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THE EFFECT OF CONDITIONED MEDIA ON HUMAN OOCYTE MATURATION AND DEVELOPMENTAL COMPETENCE

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ABSTRACT

Aim: Oocyte cytoplasmic maturation is very important during in vitro maturation (IVM) and the finding of an IVM medium that provides oocyte competence is very critical in assisted reproductive technology (ART). The aim was to determine the effects of mesenchyme stem cell conditioned media on the maturation and developmental potential of human oocyte after IVM and activation. Material and Method: 348 human germinal vesicle (GV) oocytes were collected from 191 ICSI cycles; then GV oocytes were divided into: fresh IVM (fIVM) and vitrified IVM (vIVM) which were cultivated in three maturation media: 1) mesenchymal stem cells (MSCs) conditioned media (CM) derived from human bone marrow, 2) human umbilical cord and 3) α-minimum essential medium (α-MEM) for 36 hr. Results: There were significant differences in maturation rate ($p<0.000$), arrest in 4-8 cells (0.029) and the A quality of 8-cell (0.037). The lowest maturation rate was in α-MEM vIVM and the highest was in Hu-MSCs fIVM group (71.42% vs. 85.18%, respectively).Discussion: In MSCs medium, the oocyte maturation and cleavage rate were higher in comparison with α-MEM medium. But highest oocyte development was seen in human umbilical cord MSCs medium. Probably the source of MSCs is the imperative factor to generate appropriate microenvironment consisting of essential components for inducing healthy oocyte development through paracrine mediators.

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Introduction

Successful embryonic development depends on synchronization between nuclear maturation and cytoplasmic development [1]. Different in vitro maturation(IVM) conditions and maturation media composition caused alterations in mRNA content and gene transcripts for providing capacity to survive cryopreservation; therefore, the selection of an optimal IVM culture medium is very essential. Supplementing IVM medium with various growth factors may be beneficial in oocyte maturation; through stimulating meiotic processes by means of the adjustment of antioxidants level and protein synthesis. Its optimization could have a significant influence on IVM and quality of generated embryo [2-5]. Supernatants derived from human mesenchyme stem cells (MSCs) such as bone marrow and umbilical cord have several growth factors, cytokines and vitamins which might be useful for IVM culture medium; Consequently, in vitro condition such as supplementations and formulation of the IVM medium, moreover physical environment influence on oocyte maturation rate and subsequent embryonic development [6-8]. Inducing in vitro chemical parthenogenesis using ionomycin is a direct sign for evaluation of cytoplasmic oocyte development [9] .There have been various studies comparing IVM rates among different culture media, but there is lack of data regarding ideal IVM system that has the highest efficiency of developing competent oocytes. MSCs could secrete numerous bioactive molecules such as chemokine, cytokines and growth factors which have major effects on cellular function and dynamics; accordingly, administration of MSCs conditioned medium (MSC-CM) is able to summarize the advantageous effects of MSCs including stimulating regeneration and tissue repair [10]. The aim of this study was to compare the oocyte maturation and subsequent embryo developmental potential using two culture media derived umbilical cord and bone marrow MSC-CM after activated through parthenogenesis in comparison with standard IVM medium, α-minimum essential medium (α-MEM). Also, these IVM media were assessed in fresh compared to vitrified IVM.

Materials and Methods

Study design

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This study included 348 germinal vesicle (GV) oocytes from 191 women (30.61±5.61 years old), since 2014 to 2017, who admitted to Afzalipour Infertility Center in Kerman and were consecutively on ICSI cycles. This research was done as permitted by Kerman Medical University's Ethical Committee [n: 93/692]. Fresh or vitrified GV oocytes were randomly matured in vitro in different media for 36 hr. and finally cleavage competence of the matured oocytes after artificial activation were assessed.

Oocyte preparation

Ovarian stimulation was induced according to long pituitary down regulation protocol, using administration of a combination of follicular stimulating hormone (FSH) and gonadotropin-releasing hormone (GnRH). Then, following ovarian follicular growth under trans-vaginal ultrasound, when the ovarian follicles reached larger than 18 mm, 10,000 IU of human chorionic gonadotropin (hCG) (IBSA Co, Switzerland) was administrated. The oocyte pick-up was performed through laparoscopy, 36 hr later. After oocyte retrieval, oocyte denudation was performed using chemical (80 IU hyaluronidase (Sigma Co, USA)) method and mechanical dissection with fine-bore glass pipetting. Then nuclear maturity of denuded oocytes, were examined by stereo microscope (Olympus Co, Japan). According to the first polar body extrusion, the oocytes were categorized MII or immature: germinal vesicle breakdown (GVBD) or germinal vesicle (GV); GV oocytes were used for this research [11] .

Vitrification

After washing GV oocytes in basic medium [Ham's F10+20% human serum albumin (HSA)], the GV oocytes were submerged in the equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) (Merck Co, Germany) and 7.5% dimethyl sulphoxide (DMSO) (Merck Co, Germany) in basic medium [Ham's F10+20% human serum albumin (HSA)], then it was located at room temperature, for 10 min. Next, the oocytes were transferred in the vitrification solution (VS) consisting of 15% (EG) and 15% (DMSO) with 0.5M socruse (Sigma Co, USA) in basic medium [Ham's F10+20% human serum albumin (HSA)] then was located at room temperature, for 50-60 seconds. Then, the oocytes (n:1-3) were located on the cryotops (Kitazato, Japan). These cryotops rapidly were moved into liquid nitrogen tank for two months [12].

Warming

Oocytes warming procedure was carried out in five stages; The basic medium was Ham's F10+20% human serum albumin (HSA) (Plasbumin Co, USA), 1: the warming solution [1M sucrose in basic medium] for 50-60 seconds, 2: first dilution solution [0.5M sucrose in basic medium] for 3 min; 3: second dilution solution [0.25 M sucrose in basic medium] for 3 min; 4,5: the oocytes locate in the first and second phase of washing solutions in basic medium, each for 3 to 5 min. 2-3 hr later. Viability of warmed oocytes was evaluated by stereomicroscope, then alive oocytes, randomly, were inserted in three IVM media for 36 hr [13].

IVM media

Three IVM media were used in the current study: 1. Alpha-minimum essential medium (α-MEM); 2. Supernatants of human bone marrow mesenchyme stem cells (MSCs), and 3. Human umbilical cord (MSCs) as a conditioned medium (CM), which 1-2 GV oocytes were placed in each drop [14, 15].

MSC isolation and culture

Bone marrow mesenchyme stem cells (MSCs) and human umbilical cord mesenchyme stem cells (hUCM) in the present study were provided from anatomical cell culture laboratory, in Afzalipour Medical University (Kerman-Iran). After washing the cells with PBS (phosphate buffered saline), these cells were cultured in α-MEM media as a basic medium supplemented with streptomycin 100mg/mL, penicillin 100U/mL and 10% fetal bovine serum (FBS). After three days, the medium was changed and death cells removed but adherent cells remain to achieving complete cell confluence. After 48 hr, the supernatants of these cells were removed and filtered with 0.2µm membrane which considered as conditioned medium (CM) [16].

Artificial Oocyte activation

In vitro matured oocytes were artificial activated with ionomycin according to de Fried protocol. α-MEM medium supplemented with 10 µM ionomycin (Sigma co, USA) at room temperature (37°C) for 6 min, then the oocytes were transferred to IVF medium (Vitrolife, Sweden) that supplemented with 2 µM 6-dimethylaminopurine 6 (DMAP) (Sigma co, St Louis) for 3 hr in the incubator. After this time, the activated oocytes were located in G1 medium (Vitrolife, Sweden) as a cleavage medium. After activating the mature oocyte, the fertilization pronuclear was formed after 11-18 hours and at the second day after fertilization, the 2-4 cell embryos was formed and at the third day after fertilization, the 4-8 cell embryos was seen. Compaction was performed at 68 hr after activation. The arrested embryos were labeled by stopping in the developmental phase [17, 18].

Parthenote embryos Score

Partenote embryos were scored according to Nasr-Esfahani et al. methods. The A quality called for best equal symmetrical blastomeres with minor fragmentation; the B quality called for equal blastomeres with moderate fragmentation and finally the C quality called for unequal blastomeres with severe fragmentation [19].

Statistical Analysis

The differences of maturation and developmental parameters were calculated using SPSS software (version 21, USA). Chi-square was used for categorical variables. Kruskal-Wallis test was applied as a non-parametric test. P-value <0.05 was considered significant.

Results

No significant differences ($p<0.05$) were seen between patients demographic characteristics (Table1).

Fresh and alive vitrified-warmed GV oocytes were cultured in these media. The survival rate post-warm was (88.83%, 175/197). The highest maturation rate was (85.18%) in fIVM hUCM and the lowest (71.42%) in vIVM BM- MSCs group. Oocyte maturation rate had significant differences between these groups ($p <0.000$); the highest GV arrest was in α-MEM vIVM, while the highest MI arrest was in α-MEM fIVM (Figure 1).

Cytoplasmic maturation of IVM oocytes was evaluated through chemical activation; then cleavage rate and score of parthenote embryos were compared (Figure2).

The highest degeneration after activation was in α-MEM vIVM and the lowest in B.M fIVM. The highest 2PN formation and lowest arrest in 2-4 cell was in hUCM fIVM; but the lowest arrest in 4-8 cell was in hUCM vIVM. The differences between

the 4-8 cells arrest were significant (p=0.029). The highest morula formation was in hUCM fIVM and blastocyst formation was seen in hUCM fIVM (Table2).
The Partenote embryos score were assessed in 2-4 and 4-8 cells; as the best quality was recorded as A and the lowest quality was called C. also the score was shown in figure 3.

Discussion

Several studies have shown embryonic development may be correlated with nuclear and cytoplasmic quality of oocytes, type of IVM and embryonic medium, alteration in mitochondrial membrane and mitochondrial distribution; then enrichment of IVM media could improve the oocyte development. On the other hand the oocyte developmental capacity has influence on the cytoplasmic organelles delivery, chromosomal organization, mitochondrial distribution and function; also mRNA expiration level providing critical proteins for healthy embryonic development [20-22].The main cause of embryonic arrest and fragmentation may be related to the alteration in mitochondrial membrane potential, chromosomal abnormality and suboptimal culture conditions during pre-implantation development leading to regulate the ATP production and free calcium concentrations [20, 22, 23]. In our study, embryo cell block in 4-8 cells stage was significantly different, which might be due to increase in ROS and adverse metabolite or lack of nutrients in the culture media during the culture time. However, in our study the culture media was changed and refresh to resupply their nutrients on three days after activation, at the same time with 6-8 cell proliferation in oocyte developmental stage. Furthermore, the mitochondrial shape and distribution affected the cell proliferation, differentiation and metabolism; similarly the relationship between mitochondria and smooth endoplasmic reticulum (SER) regulate the calcium signaling [24]. Full oocyte cytoplasmic maturation is including meiotic maturation, mRNA transcription, protein synthesis, reprograming genome and blastocyst formation [25]. In our study, blastocyst formation from IVM activated oocyte, was observed only in fIVM hUCM group with highest cleavage rate. During fertilization process, the sperm mitochondria are degenerated and blastomeric energy supply will be provided from the oocyte. Accordingly, the oocyte mitochondria could have a direct effect on the embryonic development [20].Our results highlight that the feasibility of hUCM as a substitute protein supplement for human oocytes maturation and great influence on oocyte quality, metabolic activity and then facility to regulate development of healthy high quality embryo.

Conclusions

Our results indicated that hUCM enhances oocyte meiotic capability with great embryo development and quality. Probably the source of mesenchyme stem cell is the important factor to generating oocyte growth factors and essential components for inducing healthy oocyte development, through paracrine mediators. These results might offer the theoretical basis of hUC-MSC for the further therapeutics clinical application.

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Conflict of interests

The authors declare that they have no conflict of interests.

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Table 1: patient's demographic characteristics

IVM medium	Mean age(y)	Male factor infertility (%)	Female factor infertility (%)	Both(male &female infertility)(%)	infertility duration (y)
α-MEM	30.72±4.34	34.3	36	29.5	6.3±3.8
BM- MSCs -CM	31.1±4.12	35.5	31.9	31.8	5.9±3.6
hUC- MSCs -CM	30.35±4.54	46.7	32.6	20.5	5.45±3.9
P value	0.63	0.36	0.97	0.53	0.48

IVM: in vitro maturation
α-MEM : Alpha-minimum essential medium
BM- MSCs- CM: Bone marrow Mesenchymal stem cells conditioned medium
hUC- MSCs -CM: human umbilical cord mesenchymal stem cells conditioned medium

Table 2: Developmental competence of activated IVM oocytes

Developmental parameter	fIVM α-MEM	vIVM α-MEM	fIVM B.M	vIVM B.M	fIVM hUCM	vIVM hUCM	P value
MII oocyte(n)	45	45	45	46	46	42	0/190
Activated oocyte(n)	25	25	25	26	22	25	0/707

Degenerated after activation	6(24%)	11(44%)	1(4%)	5(19.2%)	2(9.09%)	3(12%)	0/063
2PN (%)	76%	56%	96%	80.76%	90.9%	88%	0/168
2-4 cell arrest	63.1%	78.5%	37.5%	85.7%	25%	86.3%	0/058
4-8 cell arrest	31.5%	21.4%	54.1%	14.2%	44%	9.09%	0/029
Morula	5.2%	-	8.3%	-	15%	4.5%	0/377
Blastocyst	-	-	-	-	5%	-	0/224

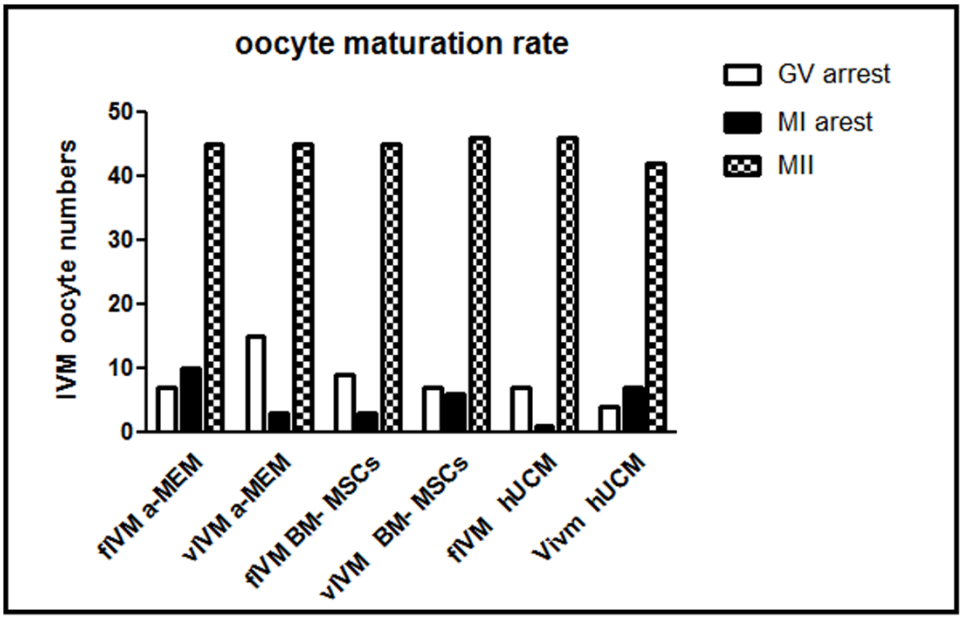

Figure 1. The comparison of oocyte maturation rate in different groups. (1) fIVM α-MEM: 62 fresh GV oocytes inserted in α-MEM (2) vIVM α-MEM: 63 vitrified GV oocytes inserted in α-MEM (3) fIVM BM- MSCs: 57 Fresh GV oocytes inserted in BM- MSCs (4) vIVM BM- MSCs: 59 vitrified GV oocytes inserted in BM- MSCs (5) fIVM hUCM: 54 Fresh GV oocytes inserted in hUCM (6) vIVM hUCM: 53 vitrified GV oocytes inserted in hUCM. The differences between GV arrest (p=0.640) and MI arrest (p=0.058) were not significant; but maturation rate had significant differences (p=0.000).

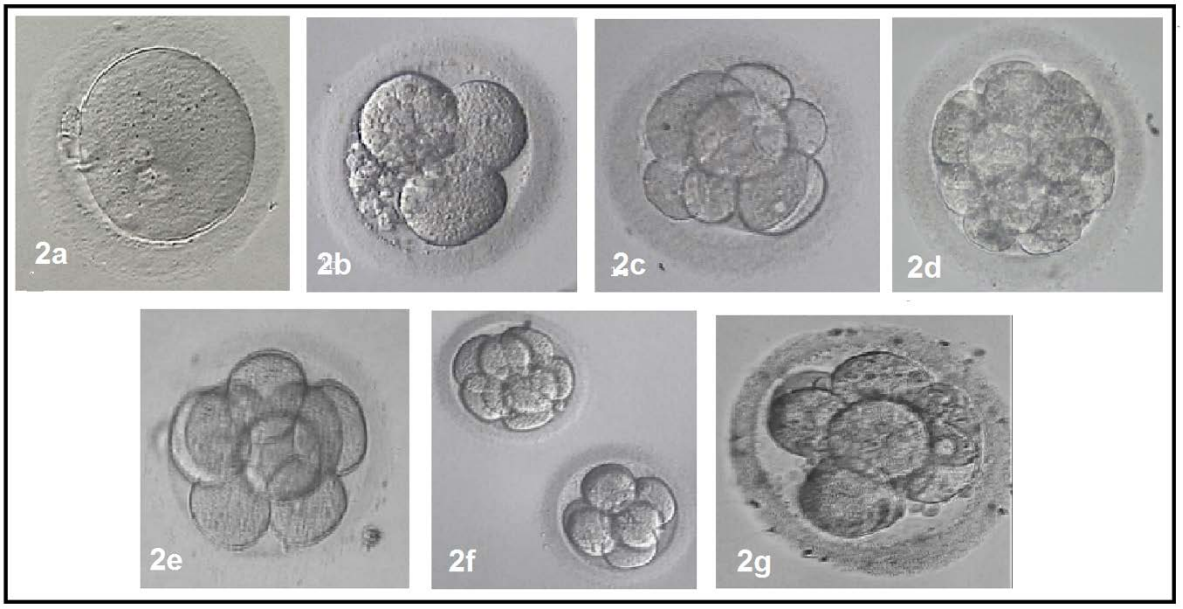

Figure 2. Assessment of the cytoplasmic maturation of MII artificial activated IVM oocytes In activation MII oocytes, the fertilization pronucleous was formed after 11-18h (fig 2a), the 2-4 cell was formed 30h after activation (fig 2b), on 3rd days,4-8 cell was formed (fig 2c);the compaction was formed 68 h after activation (fig 2d), then the blastocysts were formed on 5 days after activation .the Partenote embryos were scored by A, B and C scale (fig2e, 2f and2g); score A (fig2e), score B (fig2f) and score C (fig2g).

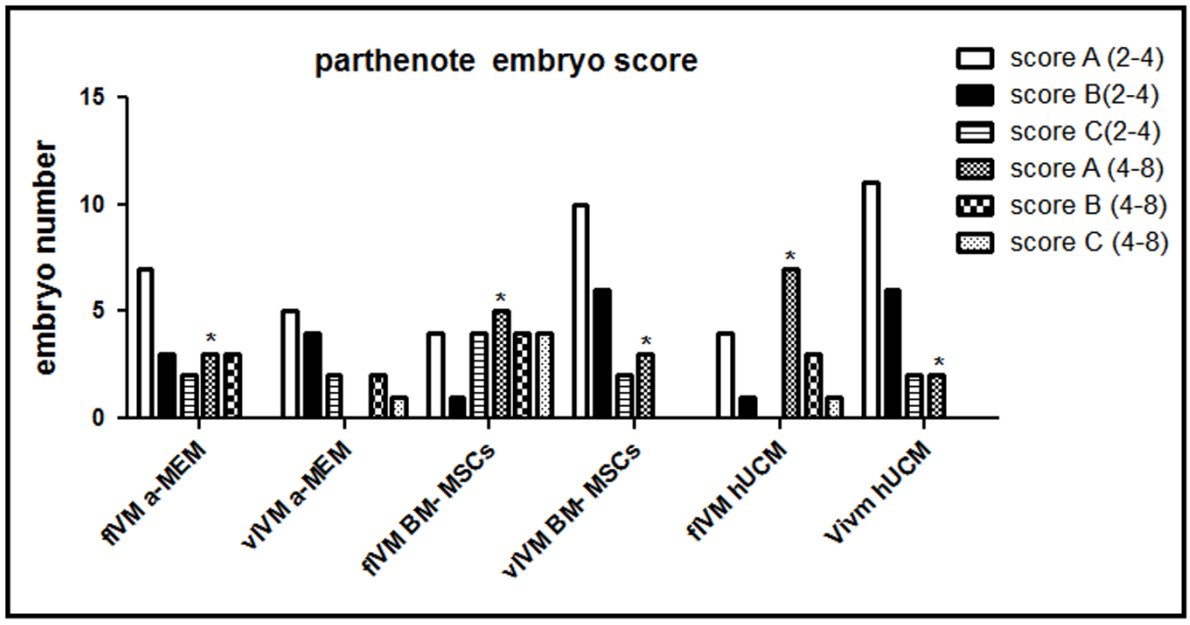

Figure3. Parthenote embryo Score. The embryo scored as A, B and C according to condition of the blastomeres and intensity of fragmentation 1) score A: equal symmetrical blastomeres + minor fragmentation. 2) score B: equal blastomeres + moderate fragmentation and 3) score C: unequal blastomeres + severe fragmentation. Differences of the A scores (4-8 cells) were significant between different IVM groups (p=0.037).