

Co-culture embedded in cumulus clumps promotes maturation of denuded oocytes and reconstructs gap junctions between oocytes and cumulus cells

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Summary

The present study was undertaken to establish an effective method for *in vitro* maturation (IVM) of denuded oocytes (DOs) by simulating the ovarian three-dimensional status *in vivo* using buffalo ovarian tissues or cumulus cells, so as to provide a model for investigating the mechanisms of oocyte maturation. Buffalo cumulus–oocyte complexes from ovaries taken at slaughter were denuded by pipetting, and then allocated randomly into four groups for IVM by direct culture in maturation medium (M1, control group), co-culture with a monolayer of cumulus cells (M2), embedded in cumulus cell clumps (M3) and ovarian tissue (M4) for 24 h. The nuclear maturation of DOs was assessed by the extrusion of the first polar body and the cytoplasmic maturation was evaluated by subsequently developmental capacity after parthenogenetic activation. More DOs matured to MII (56.89%) and developed to blastocysts (25.75%) when they were matured *in vitro* with M3 in comparison with DOs matured *in vitro* with M1 (45.14 and 15.97%) and M4 (40.48 and 13.49%). Further detection of gap junctions by injecting Lucifer yellow directly into cytoplasm of matured DOs with adherent cumulus cells and scanning with confocal microscope showed that Lucifer yellow were found in nine out of 11 the adherent cumulus cells in M3, indicating that the gap junctions between oocytes and cumulus cells was reconstructed *in vitro*. These results indicate that co-culture of DOs embedded in cumulus cell clumps can improve their nuclear and cytoplasmic maturation of DOs, possibly through the reconstruction of gap junctions *in vitro*.

Keywords: Cumulus cells, Denuded oocyte, Gap junction, *In vitro* maturation

Introduction

Oocytes matured *in vivo* are enclosed with cumulus cells (CCs). Removal of CCs before *in vitro* maturation (IVM) has been reported to be detrimental to oocyte maturation in mice (Schroeder & Eppig, 1984), rats

(Vanderhyden & Armstrong, 1989), cattle (Chian *et al.*, 1994; Zhang *et al.*, 1995), bovine (Fatehi *et al.*, 2002, 2005) and pigs (Wongsrikeao *et al.*, 2005). Co-culture with cumulus–oocyte complexes (COCs) or CCs has been found to partially restore the developmental potential of oocytes denuded of CCs (DOs), suggesting that the conventional *in vitro* maturation method is inappropriate for *in vitro* maturation of DOs. It is well known that the physiological role of gap junctions between oocytes and CCs is particularly important for normal cytoplasmic maturation of oocytes because they play essential nurturing roles in the regulation of oocyte metabolism, such as in these of energy sources (Eppig *et al.*, 2005) and the metabolism of maturation-promoting factor (MPF) (Miao *et al.*, 2005). Studies have demonstrated that denuded human oocytes exhibit accelerated meiotic resumption *in vitro*, a deficiency in the ability of the cytoplasm to maintain

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M-phase characteristics while meiosis is progressing, a propensity to activate spontaneously after M-phase arrest, and a lack of coordination between nuclear and cytoplasmic maturation (Combelles *et al.*, 2002). Synthesis of cytoplasmic glutathione by CCs, which plays a crucial role in oocyte maturation and subsequent embryonic development (Maedomari *et al.*, 2007). This effect will be absent if CCs are denuded, resulting in inadequate cytoplasmic maturation of oocytes after *in vitro* maturation, which leads to abnormality in the cell number and Oct-4 expression (Chang *et al.*, 2005), or increases DNA fragmentation in oocytes (Wongsrikeao *et al.*, 2004) during the transition from morula to blastocyst. When oocytes are not supported by CCs, it is easy for the oocytes to adhere to the bottom of the dish, and deformation of oocytes may occur, causing mechanical displacement of organelles in oocytes and injury. Additionally, adherence of oocytes to the bottom of the dish may affect material exchange between oocytes and medium. Therefore, elucidation of the function and mechanisms of CCs and establishment of an effective new method for *in vitro* maturation of DOs has great significances for further improvement of oocyte quality in the future.

It has been reported that three-dimensional *in vivo* growth model has been established using collagen-based demineralized bone matrix (DBM) (Ma *et al.*, 2007) and extracellular matrix (Combelles *et al.*, 2005; Vanhoutte *et al.*, 2009) to simulate the ovarian status *in vivo*. However, these mediators do not provide interdependent common substrates for oocytes growing *in vivo*. The aims of this study were to establish an optimal *in vitro* maturation method for DOs using buffalo ovarian tissues and CCs as mediator to simulate the ovarian status *in vivo*, so as to provide a model to investigate the mechanisms of buffalo oocyte maturation and a technical support for improving human oocyte quality in assisted reproductive technology.

Materials and methods

Collection of oocytes

Buffalo ovaries were collected from a local abattoir. Ovaries were excised within 20 to 30 min of slaughter and transported to the laboratory within 4 h in a thermos containing phosphate-buffered saline (PBS) at 35–37°C. Buffalo COCs were recovered by aspiration of buffalo follicles (diameter 2–6 mm) using a 10-ml disposable syringe with an 18-gauge needle. The COCs were collected under a stereomicroscope and the surrounding CCs were removed by manual pipetting to prepare DOs for subsequent *in vitro* maturation (IVM) experiments.

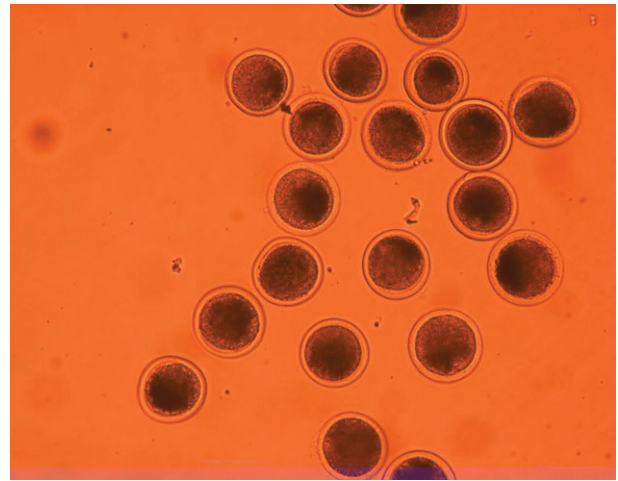


Figure 1 Direct culture of buffalo denuded oocytes in the maturation medium.

In vitro maturation of buffalo DOs

The maturation medium for buffalo DOs was TCM-199 (Gibco BRL, Paisley, Scotland, UK) supplemented with 26.2 mM NaHCO₃, 5 mM HEPES, 5% estrous cow serum (OCS, self preparation), 2% bovine follicular fluid (BFF, collected without regard to the stage of the reproductive cycle) and 0.1 µg/ml FSH (follicle stimulating hormone). The buffalo DOs were randomly divided into four groups and placed in a glass culture dish containing 1.5 ml maturation medium for IVM using following four culture systems respectively. The culture density was approximate 25 DOs per dish and culture condition was 38.5°C and 5% CO₂ in air in a humidified atmosphere.

Direct culture in maturation medium (M1, control group)

The DOs were cultured directly in the maturation medium (Fig. 1).

Co-culture with cumulus cell monolayers (M2)

The surrounding CCs of COCs matured *in vitro* were removed by gentle pipetting and harvested from the maturation medium by centrifugation (5 min at 300 g). After two washes in embryo culture medium (CM, modified TCM199 + 5% OCS), CCs were placed onto culture in a 30 mm glass culture dish at a density of $1-2 \times 10^6$ /ml. The cumulus cell monolayers were formed (Fig. 2) after culture of 48–72 h in an incubator with 5% CO₂, and then buffalo DOs were introduced to the culture dish with maturation medium.

Co-culture embedded in cumulus cell clumps (M3)

Fully expanded and cotton-like cumulus cell clumps with relatively high viscosity were collected. Then buffalo DOs were placed in the middle of the cumulus

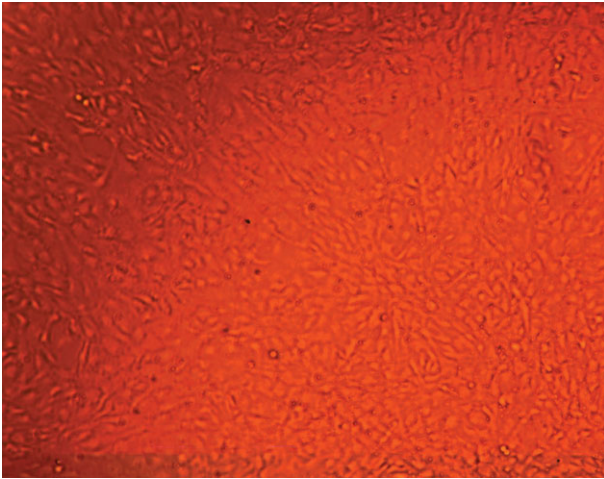


Figure 2 Cumulus cell monolayers.

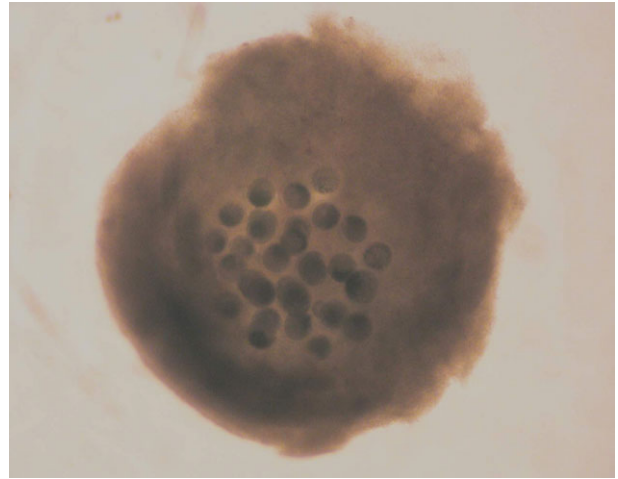


Figure 4 Co-culture embedded in the ovarian tissues.

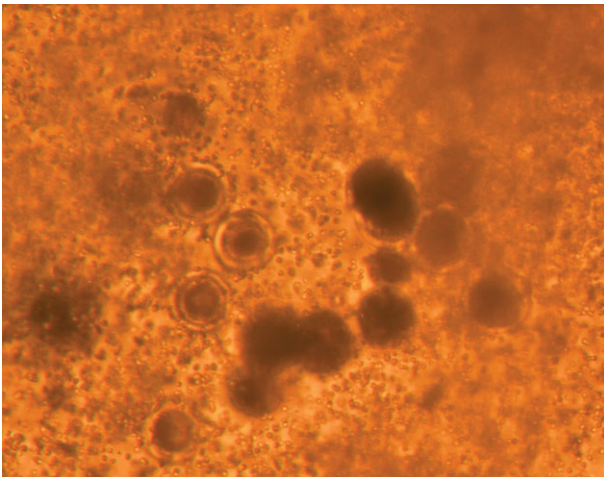


Figure 3 Co-culture embedded in the expanded cumulus cell clumps.

cell clumps and suspended in the maturation medium (Fig. 3).

Co-culture embedded in ovarian tissue (M4)

Boat-shaped thin slices of ovarian tissue were collected during recovery of oocytes. After washing with maturation medium, buffalo DOs were placed into the boat-like cavity of the slices of ovarian tissue for IVM using an ultra-thin pipette (Fig. 4).

Evaluation of nuclear maturation

After IVM for 24 h, oocytes with the first polar body (PB1) were considered to become nuclear maturation.

Evaluation of cytoplasmic maturation

Parthenogenetic activation of oocytes was performed according to the method that had been previously described (Lu *et al.*, 2005). Briefly, after 24 h IVM, the surrounding CCs of oocytes were removed by pipetting in 0.1% hyaluronidase, and the DOs were washed three times with maturation medium. Then, DOs were activated with 5 μ M ionomycin for 5 min and followed by culture in 2 mM 6-dimethylaminopurine (6-DMAP) for 3–4 h. After washing three times with CM, DOs were transferred to the monolayer of granulosa cells for culture *in vitro*.

Detection of gap junctions between oocytes and cumulus cells

Gap junctions between oocytes and CCs were detected using the procedure that has been previously described (Isobe *et al.*, 1998; Isobe and Terada, 2001). Briefly, after 24 h IVM, DOs adhered with relatively more CCs were selected and 4 μ l of 10% (w/v) Lucifer yellow CH (Sigma, St. Louis, MO) was injected into the oocyte cytoplasm via a micromanipulator. The gap junctions between oocytes and CCs were confirmed to be reconstructed if Lucifer yellow dye was observed in the surrounding adherent cumulus cells under the confocal microscopy (LSM 510 Meta, Zeiss) within 10–15 min after injection.

Statistical analysis

All data were analysed using the chi-squared (χ^2) test. Probability values $p < 0.05$ were considered to be significant.

Table 1 Nuclear maturation of buffalo denuded oocytes cultured *in vitro* for 24 h using the four methods

Maturation methods	No. of oocytes	No. of oocytes with release of the first polar body (%)
M1	144	65 (45.14) ^a
M2	92	47 (51.09) ^{a,b}
M3	167	95 (56.89) ^b
M4	126	51 (40.48) ^a

^{a,b}Within columns, values with different superscripts are significantly different ($p < 0.05$).

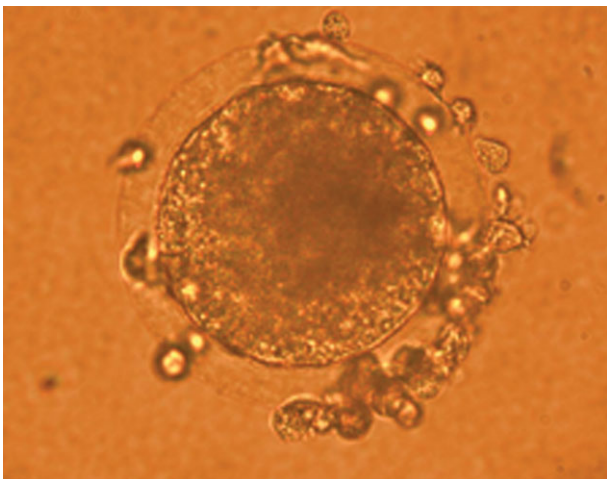


Figure 5 The oocyte after co-culture embedded in the expanded cumulus cell clumps for 24 h.

Results

Morphology of buffalo DOs matured *in vitro* for 24 h with the four methods

The DOs clustered together after 24 h IVM with M1 and M2. The cytoplasm was dark, and some oocytes showed severe deformation. The cytoplasm of oocytes matured *in vitro* with M3 was homogeneous, pale and translucent, and some of oocytes were surrounded or partially surrounded with adherent CCs and released the PB1 (Fig. 5). While the cytoplasm of oocytes matured *in vitro* with M4 was relatively dark, and adherent filamentous substances were observed in the zona pellucida.

Nuclear maturation of DOs matured *in vitro* with the four methods

As shown in Table 1, more oocytes extruded the PB1 when they were cultured *in vitro* for 24 h with M3 (56.89%) in comparison with M1 (45.14%) and M4 (40.48%; $p < 0.05$), indicating that M3 was more suitable for IVM of DOs.

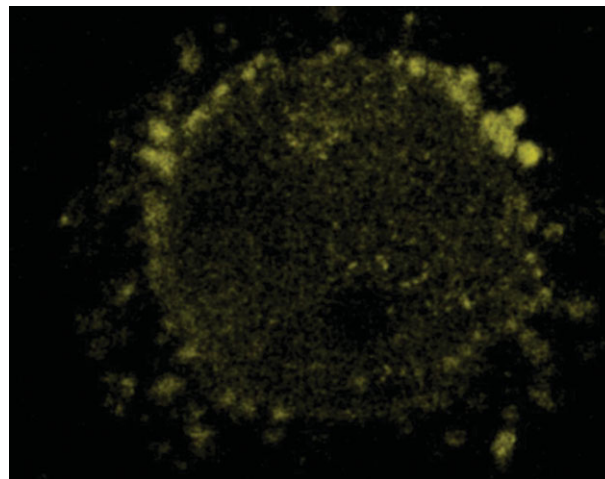


Figure 6 Lucifer yellow was observed in the cumulus cells surrounding oocytes cultured using M3.

Parthenogenetic development of DOs matured *in vitro* with the four methods

As illustrated in Table 2, the cleavage rate of oocytes matured *in vitro* with M3 after parthenogenetic activation (64.07%) was higher than that of oocytes matured *in vitro* with M4 (45.24%, $p < 0.05$), and the blastocyst yield of oocytes matured *in vitro* with M3 (25.75%) was also higher than that of oocytes matured *in vitro* with M1 (15.97%) and M4 (13.49%, $p < 0.05$). However, there was not different in the hatching rate of blastocysts among the four groups ($p > 0.05$).

Reconstruction of gap junctions between oocytes and cumulus cells during IVM

When DOs were cultured with M1, M2 and M4 for 24 h, no adherent CCs were observed around the oocytes. However, more CCs adhered to the zona pellucida of oocytes when DOs were cultured with M3. Injection of Lucifer yellow into the cytoplasm of oocytes matured *in vitro* with M3 indicated that the gap junctions between CCs and oocytes was reconstructed as Lucifer yellow was observed in the surrounding cumulus cells under the confocal microscopy (Fig. 6).

Discussion

It is well known that oocytes need to undergo cytoplasmic maturation as well as nuclear maturation to become able to support successful fertilization and embryo development (Mermillod *et al.*, 1999; Trounson *et al.*, 2001). Oocytes acquire their developmental competence (cytoplasmic maturity) after a long series of preparatory processes that involve transcription

Table 2 Parthenogenetic development of buffalo denuded oocytes matured *in vitro* using the four methods

Maturation method	No. of mature oocytes	Cleavage rate (%)	Blastocyst rate (%)	Hatching rate of blastocysts (%)
M1	144	85 (59.03) ^a	23 (15.97) ^a	17 (73.91)
M2	92	50 (54.35) ^{a,b}	20 (21.74) ^{a,b}	14 (70.00)
M3	167	107 (64.07) ^a	43 (25.75) ^b	31 (72.09)
M4	126	57 (45.24) ^b	17 (13.49) ^a	13 (76.47)

^{a,b}Within columns, values with different superscripts are significantly different ($p < 0.05$).

and subsequent translation of transcripts during the meiotic prophase, in which the cytoplasmic organelles proliferate greatly, RNA levels double, a large number of proteins are synthesized, and energy-rich substrates are accumulated continuously. In the antral ovarian follicle, CCs surround the oocyte, while mural granulosa cells form the follicular wall. The CCs communicate with each other and with the oocyte by means of gap junctions (Cha & Chian, 1998; Mermillod *et al.*, 1999; Hardy *et al.*, 2000). Further studies have demonstrated that cytoplasmic maturation of oocytes requires optimal intracellular cAMP concentrations and meiotic arrest (Vaccari *et al.*, 2008; Norris *et al.*, 2009). Direct communication between oocytes and CCs is very important for maintaining meiotic arrest. This can only be accomplished by the cumulus–oocyte complex, not DOs. In addition, CCs may promote cytoplasmic maturation of oocytes (Li *et al.*, 2000). Therefore, effective communication by gap junction between oocytes and CCs during the entire process is necessary for *in vitro* growth and development of oocytes (Canipari, 2000; Thomas and Vanderhyden, 2006; Orisaka *et al.*, 2009). In view of this requirement, researchers have used collagen-based DBM (Ma *et al.*, 2007) and extracellular matrix (Combelles *et al.*, 2005; Vanhoutte *et al.*, 2009) to establish a three-dimensional structural model successfully for IVM of DOs. However, these mediators are not substrates for intercommunication and interaction with oocytes *in vivo*. Therefore, we used ovarian tissue and CCs as the mediators to simulate the *in vivo* ovarian three-dimensional environment and create a growth pattern of oocytes similar to that of the cumulus–oocyte complex. The three-dimensional structure and morphology of oocytes may be kept intact within a certain period of culture time. In this way, it will be expected that gap junctions between oocytes and CCs may be reconstructed and a new model for study of *in vitro* oocyte maturation will be established.

In this study, we found that DOs adhered to the bottom of culture dish wall and some oocytes showed severe deformation when they were cultured directly. The possible reasons for this may be that oocytes

contact with the bottom of the dish and deform gradually due to gravity when they have lost the three-dimensional support, which will cause mechanical displacement of intracellular organelles and injury. This problem was resolved by employment of cumulus cell blocks and ovarian tissue blocks as support mediators for oocytes during culture in the present study.

Some studies have shown that removal of CCs is deleterious to the maturation of oocytes, while co-culture with cumulus cell monolayers significantly promotes cytoplasmic maturation of oocytes. The possible reasons for these findings are that CCs may regulate MPF activity, maturation and cleavage process (Ge *et al.*, 2008). However, the results of our study showed that the proportion of oocytes extruded with the PB1, cleaved and developing to blastocysts after parthenogenetic activation were not significantly improved when buffalo DOs were co-cultured with granulosa cell monolayers for 24 h. Hashimoto *et al.* (1998) reported that the medium supplemented with CCs could improve the developmental competence of bovine cumulus–oocyte complex, but had no effects on DOs. We speculate that some factors secreted by CCs may act through gap junctions to promote the maturation of oocytes, co-culture with cumulus cell monolayers does not result in the reconstruction of gap junctions between cumulus cells and oocytes.

In the ovarian tissue, the oocyte is surrounded by CCs, granulosa cells, follicle membrane and ovarian tissues, which communicate with each other and with the oocyte to regulate the maturation of oocytes by means of gap junctions (Conti, 2010). However, in the present study, when DOs were cultured by embedded in ovarian tissue *in vitro*, the release rate of the PB1 and the blastocyst rate after parthenogenetic activation were significantly lower than in the control group, indicating that embedded co-culture in ovarian tissue blocks causes inhibition of oocyte maturation rather than promotion. We speculate that ovarian tissue blocks secrete some factors that inhibit oocyte maturation. Within the body, these factors may first enter the parietal granulosa cells and CCs through the follicular membrane and undergo modifications

that they may be processed, and transformed into factors that can promote oocyte maturation. These factors act together with factors secreted by parietal granulosa cells and CCs, thereby regulate oocyte maturation. However, these important intermediary steps are missing when oocytes are directly cultured with ovarian tissue, and then the ovarian factors display inhibitory action on oocyte maturation.

Adequate maturation of the oocyte cytoplasm is necessary to ensure normal fertilization and subsequent development of the oocytes. In the present study, DOs were cultured by embedding in the expanded cumulus cell clumps and found that the release rate of the PB1 was significantly higher than in direct culture, indicating that expanded cumulus cell clumps are beneficial to the nuclear maturation of DOs. Furthermore, parthenogenetic activation revealed that the cleavage rate and blastocyst rate of oocytes were also significantly higher than directly cultured oocytes, suggesting that the expanded cumulus cell clump is also beneficial to the cytoplasmic maturation of DOs. Some studies have indicated that CCs may regulate the pH equilibrium in oocytes through a variety of pathways (Fitzharris & Baltz, 2006), and may antagonize oxidative stress during fertilization and protect oocytes (Fatehi *et al.*, 2005), induce communication between oocytes and the extrafollicular environment or reaction between oocytes and medium, or remove those components in the medium that inhibit embryo development (Fatehi *et al.*, 2002). In addition, it is possible that gap junctions are reconstructed between CCs and oocytes, and thereby maturation-inducing soluble factors secreted by the cumulus cells are transported efficiently to oocytes through these junctions, which will promote oocyte maturation and substrate accumulation for subsequent development. In this study, injection of Lucifer yellow CH into the oocytes co-cultured by embedding in the expanded cumulus cell clumps displayed that Lucifer yellow CH was observed in CCs adherent to oocytes, indicating that gap junctions were reconstructed between cumulus cells and oocytes. Then, maturation-inducing soluble factors secreted by the CCs can be transported efficiently to oocytes through these junctions, and promote oocyte maturation and substance accumulation for subsequent development.

In conclusion, co-culture of DOs embedded in cumulus cell clumps can improve their nuclear and cytoplasmic maturation, possibly through the gap junctions between oocytes and CCs reconstructed *in vitro*. This new model will be useful for studying the mechanisms of oocyte maturation, and fully utilizing the immature oocytes in intracytoplasmic sperm injection (ICSI) in human-assisted reproductive technology.

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