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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

The effect of co-culturing denuded oocytes on the *in vitro* maturation of bovine cumulus-oocyte complexes

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Abstract

The paracrine effects of oocyte secretions on the developmental competence of oocyte are still under investigation. Oocyte-secreted factors (OSFs) seem to be involved in the maturation of bovine oocytes. Therefore, this study was conducted to investigate the effects of different concentrations of endogenous OSFs on the *in vitro* maturation of bovine cumulus-oocyte complexes (COCs). For this purpose, COCs (n=1211) were aspirated from antral follicles of ovaries collected from a local abattoir. Only oocytes surrounded by complete and compact layers of cumulus cells with homogenous ooplasm were defined as well-qualified COCs (n=353), whereas, other COCs (n=858) with low quality were treated to obtain denuded oocytes (DOs). The selected COCs were randomly cultured in four groups: I) COCs cultured alone as control; II) COCs co-cultured with DOs as 1:1 ratio; III) COCs co-cultured with DOs as 1:3 ratio, and IV) COCs co-cultured with DOs as 1:6 ratio. After maturation, the two variables of cumulus expansion and nuclear status were assessed. The complete cumulus expansion rate was 80% for the control group, and 75.27%, 72.63% and 76.25% for groups of II, III, and IV, respectively. The percentage of metaphase II (MII) stage oocytes was 65.71% for the control group, and 62.67%, 64.86% and 67.60%, for groups II, III, and IV, respectively. Data analysis revealed that the rate of complete expansion of cumulus cells and the development of MII stage oocytes in experimental groups did not differ significantly as compared to the control group. In conclusion, the different concentration of native OSFs does not affect the rate of nuclear maturation and cumulus expansion of bovine COCs. However, group size of co-cultured COCs can be of important criterion for co-culturing systems.

Keywords: Oocyte-secreted factors; bovine, *In vitro* maturation; cumulus expansion; Nuclear maturation

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Introduction

The first step towards a successful *in vitro* embryo production is oocyte maturation, when oocytes undergo cytoplasmic and molecular maturation to develop fertilization capacity (Sirard et al., 2006; Gilchrist and Thompson, 2007). Since *in vitro* maturation (IVM) is not an efficient process, many attempts are undertaken to identify optimal culture conditions to improve maturation rates and oocyte competence.

In vitro maturation of oocytes is influenced by many different factors such as oocyte–somatic cell interactions and bidirectional paracrine signaling (Albertini et al., 2001; Erickson and Shimasaki, 2001; Gilchrist and Thompson, 2007). The somatic cells of the follicle, particularly the cumulus cells (CCs), play a key role in the development of oocyte developmental competence (Gilchrist et al., 2004).

Oocyte-secreted factors (OSFs) may also have an important effect on the regulation of oocyte microenvironment (Erickson and Shimasaki, 2000; Gilchrist and Thompson, 2007). Promotion of cellular growth (Eppig et al., 2002), modulation of steroidogenesis (Vanderhyden et al., 1993), regulation

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of CC expansion (Dragovic et al., 2005) and CC metabolism (Sutton et al., 2003) are all regulated by OSFs. OSFs also enhance oocyte developmental competence and improve embryo quality by increasing the total number and trophectodermal cells in blastocysts (Hussein et al., 2006; Yeo et al., 2008; Dey et al., 2012). Co-culturing denuded oocytes (DOs) with intact cumulus-oocyte complexes (COCs) during both IVM and *in vitro* fertilization (IVF) restore their complete developmental competence of oocytes (Luciano et al., 2005). Competence of goat small follicles-derived COCs can be improved by their co-culture with denuded oocytes (Romaguera et al., 2010).

These mentioned studies showed that OSFs were involved in CC function and developmental capacity of oocyte. However, for *in vitro*, the ratio of DOs to COCs is still unknown. In addition, participation of OSFs in the regulation of CC expansion in different species is not clear (Gilchrist and Thompson, 2007). This study was designed to identify the effects of different concentrations of endogenous OSFs (produced by DOs) on the rate of cumulus expansion and nuclear maturation of bovine oocytes *in vitro*.

Materials and Methods

Unless mentioned otherwise, all the culture media and chemicals were purchased from Sigma (USA).

HEPES-buffered tissue culture medium 199 (TCM-199), as aspiration medium was supplemented with 2% heat inactivated foetal bovine serum (FBS, from GIBCO), 50 µl/ml heparin, 100 IU/ml penicillin and 100 µg/ml streptomycin. Washing medium was prepared the same as the aspiration medium but without heparin. Oocyte culture medium (OCM) was prepared as bicarbonate-buffered TCM 199 with L-glutamine supplemented with 10% FBS, 1 IU/ml rhFSH (Gonal®-Merck, Serono, Germany), 1 IU/ml hCG (Pregnyl®), 1µg/ml estradiol, 0.5 mM Na-pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin sulphate. All prepared media were filtered through a 0.22 µm filter and OCM was incubated at 38.5°C under a humidified atmosphere of 5% CO_2 in air for 1 h prior to use.

Ovaries were collected from Holstein dairy cows (n=250) at the local abattoir in Mashhad, Iran, and immediately transported to the laboratory in a thermos flask containing sterile phosphate buffered saline (PBS) at 30–35°C. Then, the ovaries were washed in fresh PBS containing penicillin (100 IU/ml) and streptomycin (100 µg/ml). COCs were isolated from antral follicles with diameters of 2–6 mm by a suction unit through an 18-gauge needle connected to a bottle containing 5 ml of aspiration medium. After sedimentation, only oocytes surrounded by complete and compact layers of CCs with homogenous ooplasm were selected for IVM. The selected COCs were washed three times in the washing medium. COCs of low quality were vortexed in hyaluronidase (0.1%) to obtain DOs.

Selection and washing of COCs were carried out in the laboratory at 37.5°C on a warm stage. The COCs were randomly placed in droplets of OCM (5 oocytes in each droplet) in a 35mm petri dish and droplets were covered by sterile mineral oil. Oocytes were incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air for 24 h.

Experimental design

Culture groups were prepared as described by Hussein et al. (2006) and Dey et al. (2012). The

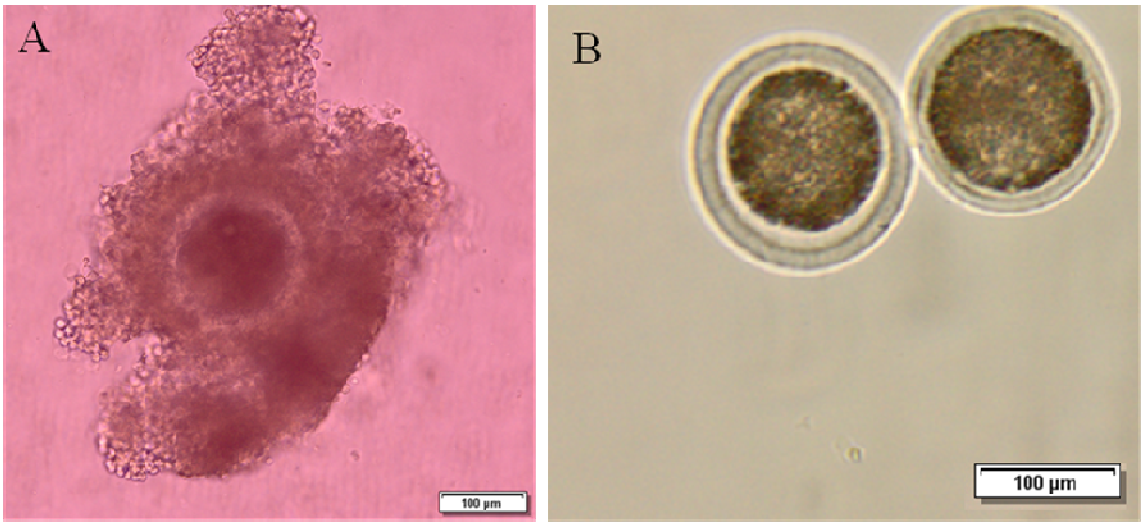

Fig. 1: A: A good selected cumulus-oocyte complex (COC) for culture (magnification 10x). B: Prepared denuded oocytes (DOs) which were used for co-culturing with COCs (magnification 10x).

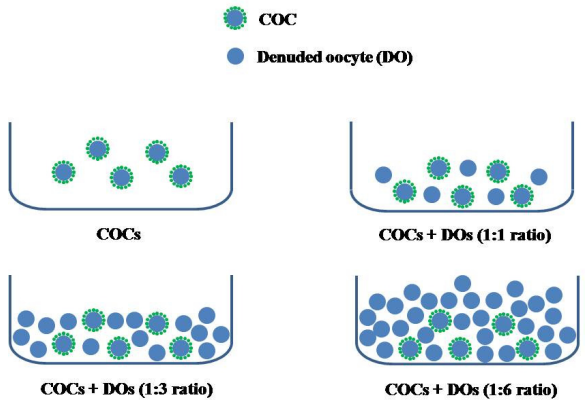

Fig. 2: Schematic illustration of culture groups for culture of COCs with denuded oocytes during IVM.

selected COCs (n=353, Fig.1A) used for co-culturing with DOs (n=858, Fig.1B) were randomly allocated to four groups; control group (I) contained COCs which were cultured in OCM in the absence of DOs, group II contained COCs which were co-cultured with DOs in OCM with a ratio of 1 DO to 1 COC; group III contained COCs which were co-cultured with DOs in OCM in 1:3 ratio; and group IV contained COCs which were co-cultured with DOs in OCM in a 1:6 ratio. Five COCs were placed in each 50µl droplet and the required number of DOs was added as illustrated in Fig. 2. Duration and conditions of incubation were same in all groups (incubated at 38.5°C under a humidified atmosphere of 5% CO_2 in air for 24 h).

Assessment of maturation

At the end of the culture period, a few criteria were considered to evaluate the cumulus expansion and to determine the stage of meiosis. For cumulus cells expansion, total expansion was defined as the expansion of all layers of CCs. Partial expansion was defined as the expansion of only the outer layers of CCs. To determine the nuclear status, the oocytes were denuded from CCs by treating with hyaluronidase (0.01% in PBS) for 5 minutes and by pipetting. Subsequently, the oocytes were fixed by paraformaldehyde (4% in PBS) for 15 minutes. After fixation, the oocytes were stained with diamidino-2-phenylindole (DAPI) by incubating in 20 µl droplets of the DAPI solution and were examined under a fluorescence microscope (Olympus BX51, Germany) to evaluate nuclear maturation. Metaphase II (MII) oocytes were considered as mature oocytes, and metaphase I (MI) and germinal vesicle (GV) oocytes were categorized as immature oocytes.

Statistics

The data regarding the effects of increasing the number of DOs on oocyte nuclear maturation and cumulus cells expansion were evaluated by Kruskal–Wallis using SPSS version 16. Data are presented as mean ± SEM for five replicates, and $P<0.05$ was considered significant.

Results

Status of cumulus expansion

After 24 hours of culture (maturation), the expansion of cumulus cells was assessed. The percentage of oocytes with different levels of expansion is presented in Table 1. It was observed that the fully expanded degree of cumulus expansion in experimental groups was lower as compared to control group but this difference was not significant statistically ($P>0.05$). Moreover, the degree of partially expanded and not expanded cumulus cells did not show any significant difference in all groups.

Status of nuclear maturation

The percentage of oocytes in different stages of meiosis is presented in Table 2. The addition of DOs with 1:6 ratios in co-culturing with COCs improved the development of oocytes to MII stage (complete nuclear maturation). However, the statistical analysis did not reveal any significant differences among groups ($P>0.05$).

Discussion

Some studies have shown that exogenous or endogenous OSFs, especially growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) could improve oocyte competence, blastocyst quality and fertility (Gilchrist et al., 2004; Juengel et al., 2004; Su et al., 2004; Hussein et al., 2006). It is well documented that presence of GDF9 and BMP15 is obligatory for the proper folliculogenesis and fertility in female mice (Erickson and Shimasaki, 2000; McNatty et al., 2004). Nonetheless, it seems that GDF9 does not play a role in competence improvement of goat small follicles-derived COCs (Romaguera et al., 2010). Dey et al. (2012) reported that co-culturing bovine DOs and COCs (1:5 ratio) improved nuclear maturation and blastocyst development of both DOs and COCs, however, the effects of OSFs as well as their concentration on cumulus expansion and nuclear maturation are still debate. Therefore, the present results provided evidence that the different concentration of endogenous (native) OSFs did not affect the rate of cumulus expansion of COCs and this is in agreement with Ralph et al. (1995). It also did not improve nuclear maturation of bovine COCs under *in vitro* condition, which is in contrast to Dey et al. (2012).

Table 1: Effect of increasing number of co-cultured DOs on the cumulus expansion of COCs

Groups	Number of COCs	Degree of cumulus expansion (%)		
		Fully expanded	Partially expanded	Not expanded
Group I	85	80.00 ± 2.68	16.47 ± 1.86	3.53 ± 1.98
Group II	93	75.27 ± 1.68	16.13 ± 2.12	8.60 ± 2.28
Group III	95	72.63 ± 2.22	14.74 ± 1.94	12.63 ± 2.32
Group IV	80	76.25 ± 1.88	8.75 ± 2.91	15.00 ± 1.67

Differences among different groups were not significant (P>0.05). Values are presented as mean ± SEM of five replicates.
Group I: Control (only COCs); Group II: COCs + DOs (1:1 Ratio); Group III: COCs + DOs (1:3 Ratio); Group IV: COCs + DOs (1:6 Ratio)

Table 2: Effect of increasing number of co-cultured DOs on nuclear maturation of COCs

Groups	Number of stained oocytes	Stage of nuclear maturation (%)		
		MΠ	MI	GV
Group I	70	65.71 ± 2.35	20.00 ± 1.09	14.29 ± 2.21
Group II	75	62.67 ± 1.97	17.33 ± 1.64	20.00 ± 1.58
Group III	74	64.86 ± 2.41	10.81 ± 1.84	24.33 ± 1.26
Group IV	71	67.60 ± 1.01	11.27 ± 2.28	21.13 ± 2.10

Differences among groups were not significant (P>0.05). Values are presented as mean ± SEM of five replicates. MII: Meiosis II; MI: Meiosis I; GV: Germinal vesicle.
Group I: Control (only COCs); Group II: COCs + DOs (1:1 Ratio); Group III: COCs + DOs (1:3 Ratio); Group IV: COCs + DOs (1:6 Ratio)

It is known that maturation of the oocyte and the acquisition of oocyte developmental competence are essentially dependent on the oocyte-cumulus association (Gilchrist and Thompson, 2007). Gap junctions, paracrine signaling (Gilchrist et al., 2004) or diffusible factors (Luciano et al., 2005) are the communication channels between oocyte and CCs. Cumulus cells as well as oocytes secrete some factor (s) play a crucial role during oocyte maturation (Tanghe et al., 2002; Gilchrist et al., 2004; Gilchrist and Thompson, 2007). Moreover, only intact COCs can improve the developmental capacity of DOs, indicating the importance of oocyte in supporting CCs (Luciano et al., 2005). There is an absolute requirement for an oocyte-secreted factor(s) to expand CCs in mouse (Buccione et al., 1990) presumably by formation and stabilization of the mucoid matrix molecules (Gilchrist et al., 2004). Dragovic et al. (2005) suggested that both GDF9 and BMP15 secreted from oocytes were involved in mouse CC expansion. In this study, however, the different concentration of native OSFs did not affect the rate of bovine cumulus expansion. It has been reported that bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor (Ralph et al., 1995). This observation may support our result and explain why native OSFs did not alter the rate of bovine cumulus expansion. Moreover, it has been indicated that bovine oocyte conditioned medium enables FSH-induced expansion of mouse complexes (Sirard et al., 2006). Thus, it can be deduced that bovine oocytes secrete the factor (s) which are involved in cumulus expansion, while they are not obligatory for expansion of its own COCs.

The molecular mechanism for oocyte nuclear maturation includes several pathways such as alteration of protein phosphorylation, cyclic adenosine monophosphate (cAMP) and calcium levels. It is well established that a protein called maturation promoting factor (MPF) is responsible for the onset of oocyte maturation (van den Hurk and Zhao, 2005). In addition, intra-oocyte cAMP levels play an important role in the maintenance of meiotic arrest at diplotene (GV) stage in mouse oocyte (Mehlmann 2005; van den Hurk and Zhao, 2005). However, results obtained in cattle (Sirard et al., 1992) have indicated that cAMP accumulation in the oocyte might not be the principal physiological pathway to maintain the meiotic arrest. These data suggests that some different molecular mechanisms may be involved in nuclear maturation in various species. In this study, the results showed that different concentrations of bovine OSFs did not affect the progression of meiosis from GV to MII phase *in vitro* which was in contrast to results of Dey et al. (2012) who used 12 DOs with 60 COCs (total of 72) in a 1 to 5 ratio and observed the improvement of nuclear maturation. Moreover, O'Doherty et al. (1997) showed that cultured oocytes in groups had superior developmental capacity and the group size also improved the success of their *in vitro* maturation, fertilization and culture. The inconsistencies within those results might be related to co-culturing ratio or the size of cultured oocytes.

Hussein et al. (2006) reported that the capacity of IVM oocytes to proceed to the blastocyst stage improved by treating COCs during IVM with natural or recombinant OSFs. Developmental competence is mainly acquired during oocyte growth by an unknown mechanism (Sirard et al., 2006). It has been suggested that specific instructing and molecular maturation factors are essential to promote the development of

oocyte to the blastocyst stage (Sirard et al., 2006). Su et al. (2004) showed that spontaneous *in vitro* oocyte maturation occurred normally in double mutant (GDF9 and BMP15) mouse, but oocyte fertilization and preimplantation embryogenesis were significantly decreased. It is likely that bovine OSFs affect molecular maturation aspects of oocyte maturation rather than cumulus expansion and even nuclear maturation, and hence influence oocyte competence for embryo development.

In conclusion, based on our results it seems that the different concentration of native OSFs does not affect the bovine COCs quality regarding cumulus expansion and meiotic progression *in vitro*. However, group size of co-cultured COCs can be of important criterion for co-culturing systems.

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