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ORIGINAL ARTICLE

Cumulus co-culture system does not improve the in-vitro maturation (IVM) of oocytes in mice

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KEYWORDS

In vitro maturation;
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Abstract *Objective:* To examine the effect of co-culture system with CCs on the in vitro maturation (IVM) of immature oocytes retrieved from mice.
Design: This is an experimental study. *Setting:* Research and Clinical Center for Infertility of Yazd.
Sample: A total of 144 oocytes from 5 to 6 weeks mice.
Material: After dissecting ovaries, denuded GVs (DGVs) were considered as control group and put in IVM medium. In cumulus oocyte complexes (COCs) as exp I, intact COCs were put in IVM medium and observed for maturation after 24 h and 48 h. In exp II, GVs were co-cultured with mature CCs. For exp II, COCs were incubated for 30 min before removing oocytes. Then, denuded mature oocytes were removed and the remaining CCs were used to culture denuded GVs recovered from ovaries.
Main outcome measures: Maturation rates of oocytes after 24 h and 48 h.
Results: The rate of oocyte maturation was 77.36 ± 14.4 in control group. In exp I and exp II, the rates of maturation were 91.32 ± 22.5 and 63.33 ± 7.4, respectively ($P = 0.04$). However, maturation rate did not increase after 24 h. The maturation rate in metaphase I (MI) oocytes was as low as 15% in different groups at 24 h and did not increase by 48 h. Degeneration rate was increased from time 0 to 48 h, but not significant in different times.
Conclusion: Presence of intact COCs significantly improved the oocyte maturation rates in animal model. However, co-culture of GVs with mature CCs did not improve IVM program in mice.

1. Introduction

The oocyte is coupled with cumulus cells (CCs) via gap junctions that allow inorganic ions, second messengers and metabolites to pass from cell to cell. This allows signal transmission to regulate development of oocytes from MI to MII stage and early embryonic development (1). Process by which follicular somatic cell secretions influence complete oocyte maturation is due to maintaining a functional intercellular coupling between CCs and the oocyte (2). The CCs are also

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important during the process of fertilization and sperm–oocyte binding and play an important role in preventing polyspermy (3). Also, CCs may neutralize the damaging processes that reactive oxygen species (ROS) may exert on oocytes during the course of maturation (4). Acquisition of developmental competency in cumulus oocyte complexes (COCs) is derived from endocrine hormones and oocyte secreted factors (5). Acting together, bone marrow promotion (BMP15) and growth differentiation factor (GDF9) function at different stages of follicular development promote normal CCs expansion (6). CCs also suppress free intracellular zinc in oocyte through the production of a paracrine factor (7). Moreover, the beneficial effect of prolactin (PRL) on the oocyte developmental capacity is achieved via CCs containing receptors of PRL (8). Expansion of COCs is dependent upon two signaling events; stimulation by gonadotropins or epidermal growth factors (EGFs) and paracrine signals secreted by the oocyte, which act on neighboring CCs, enabling them to respond (9).

In terms of in vitro maturation (IVM), co-culture system is an alternative method used to improve in vitro development of oocyte and embryo (10). Many factors influence the karyoplasmic and cytoplasmic maturation of oocytes in vitro, including co-culturing with follicular cells, such as CCs, duration of maturation and type of IVM media (11). Some studies reported comparable rates of maturation to MII and similar organization of spindles and chromatin between oocytes matured in microdrops or in a co-culture system. Culture conditions, including formulation of the base medium, supplementations, and in-vitro physical environment, such as the oxygen tension and presence of CCs influence events that are paramount to oocyte maturation and subsequent embryonic development. Although, a variety of culture media have been utilized for IVM, no single system has been shown to be superior for the production of developmentally competent oocytes (12). The objective was, therefore, to examine the effect of co-culture system using mature CCs on the IVM of mouse oocytes.

2. Materials and methods

This experimental study was approved by ethics committee of Shahid Sadoughi of Medical Sciences. Study was done in three groups of control and two experimental groups (exp I and exp II) (Fig. 1). In control group, 5–6 week old BALB/c strain female mice were killed by cervical dislocation at 46 h after 5 IU hCG (BioScience GmbH, Germany) administration. Then, the ovary was dissected to release immature follicles. Oocytes were pipetted into hyaluronidase drops (Sigma Co, St Louis, USA). Denuded GVs (DGVs) were put in IVM medium and observed for maturation after 24 h and 48 h.

In exp I, Only COCs (GVs with intact immature CCs) with >3 layers of unexpanded CCs and containing oocytes >70 mm in diameter were chosen. The intact COCs were put in IVM medium and observed for maturation after 24 and 48 h. In exp II as GVs co-cultured with mature CCs, 6–8 weeks old mice were superovulated with pregnant mare serum gonadotropin (PMSG, 10 IU.IP, Lab HIPRA, SA, Spain) and human chorionic gonadotropin (hCG, 10 IU, IP) 48 h later. The mice were killed, and ampulla was ruptured to release COCs in IVM drops. COCs were incubated for 30 min before removing oocytes. Then, denuded mature oocytes were removed and the remaining CCs were used to culture denuded GVs recovered before. Maturation was observed 24 h and 48 h later. All culturing drops were 25 µl at 37 °C in

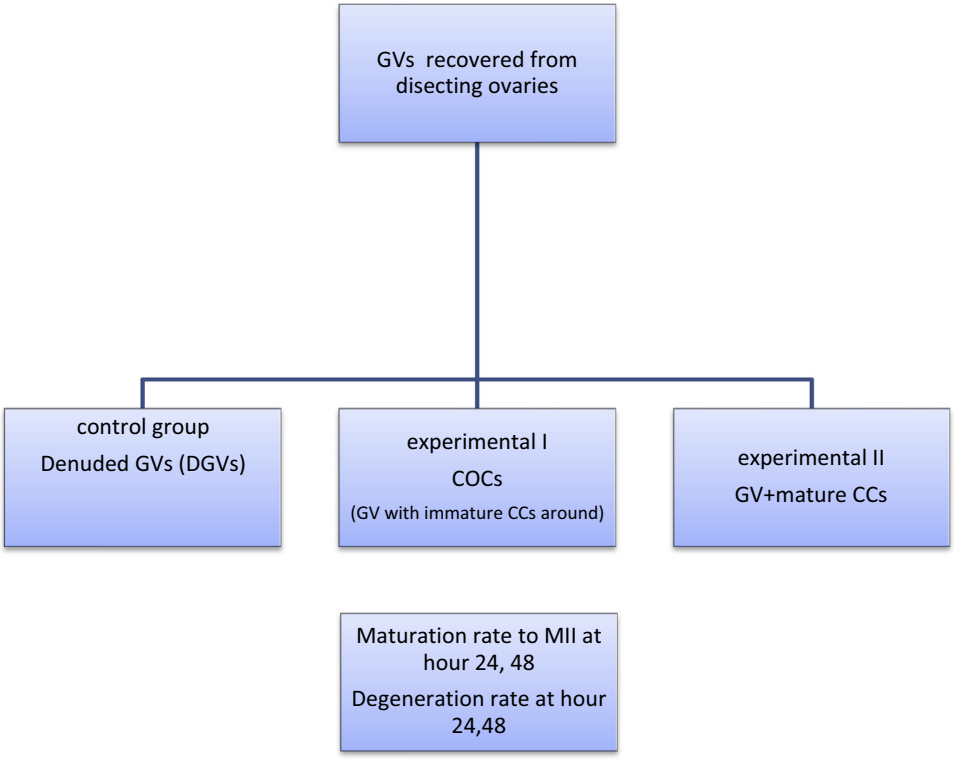

Figure 1 Flowchart of three different IVM groups.

incubator with 5% CO_2 and high humidity. Oocyte maturation was assessed by the presence of the first polar body under inverted microscopes (Olympus), SDF PLAPO 1XPF and CKX 41.

2.1. IVM culture medium

Denuded oocytes were washed in 3 drops of culture medium, then cultured in IVM medium consisting of: Ham's F10 (Biochrom Co, Germany) supplemented with 0.75 IU LH, 0.75 IU FSH (Menogon, FERRING, Germany) and 40% follicular fluid (FF) (13). Briefly, FF was centrifuged at 300 g for 10 min to remove blood and granulosa cells. After inactivation at 56 °C for 30 min, the clear FF was filtered with 0.22 μm filter and stored at −20 °C before use.

2.2. Statistical analysis

The total estimated sample size in different groups was $n = 144$ (3). Statistical analysis was performed using the SPSS (SPSS 18.0, Chicago, USA) software. Data were analyzed using the descriptive test to show Comparison between different groups regarding time variables (Table 1) and one-way ANOVA test to compare different variables in three groups (Table 2). P value of <0.05 was considered significant.

Table 2 Maturation rates of oocytes in different groups.

	Mean ± SD (%)	F	p value
Maturation rate to MII at hour 24			
Control	77.36 ± 14.4	3.935	.044[a]
Exp I	91.32 ± 22.5		
Exp II	63.33 ± 7.4		
Maturation rate to MII at hour 48			
Control	77.36 ± 14.4	2.532	.115
Exp I	82.50 ± 21.6		
Exp II	60.88 ± 9.6		
Degeneration rate in hour 24			
Control	176.66 ± 32.4	2.322	.140
Exp I	191.66 ± 20.4		
Exp II	225.00 ± 50.0		
Degeneration rate in hour 48			
Control	283.33 ± 110.5	.032	.968
Exp I	283.33 ± 93.0		
Exp II	300.00 ± 141.4		

ANOVA test was done followed by Scheffe, df total = 16. $F = 3.9$.
p-value of <0.05 was considered statistically significant.
Control group: Denuded GVs in IVM medium.
Exp I: COCs (GV with immature CCs around).
Exp II: GV plus mature CCs.
[a] Difference between COCs and mature cell was significant.

3. Results

The number of oocytes ($n = 144$) in different developmental stages is shown in Table 1. The presence of oocytes was comparable in different study groups. In control group, the rate of maturation was 77.36 ± 14.4. In exp I, the rate of maturation noticeably increased to 91.32 ± 22.5. However, the rate of maturation in exp II was as low as 63.33 ± 7.4. Differences in maturation rates were significant between two groups of exp I and exp II ($P = 0.04$) at 24 h (Table 2). The number of MI oocytes was the same at 24 and 48 h in aforementioned groups. However, maturation rate did not increase after 24 h, even the number of MIIs decreased because of degeneration which was insignificant among the groups. The average of maturation rate in MI was as low as 15% in different groups at 24 h, which did not increase by 48 h. Degeneration rate was increased from time 0 to 48 h, but not significant at different times (Table 1). Numbers of normal MII oocytes were 58%, 50% and 45% in control, exp I and exp II groups, respectively.

4. Discussion

In exp I, when COCs as GVs containing immature CCs around were matured, the rate of maturation was more than the other two groups at 24 h. Maturation rate was not significantly different between exp I and control groups, but this rate was significantly higher in exp I comparing to exp II. Haberle et al. showed that out of 131 GV oocytes, 64.9% were matured in co-culture group and 73.8% in the control group (14). In another study, the maturation rates (77.4–88.3%) did not differ among different culture systems (15). In Song and Lee study, proportion of MII oocytes was increased by co-culturing compared to control oocytes (98% vs. 94%). Upon 24 h of IVM culture, final maturation rates were equal among the experimental groups (11). Our findings showed maturation rate did not increase after 24 h. No difference was observed during the subsequent IVM culture, up to 48 h (4). However, Song and Lee reported that nuclear maturation of oocytes matured for 39 or 42 h was higher than oocytes matured for

Table 1 The number of oocytes in different developmental stages in different groups.

	GV (N)			P value	M I (N)			P value	M II (N)			P value	Degeneration (N)			P value
	Control	Exp I	Exp II		Control	Exp I	Exp II		Control	Exp I	Exp II		Control	Exp I	Exp II	
0 h	47	46	49	.490	15	25	17	.118	0	0	0	–	9	9	6	.800
24 h	2	0	4	.173	18	30	26	.194	35	38	31	.861	17	17	14	.998
48 h	0	0	0	–	15	27	20	.111	35	34	30	.943	24	24	20	1.000

*ANOVA test was done followed by Scheffe.
p-value of <0.05 was considered statistically significant.
Control group: Denuded GVs.
Exp I: COCs (GV with intact immature CCs).
Exp II: GV co-cultured with mature CCs.

36 h (96% vs. 79%) (11). Promoting the developmental potential of oocytes was mentioned by increased CCs layers, suggesting that cumulus layer removal does not affect oocyte maturation potential. However, this decrease was not statistically different and partial removal of CCs layers from COCs significantly decreased the rates of fertilization and embryo development compared with COCs (3). The data showed that the rate of maturation was significantly more in exp I comparing to exp II, in which the presence of mature CCs could not promote IVM condition. In another approach, an interaction has been observed between denuded oocytes co-cultured with COCs. In one model, there was benefit to meiotic maturation and fertilization of denuded oocytes during co-culture with intact COCs in the same micro-drop. Spontaneous meiotic resumption was inhibited in the absence of FSH/LH during IVM by CCs removal or disruption of cumulus-oocyte communications. Although, gonadotropins could stimulate the denuded oocytes or the disrupted COCs to resume meiosis, maturation beyond the MI stage could not be achieved. Also, denuded oocytes co-cultured with CCs can partially recover meiotic and developmental competence as revealed in human, mouse, and cow. However, results showed there was no advantage to the cat COCs in being co-cultured with denuded oocytes (16). This was not in agreement with previous study revealing that oocytes could appear to be secreting beneficial factors even without CCs (17). Use of COCs as co-culture could promote fertilization and cleavage rates. The number of normal MII oocytes was found to be decreasing in exp II comparing to exp I and control group. Developmental potential correlates highly between COCs morphology. Studies also showed that COCs with good morphology tended to develop better in vitro (18).

Developing culture systems that support full oocyte development and differentiation would require defining the conditions for producing oocytes that express RNA and protein patterns close to oocytes grown in vivo (19). CCs may play the protective role by reducing cystine to cysteine and promoting the uptake of cysteine in the oocyte is another possibility to support their results (11). In addition, the overall effects of midkine (MK), a heparin-binding growth differentiation factor, are mediated by CCs during IVM on each developmental rate. MK may promote the production of such factors in part by its anti-apoptotic effects on CCs. Besides, COCs freshly isolated from ovarian follicles could interact with EGF to induce pulsatile efflux of Ca^2 from the complexes, but not from isolated oocytes (1). Compounds such as glutathione, a major non-protein sulfhydryl compound present in cells such as COCs, play an important role in protecting cells against ROS (20). CCs secret hyaluronan synthase 2 (HAS2) that is an enzyme required for the production of hyaluronic acid and fundamental component of the cumulus matrix (9). The role of cGMP-specific phosphodiesterase in GV oocytes has been prevented by removal of CC and proposed in the resumption of meiosis (21).

Zinc homeostasis is another process regulated in oocytes by CCs. Moreover, activation of EGF signaling abolishes the ability of CCs to suppress free intracellular zinc, thereby allowing levels of zinc to increase in the oocyte during maturation. Co-culture of denuded mature oocytes with COC lowers free intracellular zinc in mature oocytes. Thus, when connection is disrupted with increasing age or by removal of CCs, regulating oocyte zinc homeostasis has been damaged (7). Oocyte maturation could be activated in cultured COCs, but not in denuded oocytes. Also, embryonic development of IVM oocytes could be affected neither by cumulus nor by fibroblast co-culture. As oocytes need to reach their competence to achieve both nuclear and cytoplasmic maturation to support further development, co-culture system could not induce cytoplasmic maturation in oocytes. It could be probably due to decreased potential of co-culture system during long culturing period (10). In contrast, some data showed that co-culture with a monolayer of CC could enhance the development of porcine denuded oocyte after fertilization (22). Some findings have stressed the role of the oocyte by demonstrating that expansion of the CCs depends on the presence of an intact oocyte. These intercellular connections were not formed with cells of CCs not containing an oocyte, indicating that the proximity of an intact oocyte is essential for normal CCs function. The low developmental capacity of IVM oocytes may be caused by the lack of support by surrounding and nursing CCs (23).

In conclusion, presence of co-culture system with mature CCs did not improve maturation in denuded GV oocytes. However, the presence of intact COCs (exp I) significantly improved the oocyte maturation rates in animal model.

Disclosure of interest

All investigators disclose no conflict of interest in this study.

Details of ethics approval and funding

This study was approved by ethics committee of Shahid Sadoughi of Medical Sciences. Shahid Sadoughi University of Medical Sciences funded this study.

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