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The Effect of The Conditioned Medium from Human Embryonic Stem Cells on Mouse Oocytes *In Vitro* Maturation

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Abstract

Objective: Some reports have indicated that conditioned medium from growing mouse embryonic stem cells (ESCs) provides a supportive condition for small follicles growing, oocyte maturation, and following embryo growth. The aim of this study is assessing *in vitro* maturation (IVM) and consequent *in vitro* fertilization (IVF) outcome of immature mouse oocytes using human embryonic stem cells conditioned medium (HESCM).

Materials and Methods: In this experimental study, 240 germinal vesicle (GV) oocytes were took from NMRI female mice, aged 4-6 weeks, 48 hours before injection of 5 IU pregnant mare serum gonadotropin (PMSG). 120 GV oocytes without cumulus cells were cultured in each of the groups. 120 GV were cultured in HESCM as test groups and also 120 GV cultured in human embryonic stem cells medium (HESM) as control groups. After evaluating the metaphase II (MII) oocyte maturation rate at 8, 16 and 24 hours, the MII oocytes subsequently were fertilized *in vitro* and the two-cell embryo development rate was recorded at days 1, 2, and 3. Statistical analysis was performed by using the generalized estimating equations (GEE) method that calculated their rate ratio.

Results: Our data indicated there are significant differences between the maturation rates in HESCM and HESM (P=0.004), also the two-cell embryo development was significant between two culture media (P=0.00).

Conclusion: Similar to some other studies, the secretome of the HESCM showed a significant impact on the IVM outcomes in mice.

Keywords: Assisted Reproductive Techniques, Conditioned Medium, Germinal Vesicle, Human Embryonic Stem Cells, *In Vitro* Maturation

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Introduction

Since the first attempts for *in vitro* maturation (IVM) of mammalian oocytes by Pincus and Enzmann in 1935, on immature rabbit oocytes (1), studies are carrying on to improve this technology. IVM is one of the assisted reproductive techniques (ART) inducing the meiotic IVM of oocytes retrieved from small follicles of unstimulated ovaries. Since defect of oogenesis is the cause of infertility in some infertile couples, IVM of germinal vesicle (GV) oocytes is an important method in infertility treatments (2, 3).

Retrieval of immature oocytes and applying IVM is used to help polycystic ovarian syndrome cases in infertility treatments (4, 5). IVM improvement is important and is the only option for replacement with routine stimulation methods by using drugs and hormones in the future (6). One of the problems of the growth and maturation of immature oocytes is the preparation of an efficient medium that makes *in vitro* conditions similar to the *in vivo* condition (3, 4). One of the strategies which have been employed is using conditioned medium from different sources of growing cells to improve IVM (7, 8). Conditioned medium contains growth factors and hormones secreted by growing cells which can be used to stimulate the growth of other cells (6).

Embryonic stem cells (ESCs) are pluripotent cells resulting from the inner cell mass (ICM) of preimplantation embryos in blastocyst stage (9, 10). The ESCs can secrete biological products and activated proteins that

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subsequently provide an medium culture with mitogenic factors, growth factors, cytokines, and chemokines (11, 12) which might be useful to improve IVM outcomes.

In this study, the aim was to determine whether IVM of mouse GV oocytes can be improved by using human embryonic stem cells conditioned medium (HESCM) or not.

Materials and Methods

Ethical consideration

All animals were preserved according to the ethical guidelines provided by the Yazd Reproductive Sciences Institute Ethical Committee for animal studies (IR.SSU.REC.1397.087).

Materials

Our study was an experimental study. Chemicals and reagents were purchased from Sigma Aldrich Co. (UK). Culture media and supplements were purchased from Invitrogen Co. (UK) unless otherwise stated.

Preparation of HESM and HESCM

The human ESC growing medium was provided from the stem cell biology research center, Yazd reproductive sciences institute as explained elsewhere (10). HESCM was obtained from a HESCs (Fig.1A, B) cultured in a microdrop system (Fig.1C). After sufficient growth of undifferentiated colonies within a microdrop and before passage, the culture medium around the colony was collected and used as a conditioned medium (HESCM) for mouse oocytes IVM.

Animals

The animals used for the experiments were the Naval Medical Research Institute (NMRI) female mice, aged 4-6 weeks. Similar to our previous reports (7, 8), these mice were housed and bred in the animal house at the Biotechnology Unit of the Yazd Reproductive Sciences Institute. The mice were kept on a 12 hours' light dark cycle, a temperature range of 22 to 25℃, humidity 40 to 60% and enough nutrients.

Immature oocytes collection and their *in vitro* maturation

The immature oocyte collection and IVM process was done as explained elsewhere (7, 8). But the test and control medium are HESCM and HESM in the current study.

In this way, twenty 4-6 weeks old female mice received an injection of 5 IU pregnant mare serum gonadotropin (PMSG). Fourty-eight hours after injection, immature GV oocytes from the ovaries were taken out. The GV oocyte retrieval was done by scratching the ovaries with a sterile 28-gauge needle under a stereomicroscope. GV oocytes (Fig.2A) were individually cultured in micro drops (Fig.1C) of HESCM (test group) and of HESM (control group). In the present study, 240 GV oocytes (120 per group) were included and incubated at 37°C in a 5% CO_2 incubator for 24 hours. At 8, 16, 24 hours Oocyte maturation was evaluated by using stereo microscopy, only those oocytes that showed the first polar body in the perivitelline space were selected as metaphase II (MII) for IVF.

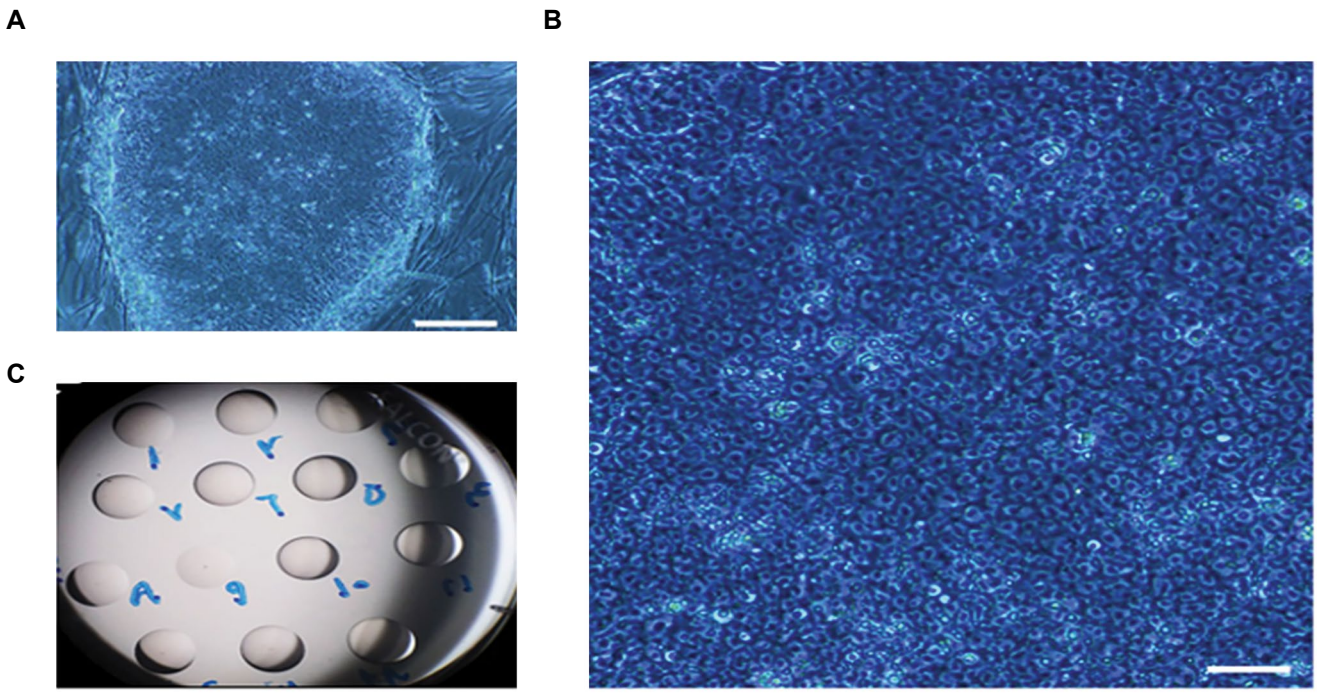

Fig.1: HESCM preparation. **A, B.** HESM from growing Yazd2 hESC line was collected and **C.** Microdrops of HESCM prepared for IVM of mouse oocytes (scale bars of A: 500 µm and B: 100 µm). HESCM; Human embryonic stem cell conditioned medium and IVM; *In vitro* maturation.

In vitro fertilization and embryo development

Following IVM, the two-cell embryo development potential rate of MII oocytes was evaluated by IVF. Male mice spermatozoa were obtained from the cauda epididymis that capacitated for 1 hour at 37°C. MII oocytes were incubated with sperms in the GIVF medium (Vitrolife, Sweden) for 4 hours. For the removal of additional spermatozoa, oocytes were washed and then cultured in a G1-plus droplet (Vitrolife, Sweden; 5-6 oocytes in one drop) and in 37°C and 5% CO_2 in air for 3 days. The embryo development rate was checked and evaluated everyday by using a stereomicroscope.

Statistical analysis

The trend of changes in oocyte maturation and embryo development rate in mice oocytes were evaluated at each stage and compared between test (HESCM) and control (HESM) groups. Generalized estimating equations (GEE) method was used for comparing the trend change of each outcome variable through different time. Chi-square and Fisher exact-test were used for comparison of the frequencies of each outcome variable between HESM and HESCM groups. A significant level was considered as $P \leq 0.05$.

Results

In vitro maturation outcomes using human embryonic stem cells conditioned medium

Mice GV oocytes (Fig.2A) were cultured in HESCM (test group) and HESM (control group) for further maturation *in vitro*. IVM of mice oocytes was assessed during 24 hours. Resumption of meiosis from GV (Fig.2A) to the MI (Fig.2B) and MII (Fig.2C) stage was considered to be oocyte IVM. However, our data shows that HESCM provides more supportive conditions for maturation of mouse immature oocytes to the MII stage after IVM (P=0.004).

In two groups some of the GV oocytes were developed to MI stage after 8 hours (Table 1). The number of MI oocytes in HESCM group after 8 hours was 21(17.5%) from 120 oocytes and in control group was 27 (22.5%) from 120. The number of the MII oocytes in HESCM was 3 (2.5%) from 120 and in control group was 1 (0.08%) from 120 (Table 1). The number of MI and MII oocytes in HESCM after 16 hours [respectively 49 (40.83%) and 11(9.16%) from 120] was higher than control group [respectively 41 (34.16%) and 8 (6.66%) from 120] (Table 1). In test group after 24 hours 17 (14.16%) of oocytes developed to MI and 32 (26.66%) oocytes reached to MII, whereas in control group only 30 (25%) from 120 oocytes developed further to MI stage and 28 (23.33%) of them developed to MII stage (Table 1). Significant difference was existed between the MII maturation stage rates in HESCM and HESM (P=0.004). Two groups support the further development of the GV oocytes to MI stage which this progress was insignificant in both groups. MI formation rate in HESCM and HESM has increasing and then decreasing trend that is because of the maturity of the oocytes to MII after 24 hours (Table 1). It is notable that the GV oocyte maturation to MII increased after 24 hours that shows the positive effect of the time on oocyte in HESCM group (Table 1) Similar to development of oocytes to MI stage in control group.

In sum, HESCM support the maturation of GV oocytes to MII stage. This data shows that there are factors in human ESCs which support IVM process in mouse.

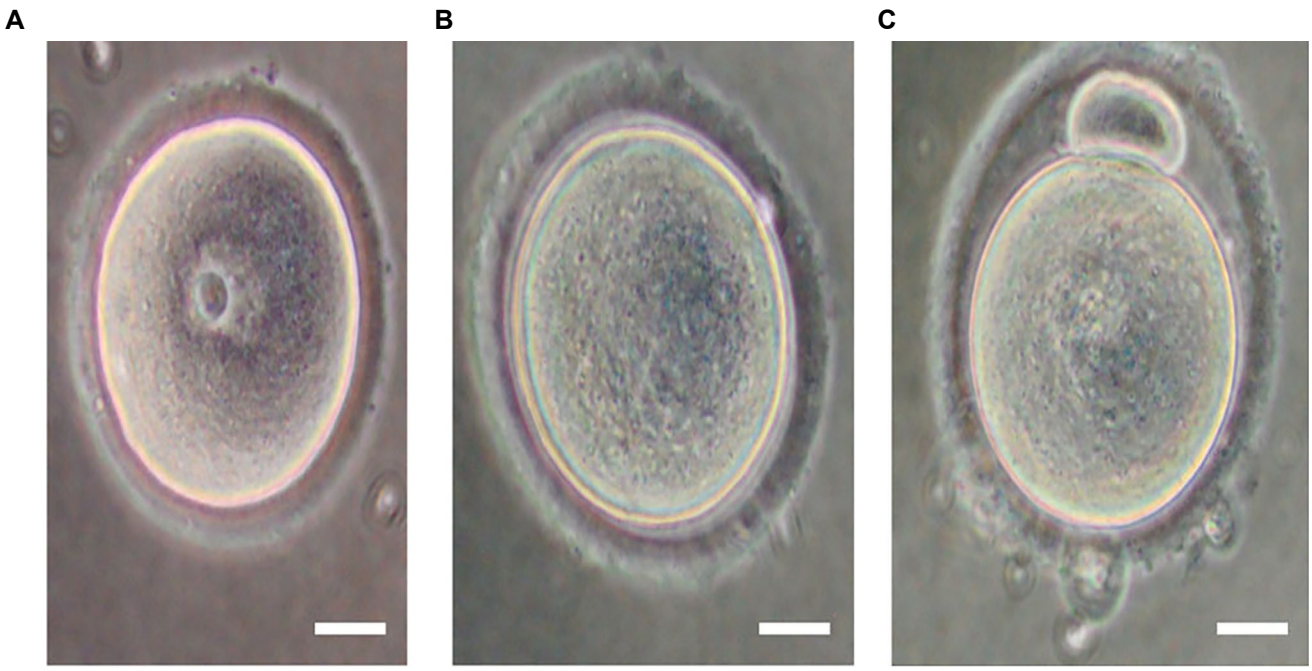

Fig.2: Different stages of mouse oocyte during *in vitro* maturation in our study. Each experiment was repeated for 120 oocytes. **A.** GV oocyte, **B.** MI oocyte, **C.** MII oocyte (scale bar: 15 µm). GV; Germinal vesicle, MI; Meiosis I; and MII; Meiosis II.

Table 1: IVM rate at 8, 16, and 24 hours in the HESM and HESCM groups

Group	MI			MII		
	8 hours	16 hours	24 hours	8 hours	16 hours	24 hours
HESM	27 (22.5)	41 (34.16)	30 (25)	1 (0.08)	8 (6.66)	28 (23.33)
HESCM	21 (17.5)	49 (40.83)	17 (14.16)	3 (2.5)	11 (9.16)	32 (26.66)
P value		0.52			0.004	

Data are presented as n (%). MI, MII, and developmental rate at deferent times at test and control medium at 8, 16 and 24 hours. GEE method was used for change trend in MI and MII. P value of Chi-square test for MI and MII was 0.52 and 0.004 respectively (statistical test: Chi-square). HESM; human embryonic stem cell medium, HESCM; Human embryonic stem cell conditioned medium, MI; Meiosis I, MII; Meiosis II, GEE; Generalized estimating equations. Each experiment was repeated for 120 oocytes.

Embryo development following *in vitro* fertilization of *in vitro* maturation oocytes

The developing competence of IVM oocytes was evaluated by subsequent IVF and embryo culture to the 2-cell cleavage stage (Fig.3).

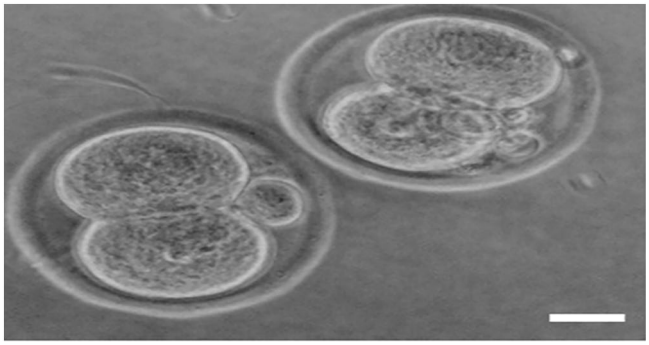

Fig.3: Embryo development following IVF of MII oocytes. Picture of two cells embryos after 48 hours and 72 hours (scale bar: 15 µm). Each experiment was repeated for 32 MII oocytes obtained from HESCM medium and for 28 MII oocytes obtained from HESM medium. IVF; *In vitro* fertilization, MII; Meiosis II, and HESM; Human embryonic stem cell medium.

26.66% of oocytes that matured in HESCM were fertilized in GIVF medium. The IVF success rate was determined according to the number of embryos that reached the 2-cell stage at 24 hours after IVF. Number and Percentage of two-cell embryo development from HESCM after 1, 2 and 3 days were respectively 0 (0%), 7 (21.87%), and 7 (21.87%). Table 2 shows results for the percentage of IVF success at 1, 2, and 3 days in the HESCM. Table 2 indicates the two-cell embryo development rate increased until the second day after IVF, and then remained at this stage on the third day. 23.33% of oocytes (28 from 120 GV) that matured in HESM were fertilized in GIVF medium. Percentage of two-cell embryo development rate from HESM at 3 days was 3 (10.71%, Table 2).

Table 2: Two-cell embryo development following IVF of MII oocytes

IVF	Two-cell embryo development		
	1 day	2 days	3 days
HESM	0 (0)	3 (10.71)	3 (10.71)
HESCM	0 (0)	7 (21.87)	7 (21.87)
P value	0.000		

Data are presented as n (%). Each experiment was repeated for 32 MII oocytes obtained from HESCM medium and for 28 MII oocytes obtained from HESM medium. P value=0.000 (statistical test: Chi-square). IVF: *In vitro* fertilization, MII; Meiosis II, HESM; Human embryonic stem cell medium, and HESCM; Human embryonic stem cell conditioned medium.

Discussion

Since many factors are involved in the maturation and development of oocytes, much research has been done based on the selection of more appropriate culture medium containing these factors (11-13). For example, factors such as EGFs have been shown to increase the rate of oocyte maturation (13). Some studies have shown that the addition of a series of substances secreted from oocytes to the IVM culture medium of mice (14) and bovine (15, 16) significantly increases the maturity of oocytes.

There are some factors secreted by ESCs (EGF, LIF, TGF-β, IGF) in the culture medium which supports IVM of oocytes (12). The effect of these factors on IVM, have been verified in several species (17, 18). On the other hand, Giuffrida and colleagues have shown that human ESCs (hESCs) secrete soluble factors that inhibit cancer cells growth (19). Therefore, in this study, we prepared a conditioned medium from ESCs culture to be containing these factors.

In this study, the maturation and two-cell embryo development rate of mouse immature oocytes in HESCM, and HESM were evaluated. Our data indicated that HESCM to be more supportive than HESM in IVM 32(26.66%) MII from 120(100%) GV vs. 28(23.33%) MII from 120(100%) GV and two-cell embryo development 7(21.87%) from 32(26.66%) MII oocytes vs. 3(10.71%) embryos from 28(23.33%) MII oocytes in HESCM, and HESM groups respectively, and this difference was statistically significant.

Previously, we have shown that conditioned media from human testicular cells (7) and human cumulus cells (8) support IVM in mice. In compare with our previous studies after 24 hours human testicular cells conditioned medium (hTCCM) is the most supportive and then HESCM and at last is human cumulus cell conditioned medium (hCCCM) with 31.67% (7) vs. 26.66% vs. 24.16% (8) IVM rate respectively. On the other hand, interestingly HESM seems to be more supportive than DMEM+20%FBS as the basal medium in previous study, to support IVM in mice (23.33% vs. 0%).

Chian and Tan (20) showed that immature human oocytes without cumulus cells are also able to support the early stages of embryonic development following IVM in a suitable culture medium. In our study, in order to investigate the effect of HESCM on IVM in mouse GV oocytes, immature oocytes without cumulus cells were used for IVM in both test and control groups.

It has also been reported in some studies that the rate of maturation in GV resulting from stimulation cycles (21) is higher than that without stimulation cycles in the laboratory (22). In our study; 5 IU of PMSG was used to stimulate the ovaries 48 hours before taking an immature oocyte.

In other studies, cross species experiments have shown the conditioned medium from different species support IVM of GV oocytes from other species (7, 8, 23). For instance, IVM of canine oocytes was evaluated by using conditioned medium from mouse (23) or bovine (24) sources. In our study, the effect of human source of ESCs was investigated on the IVM of mouse oocytes.

In sum, our data supports the previous report of the supportive effect of mouse ESCCM on mouse IVM (12), however, this supportive effect in our results was less than the study, which might be due to the human source of the conditioned medium used in our work. Moreover, from our experiences at the same laboratory (7, 8) HESM as a basal medium has a better impact on IVM in mice in comparison with DMEM+20% FBS. The other issue is that we have used a single culture for each individual GV oocyte whereas in some of the previous studies GV oocytes (12) were cultured in groups which may have an impact on the result. Additionally, in some reports GV oocytes were not completely denuded from granulosa cells which also might cause some variations between different studies. In addition, several studies have reported beneficial effects of conditioned media for enrichment IVM culture medium (25).

Conclusion

This study demonstrated the advantageous effect of the HESCM on the IVM outcomes in mice providing a framework for further investigation.

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Authors' Contributions

R.R.R.S., B.A.; Conceptualization, Methodology, and Software. R.R.R.S.; Data curation, Writing, and Supervision. J.G., F.M., M.A.; Visualization and Investigation. M.I.; Software and Validation. B.A., M.D.A., S.M.S.; Were responsible for overall supervision, study design, and data analysis. R.R.R.S.; Drafted the manuscript, which was revised by B.A. All authors read and approved the final manuscript.

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