Global Veterinaria 18 (1): 74-79, 2017
ISSN 1992-6197
© IDOSI Publications, 2017
DOI: 10.5829/idosi.gv.2017.74.79

Interaction Between Oocyte Type and Cumulus Cells in Maturation Medium on Developmental Competence of Rabbit Embryos

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Abstract: The current study aimed to investigate the effect of adding cumulus cells separated from the other oocytes (CCs^1) or same oocytes (CCs^2) to maturation medium (MM), on rate of maturation (IVM), *in vitro* fertilization (IVF) and morulae/blastocysts (MOR/BLR) formation, of compact cumulus (COCs), denuded (DOs) and mechanically denuded (MDOs) rabbit oocytes. Oocytes were recovered from 24 mature NZW doe rabbits. The COCs and DOs were *in vitro* matured in MM with or without CCs^2, but only MDOs were matured without or with CCs^1 and CCs^2. Results show that maturation rate (MR) was higher ($P<0.05$) for COCs than DOs or MDOs, regardless adding CCs in MM. Adding CCs^1 to MM improved ($P<0.05$) IVM of DOs and MDOs, but had no effect on COCs. Adding CCs^2 increased ($P<0.05$) IVM of MDOs as compared to DOs and MDOs with CCs^1, but intact COCs still to have higher ($P<0.05$) IVM than MDOs with their CCs^2. Fertilization rate (FR), MOR and BLR were the highest ($P<0.05$) for COCs, moderate for MDOs, while the lowest for DOs. Adding CCs^1 improved FR of matured COCs and MDOs ($P<0.05$) and DOs ($P=0.05$). Adding CCs did not affect MOR and BLR. The FR of MDOs with CCs^1 or CCs^2 increased ($P<0.05$) as compared to without CCs, but FR, MOR and BLR were not affected with CCs^1 or CCs^2. In conclusion, compact oocyte complex reflected the highest rate of maturation, fertilization and morulae/blastocysts formation. Co-culture of denuded or mechanically denuded oocytes required addition of cumulus cells to maturation medium to improve *in vitro* embryo production. Adding cumulus cell of the same collected oocytes have more impact than those of strange oocytes on nuclear maturation.

Key words: Rabbit Oocytes · Cumulus Denudation · Nuclear Maturation · Developmental Competence

INTRODUCTION

Embryo production *in vitro* (EPI) is depending mainly on oocyte maturation as a fundamental process for species livability, during which oocytes acquire their intrinsic capacity to fertilize and develop to live offspring [1].For improving IVM processes, it is important to understand the interactions between follicle cells and oocytes, including intrinsic cumuli quality of oocytes [2], dialog with neighboring somatic compartment [3], presence cumulus cells (Ccs) around the oocytes [4] and functional gap junctions [5].

Oocytes recovered from the antral follicles, including COCs, Dos and partial denuded oocytes are surrounded by CCs. The CCs play important roles in their *in vitro* cytoplasmic and nuclear maturation [6, 7]. Also, physical communication between oocyte and CCs is essential for transferring nutrition, growth and regulation of meiotic progression, as essential factors for development of oocytes [8]. Corona rediata cells, first layer of CCs, penetrate through the zona pellucid and communicate with oocyte through gap junctions (GJ) which are essential for IVM and IVF as reported by Zhou *et al.* [9].

In addition, GJ between the oocyte and the CCs is a useful to investigate CCs function during IVM without any disturbance in penetration ability of oocytes [10]. During IVF, the developmental competence of immature oocytes positively correlated with initial quality and size of CCs, in term of CCs layers number. Although a smaller number of CCs layers is not strictly limiting for oocyte maturation, it unfavourably influences early embryonic development, in comparison with a high quality CCs covering the oocytes [11].

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The role of CCs in MM was studied in mouse by Mahmodi *et al.* [12] to be responsible for IVM, IVF and embryonic development to blastocyte stage. In this respect, CCs remove during IVM or shortly before IVF adversely affect subsequent IVM, IVF and embryonic development of bovine oocytes [13]. In rabbits, COCs showed higher IVM and developmental capacity compared with mechanically denuded cumulus oocytes (MDOs) [14].The possibility of improving IVM, IVF and EPI from denuded oocytes was studied in bovine by Modina *et al.* [15].

Generally, embryonic developmental competence and blastocyst quality of *in vitro* matured oocytes depends on successful rate of nuclear maturation, zona pellucida hardening, poly-spermic zygotes, chromosomal abnormalities and glutathione peroxidase in oocytes [1], but information on the role of CCs in *in vitro* production of rabbit embryos is rare.

Therefore, the current study was designed to investigate the effect of adding CCs, separated from the same oocytes or other oocytes to MM, on rate of IVM, IVF, MOR and BLR formation, of COCs, DOs and MDOs rabbit oocytes.

MATERIALS AND METHODS

All types of chemicals and media were purchased from Sigma-Aldrich Company (St. Louis, MO).

Type of Oocytes: Immature Oocytes for IVM were recovered from mature New Zealand White (NZW) doe rabbits (n=24) weighing 3.5 kg LBW. Doe rabbits were taken from a private rabbit farm to Laboratory of Physiology and Biotechnology, belonging to Department of Animal Production, Faculty of Agriculture Mansoura University.

Does were slaughtered and ovaries were separated, washed by 0.9% NaCl solution and dried. Oocytes were harvested from visible follicles by slicing method in phosphate buffered saline (PBS) supplemented with 10% (v: v) fetal calve serum (FCS), 10 mM of HEPES and gentamycin (50 µg/ml) in tissue culture dishes.

After slicing, oocytes were evaluated under stereomicroscopy according to the intact CCs layers and cytoplasmic homogeny, into COCs, DOs and partial denuded oocytes.

The COCs were mechanically denuded by gentle repeated pipetting in PBS containing hyaluronidase (0.2 mg/ml) solution to remove CCs and obtaining MDOs after the method of Hammad *et al.* [16].

Oocyte Maturation: All types of oocytes (COCs, DOs and MDOs) were rapidly washed 3 times in droplets of tissue culture media (TCM-199) with FCS (10%). The MM included TCM-199 plus bovine serum albumin (BSA, 3 mg/ml), estradiol-17â (1 µg/ml), PMSG (10 IU/ml), hCG (10 IU/ml) and gentamycin (50 µg/ml). Different types of oocytes were *in vitro* matured in MM without or with CCs as the following:

Treat.	Type of oocyte	Supplementation	Oocytes (n) Maturation	Oocytes (n) Fertilization
T1	COCs	Without CCs	95	75
T2	COCs	With CCs of other oocytes (CCs[1])	100	75
T3	DOs	Without CCs	100	75
T4	DOs	With CCs of other oocytes (CCs[1])	85	75
T5	MDOs	Without CCs	70	75
T6	MDOs	With CCs of other oocytes (Ccs[1])	70	75
T7	MDOs	With CCs of same oocytes (Ccs[2])	80	75
		Total	600	525

Each treatment of oocytes was matured in 500 µl of MM under paraffin oil in a petri dish culture and incubated for 18-20 h as maturation period at 37.5 °C under 5% CO_2 in air with maximum relative humidity. Then, oocytes were fixed with acetic alcohol for 24h, stained with orcein (1%) in acetic acid (45%) and examined under inverted microscopy to determine morphological changes in the nucleus or extrusion of first polar body (PB). Percentage of oocytes showing PB (Metaphase II, MII) extrusion was served as nuclear maturation rate.

***In vitro* Fertilization (IVF):** Mature-fertile NZW bucks (n=5) were used for fresh semen collection for achieving IVF in this study. Semen with sperm density of $1x10^6$ sperm/ml was capacitated in HEPES-TALP medium plus BSA (3 mg/ml) and 50 µg/ml gentamycin. Oocytes matured by different types of MM were incubated with capacitated spermatozoa in 50 µl of IVF-TALP containing BSA free from fatty acids (6 mg/ml), Na-pyruvate (20 µg/ml), heparin (25 µg/ml) and gentamycin (50 µg/ml) for 2 h. After incubation, the oocytes were washed 3 times with IVF-TALP medium and cultured with spermatozoa in100 µl of IVF-TALP under paraffin oil at 37.5 °C for 24 h in 5% Co_2.

***In vitro* culture (IVC):** Following IVF, fertilized oocytes were gently pipetting, then washed twice in PBS and cultured in 100 µl of SOF medium with FCS (20%) overlaid

with paraffin oil and incubated at 37.5 °C with 5% CO_2 for 5 days to assess FR and production of embryos at morula and blastocyst stages.

Statistical Analysis: Data were analyzed by one-way ANOVA using GLM procedures of SAS [17].Duncan's Multiple Range Test according to Duncan [18] was set at P=0.05 to determine the significant differences among means.

RESULTS AND DISCUSSIONS

Effects of Oocyte Type with or Without CCs on:

***In vitro* Maturation:** Nuclear maturation rate was significantly (P<0.05) higher of COCs than of DOs or MDOs, regardless adding CCs in MM. This trend indicated marked effect of oocyte type on IVM. Adding CCs of other oocytes to MM significantly (P<0.05) improved IVM of DOs or MDOs, but had no effect on COCs. It is worthy noting that adding CCs of the same oocytes significantly (P<0.05) increased IVM of MDOs as compared to that of DOs or MDOs with other CCs, but intact COCs still to have significantly (P<0.05) higher IVM than in MDOs with their CCs (Table 1).

These results may suggest that adding CCs of the same oocytes in MM had bifacial effects on its IVM as compared to CCs of other oocytes. Also, intact COCs had better IVM than that of MDOs with their CCs.

In accordance with the present results, Lu *et al.* [14] observed that removal CCs before IVM was detrimental to oocyte maturation. Co-culture with COCs or CCs improved IVM and developmental potential of MDOs of rabbits. The obtained results on rabbits in this study are in agreement with the results of several authors on rats.

In this respect, Zhou *et al.* [9] showed the highest (P<0.05) nuclear maturation rate of COCS as compared to oocytes at other categories. They added that CCs remove from the COCs significantly (P<0.05) reduced IVM and this reduction was not reversed by co-culturing CCs with IVM of oocytes. Also, Mahmodi *et al*. [12] indicated that CCs presence in MM during IVM is responsible for nuclear maturation of oocyte.

Many authors recorded that percentage of oocytes at MII stage was affected by the time when the surrounding CCs were removed from the COCs in pigs [19] and MR was significantly higher in COCs than in DCOs for rabbits and humans [20,21].

Thus, only oocytes with an intact cover of CCs in a sufficient number of layers are used for IVM [22]. During oocyte growth and maturation, CCs are important for meiotic and developmental competence acquisition [23; 24]. Number of CCs layers, cumuli quality and cumulus expansion intensity are decisive in the success of oocyte IVM and required for matured oocyte viability [25,26]. The CCs locally produced glyosaminoglycans and steroid hormones, which are responsible for male pronucleus formation, monospermic fertilization and subsequent embryo development [6, 7].

The Ccs of corona rediata cells penetrate through the zona pellucida and communicate with oocyte via GJ channels which essential for IVM and IVF [9]. Oocyte-CCs communication by GJ increased oocyte growth, differentiation and maturation stages [27]. The GJ are main channels, which allow the exchange of ions and small molecules (purines and cAMP) to inhibit resumption of premature oocyte meiotic progression [28]. During nuclear maturation of oocytes, GJ have been considered necessary for the transfer of molecules smaller than 1 kDa such as ions, nucleotides and regulatory molecules from CCs into the oocyte [29]. Also, GJ is thought to play an important role in regulating ooplasmic factors involved in the removal of sperm nuclear envelopes as well as in GSH transportation [30].

Table 1: Effect of oocyte type with or without cumulus cells supplemented to maturation medium on *in vitro* nuclear maturation.

Type of oocytes	Co-culture medium	Total Oocytes	Maturation rate (%)	Degenerated (%)	Immature oocytes (%)
COCs	Without CCs	95	87.50^{a}	5.25^{b}	7.25^{d}
	With CCs^{1}	100	87.00^{a}	3.00^{b}	10.00^{d}
Dos	without CCs	100	45.00^{d}	20.00^{a}	35.00^{ab}
	with CCs^{1}	85	58.89^{c}	20.13^{a}	20.99^{c}
MDCOs	without CCs	70	50.00^{d}	8.75^{b}	41.25^{a}
	with CCs^{1}	70	63.34^{c}	5.84^{b}	30.84^{b}
	with CCs^{2}	80	75.00^{b}	3.75^{b}	21.25^{c}
±SEM		600	2.66	1.78	3.02

$^{a, b, c \text{ and } d}$: Means within the same column with different superscripts are significantly different at P=0.05. COCs: Compact cumulus oocytes. DCOs: Denuded oocytes. MDOs: Mechanically denuded oocytes. CCs: Cumulus cells from other oocytes $^{(1)}$ or from the same oocytes $^{(2)}$.

Table 2: Effect of oocyte type with or without cumulus cells supplemented to maturation medium on fertilization rate and developmental competence rate of oocyte.

				Formation rate (%)		
Type of oocytes	Co-culture medium	Total oocytes	Fertilization rate (%)	Morulae	Blastocysts	Others
COCs	Without CCs	75	50.67[b]	26.49[ab]	13.24[a]	60.26[bc]
	With CCs[1]	75	61.33[a]	32.44[a]	16.78[a]	50.78[c]
Dos	without CCs	75	24.00[d]	10.32[c]	0.00[b]	89.68[a]
	with CCs[1]	75	32.00[d]	12.63[c]	8.93[ab]	78.44[ab]
MDOs	without CCs	75	29.33[d]	18.78[bc]	4.76[ab]	76.45[ab]
	with CCs[1]	75	41.33[c]	16.06[bc]	13.03[a]	70.91[ab]
	with CCs[2]	75	44.00[bc]	21.06[bc]	12.17[ab]	66.77[bc]
±SEM		525	2.81	3.42	3.73	5.97

[a, b, c and d]: Means within the same column with different superscripts are significantly different at P=0.05.

Fertilization Rate and Developmental Competence: Regardless adding CCs in MM, FR and formation rate of MOR and BLR were significantly (P<0.05) the highest for COCs, moderate for MDOs and the lowest for DOs. Such finding supported the results of IVM, indicating remarkable influence of oocyte type on FR, MOR and BLR. Adding CCs of other oocytes to MM improved FR significantly (P<0.05) for matured COCs and MDOs, while insignificantly for FR of matured DOs. On the other hand, adding CCs to MM did not affect MOR and BLR. It is of interest to observe that adding CCs of the same or other oocytes significantly (P<0.05) increased FR of matured MDOs as compared to without CCs, but failed to affect FR, MOR and BLR as compared to adding CCs of the same or other oocytes (Table 2).

These results may suggest that adding CCs in MM had impact on IVF, but did not affect MOR or BLR.

In agreement with the present results, Zhou *et al.* [9] found that the presence of CCs during insemination improved the FR of mice. Also, Mahmodi *et al.*[12] showed that the presence of CCs in MM is responsible for cleavage and developmental competence to blastocytes of mouse oocytes. In the same line, Ju and Rui [26] found that the CCs presence significantly (P=0.05) influences the 1st division of the zygote, blastocyst stage and viability of embryos. In rabbits, FR and development to blastocyst stage were significantly higher for COCs than DOs [21]. In buffalo, the subsequent fertilization and cleavage rates in oocytes with CCs were better than oocytes without CCs [31].

The present results indicated the role of CCs in improving FR by regulating a wide array of events during IVF that include directing spermatozoa towards the oocyte, capacitation and facilitating acrosomal reaction. However, absence of CCs effect on MOR and BLR may be related to that the embryonic developmental competence and blastocyst quality depends on increasing nuclear maturation rate, zona pellucida hardening, poly-spermic zygotes, chromosomal abnormalities and glutathione peroxidase in oocyte [13].The association between the oocyte and CCs, which form the COCs, is sustained during oocyte growth, differentiation, maturation and fertilization stages [22].The present study indicated higher FR when CCs were added to MM, which supported the hypothesis of Guidobaldi *et al.* [32], which may be due to factors secreted from the CCs. Direction of spermatozoa towards the oocytes was attributed to secretion of chemotactic factors from the oocytes [33], or/and secretion of substances that promote penetrability of the oocyte by sperm during IVF [8].

CONCLUSION

Based on the foregoing results, oocyte type in term of compact oocyte complex reflected the highest rate of maturation, fertilization and morulae/blastocysts formation. Co-culture of denuded or mechanically denuded oocytes required addition of cumulus cells to maturation medium to improve *in vitro* embryo production processes. Adding cumulus cell of the same collected oocytes have more impact than those of strange oocytes on nuclear maturation.

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