researches presume cytokines and growth factors secreted by MSCs play the key role in improvement of developmental components (13). Some of these secreted bioactive factors

improve meiotic maturation in vitro and subsequent embryo developmental potential directly or via cumulus cells (14, 15). It has been reported that the rate of meiotic progression of bovine oocytes enclosed with cumulus cells enhanced with epidermal growth factor(EGF) and insulin-like growth factor-1(IGF-I) (16, 17). The time required for GVBD of denuded oocytes in mice decreased when they treat with epidermal growth factor (EGF) during maturation culture (18) and it has a maturation-promoting effect on denuded bovine oocytes (19). So we can suppose cytokines and growth factors secreted by MSCs in CM are effective in oocyte maturation and embryo development.

In a study, embryo development after IVM in CM culture showed a significant portion of the oocytes (which underwent GVBD) cleaved to the two-cells stage after insemination (13). In this study we used intracytoplasmic sperm injection (ICSI) method to assess the developmental competence of IVM oocyte.

In some IVM systems, cytoplasmic maturity was not fully achieved, which might cause reduced meiotic and developmental competence after fertilization (20, 21). Thus, improved meiotic maturation rate did not translate into any significant improvement in developmental competence, which assessed by IVF and blastocyst formation.

When we compared two IVM mediums, we found that embryo formation of matured oocytes in CM is significantly more effective than matured oocytes in commercial IVM medium ($p<0.05$)

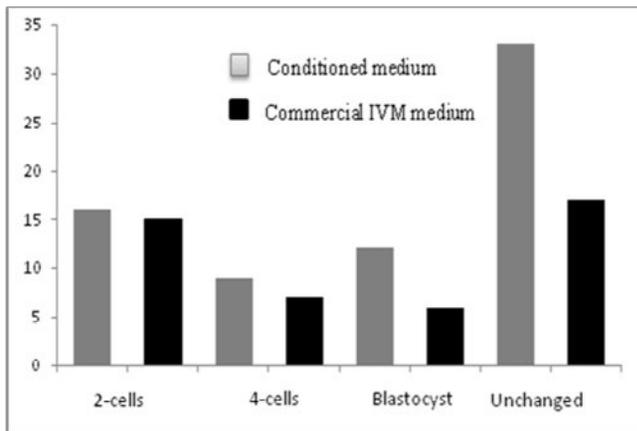


Figure 4. Comparison of 2-cells, 4-cells, blastocyst and unchanged embryos in two conditioned and commercial IVM mediums.

In general, comparison of CM and commercial medium in order to in vitro maturation of GV oocytes and surveying their developmental competence after ICSI procedure, showed that CM had a better effect on the immature oocytes to resume meiosis, although there weren't significant difference in number of successful maturation between two groups. But about embryo formation their difference was significant ($p<0.05$). Regarding morphological and not molecular assay in this study, it means that in terms of the quality matured oocyte in CM are more efficient in embryo formation (Figure 4).

As a conclusion in vitro maturation and fertilization of oocytes from natural cycle is an easy, cheap and realistic method to overcome infertility (22), and it would be more effective if a natural medium like CM of MSCs be use for maturing the GV oocytes.

Conclusion: Obtained results have shown that CM in comparison with commercial IVM medium is a more effective medium for maturation of oocyte and didn't disturb subsequent embryonic development.

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