

Cytoskeletal Patterns, *In Vitro* Maturation and Parthenogenetic Development of Rabbit GV Oocytes

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ABSTRACT : The purposes of this study were to optimize the *in vitro* maturation (IVM) and culture (IVC) systems of rabbit oocytes. Cytoskeletal structures in the germinal vesicle stage (GV) and during IVM are also investigated. Ovaries were transported from local slaughterhouses and the cumulus-oocyte complexes (COCs) were collected from ovarian follicles (≥ 1 mm). COCs were randomly allocated to TCM199-based medium (T₁, TCM-199) supplemented with NaHCO₃ glucose, sodium pyruvate and FSH (T₂), T₂+E₂+LH (T₃), T₃+FBS (T₄), or T₁+E₂+LH+FSH+FBS (T₅), for IVM. In Experiment 1, COCs were retrieved from the follicles and 51 GV oocytes were fixed in the fixative (MTSB-XF) for nuclear and cytoplasmic examinations. In Experiment 2, progressive changes of both the nucleus and the cytoskeleton were examined at 0, 6, 16, and 20 h after IVM. Maturation (MR) and developmental rates were assessed in Experiment 3. Cytoplasmic microtubules (MT) were clearly observed in rabbit GV oocytes. To our knowledge, this is the first report that describes the appearance of MT structures in the GV stage ooplasm. Tremendous variations in cytoskeletal alterations were observed among treatments with the exception of the vitelline ring (VR), which is constantly visible and unchanged during maturation. Germinal vesicle breakdown (GVBD) does not occur at 6 h after onset of maturation culture. When the oocytes for IVM were collected within 2 h, results from Experiment 3 showed that rates of nuclear maturation were 42, 8, 42, 37 and 65% at 16 h of IVM for T₁ through T₅, respectively, in which T₁, T₄ and T₅ had significantly greater MR than those in other groups ($p < 0.05$). Morula/blastocyst development after parthenogenetic activation ranged from 20 to 63% with significantly greater rates in T₃, T₄ and T₅ ($p < 0.05$). These results suggested that oocytes recovered from slaughterhouse ovaries can be matured and parthenogenetically activated *in vitro*, but the MR remained low in this study. Addition of E₂ and LH in the medium may be beneficial for cytoplasmic maturation, but FBS exerts a negative role in the subsequent development of parthenogenetic embryos when energy substrates are provided in the IVC media. More studies are required for improving the MR and further development of the GV stage rabbit oocytes. (*Asian-Aust. J. Anim. Sci.* 2002, Vol 15, No. 12 : 1695-1701)

Key Words : Germinal Vesicle, Cytoplasmic Microtubule, *In Vitro* Maturation, Cytoskeleton, Rabbit

INTRODUCTION

Due to the advantages of small body size, short reproductive life span, ease of manipulation, and an induced ovulator, rabbits have been one of the most popular model animals in scientific research (Hahn, 1984; Lorenzo et al., 1997). Rabbits are also unique in their reproductive characteristics such as the estrous cycle, physiology of pre- and post-implantational development, and gamete biology. It has been well-known that oocytes undergo meiosis but remain in the GV stage, i.e. the G2-like phase of the first meiotic prophase, during oogenesis. The features of meiotic prophase including leptotene, pachytene, zygotene, diplotene, and diakinesis had been described in various species (McGaughey et al., 1979; Ocampo et al., 1991). However, detailed configurations of the GV nucleus and characteristics of its cytoskeletal structures vary from species to species. Condensed bivalent chromosomes of a fully grown rabbit oocyte are visible in the GV stage (Motlik and Fulka, 1986; Motlik et al., 1989; Sutovsky et al., 1993; Jelinkova et al., 1994) but not in other species.

This might contribute to a slightly shorter maturation time for rabbit oocytes than that in other species. Although being widely used for various experimental procedures, little information is available on IVM and the subsequent developmental competence after *in vitro* activation (IVA) or fertilization (IVF) in rabbit oocytes. Jelinkova et al. (1994) reported a low maturation rate (64%) of preovulatory rabbit oocytes (follicle size=1.5-2.0 mm) cultured in M199 medium supplemented with NaHCO₃, glucose, sodium pyruvate and FSH. If the IVM system of rabbit GV oocytes can be further optimized, more *in vitro* produced rabbit embryos could be used for both production and research purposes as that in other domestic species. In addition, no information has been available for the changes of cytoskeleton during IVM in rabbit oocytes yet. In this study, we compared different culture media and examined the nuclear and cytoplasmic changes during maturation of rabbit oocytes. The developmental competence of the IVM-derived oocytes was also evaluated by parthenogenetic activation.

MATERIALS AND METHODS

Chemicals and oocyte collection

All chemicals or reagents were purchased from Sigma Company unless otherwise indicated. Ham's F-10-based

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Received April 12, 2002; Accepted June 18, 2002

medium supplemented with 0.4% BSA plus 1% rabbit serum (RS) was used as flushing medium. Follicular ovaries were obtained from local abattoirs and transported back to the laboratory in Dulbecco's phosphate buffered solution (DPBS) in 2 h after being slaughtered. Cumulus-oocyte complexes (COCs) were aspirated from the follicles by a syringe attached to an 18G needle. COCs were gathered and quickly rinsed with DPBS for several times prior to IVM treatments.

IVM treatments

COCs were selected and randomly allocated into any one of the following five TCM199-based media, i.e., TCM-199 (T₁) supplemented with NaHCO₃ (2.45 mg/ml), glucose (5.5 mg/ml), sodium pyruvate (0.09 mg/ml) and FSH (1 IU/ml) (T₂, Jelinkava et al., 1994), T₂+E₂+LH (T₃), T₃+FBS (T₄), or T₁+E₂+LH+FSH+FBS (T₅, Ju et al., 1999) and were IVM for various length of time (0, 6, 16 and/or 20 h after IVM). Osmolarity (Osm) was measured in each treatment group. Gonadotrophins for IVM were obtained from the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Disease (NIDDKD), the National Institute of Child Health and Human Development (NIH), and the U.S. Department of Agriculture (USDA).

Nuclear staining and immunocytochemical labeling of the cytoskeleton

Preparation and compositions of the fixative were based on the protocols described by Albertini and Clark (1981) with some modifications (Aman et al., 1994; Liu et al., 1998). Microtubule stabilization buffer (MTSB) was prepared as a 5x stock solution (pH=6.9) containing 0.5 M PIPES (P-6757), 25 mM magnesium chloride (Fisher Scientific, M33-500), and 12.5 mM EGTA (E-4378). Working solution (MTSB-XF) was prepared by mixing 2 ml MTSB stock solution with 1 µl aprotinin (0.01%; A6279), 10 µl dithiothreitol (1 mM; D9779), 5 ml deuterium oxide (50%; D4501), 10 µl paclitaxel (1 µM; T7402), 500 µl Triton-X (0.1%), 540 µl formaldehyde (2%), and made up to 10 ml with distilled water. Mounting medium contained Hoechst 33342 (10 µg/ml), glycerol (50%), and sodium azide (25 mg/ml) in DPBS. Oocytes from both experimental series were transferred into the prewarmed fixative (38°C) in an Eppendorf tube. The oocytes were incubated in the fixative at 39°C for 1 h and then left overnight at 4°C before immunocytochemical staining.

The fixed oocytes were exposed to anti- α (1:400; T-5168) and anti- β (1:400; T-5293) tubulin primary antibodies at 4°C overnight, then washed 3 times (20-30 min interval) with wash solution containing 2% BSA (S-9647), 2% goat serum, 0.2% milk powder, 0.2% sodium azide (S-8032), and 0.1% triton in DPBS without calcium chloride

(Gibco 11500-048). Oocytes were subsequently incubated with the FITC-conjugated secondary antibody (1:200; Cappel #55514) at 39°C for 2 h, then washed 3 times as with primary antibodies. Oocytes were then stained with rhodamine-phalloidin (Molecular Probes R415) for 1 h and washed 3 times as described previously. Finally, the oocytes were mounted on a slide with 10 µl mounting medium containing Hoechst 33342 (10 µg/ml), and then sealed with clear fingernail polish.

Observation in nuclear and cytoplasmic changes

Nuclear dynamics or MR was examined based on the morphological progression as reported previously (Ju et al., 2001). In addition to metaphase I (MI), anaphase/telophase I (A/TI), and metaphase II (MII), the GV nucleus was classified into 3 types based on their chromatin configurations (Figure 1). Type I is a pachytene-like chromatin configuration, in which big clumps of chromatin are predominant (Figure 1a). Type II is characterized by the diplotene-like stage, in which thread-like chromatin with

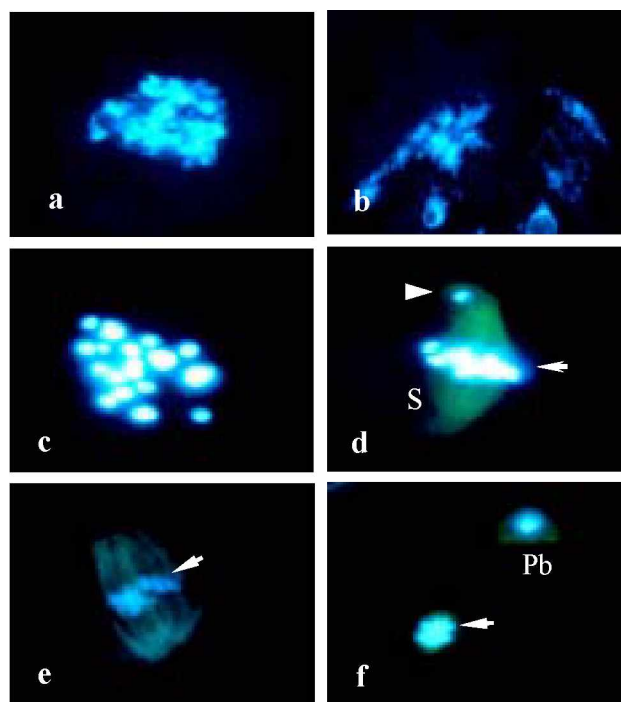


Figure 1. Classifications of nuclear morphology of rabbit oocytes during *in vitro* maturation. (a) Type I GV nucleus is characterized as a big clump of chromatin mass with hardly distinguishable chromatin fibers. (b) Type II GV nucleus appears distinguishable chromatin fibers or thread-like chromatin and nucleolus-like structures are commonly found. (c) Type III GV nucleus is characterized with many separated or condensed chromatin. (d) Metaphase I (MI) chromosome alignment (arrow) with aberrant chromatin (arrow head) and spindle morphology (S). (e) The anaphase I (AI) stage oocyte. (f) The mature oocyte (Metaphase II, MII) with a polar body (Pb) and MII chromosomes on the spindle (arrow).

distinct nucleoli is commonly visible (Figure 1b). Separation of chromatin is evident in the type III nucleus, which is around the diakinesis immediately before GVBD (Figure 1c). Dynamics and amount of cytoplasmic cytoskeleton were observed and judged by the intensity of the microtubular (+, ++, +++) and microfilament staining (+, ++, +++) under a fluorescence microscope (Olympus AF-70)(Figures 2a-f).

***In vitro* activation (IVA) and culture (IVC) of the parthenotes**

Morphologically normal metaphase II (MII) oocytes were activated with ethanol (7%) and 6-DMAP (2.5 mM) combined treatment at the end of IVM, as described in previous study (Liu et al., 2002). Oocytes after activation treatment were cultured in Ham's F-10 supplemented with 1.5% BSA and 10% RS for 4 to 6 days at 38°C in a humidified atmosphere with 5% CO₂. Cleavage rates were determined on day 2 after IVC and blastocyst rates were recorded at the end of culture.

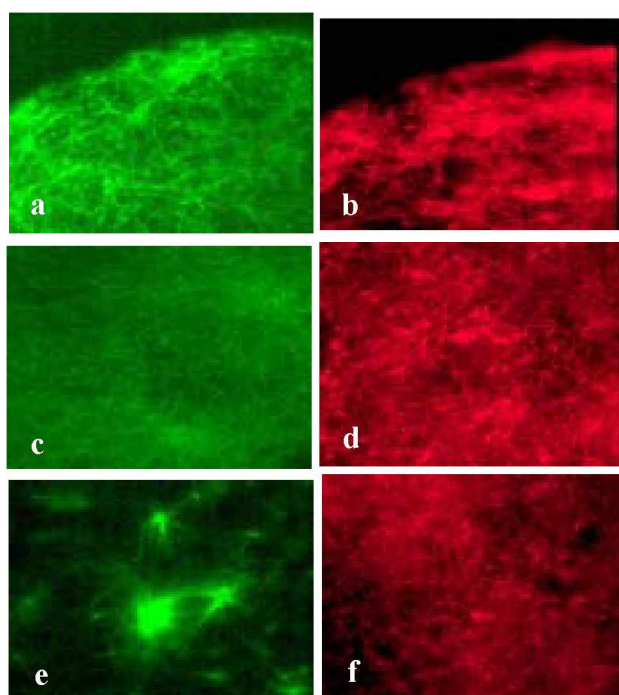


Figure 2. Patterns of the microtubule and microfilament of GV stage oocytes and during *in vitro* maturation of rabbit oocytes. (a) More intensive and coarse microtubular networks were observed in this type of oocytes, which were classified as “+++”. (b) Oocytes with strongly stained microfilaments were also designated as “+++”. (c) Finer microtubular filaments were observed in this type of oocytes (+) (d) A slightly weaker staining of microfilaments compared to that in (b) was designated as “+”. (e) Sparkle or astral-like microtubular structure were found in this group of oocytes, which is designated as “+”. (f) Oocytes with much weaker microfilament staining were classified into this group (+-).

Statistical analysis

Data were analyzed using General Linear Model (GLM) and Chi-square test in the Statistical Analysis System (SAS, 1989).

RESULTS

Viability, nuclear and cytoskeletal configurations of the GV stage oocytes

To identify the viability of oocytes, the GV oocytes derived immediately from the follicles were examined using FDA staining. A total of 73 oocytes in two batches were evaluated, of which an average of 92% (37/41 and 30/32) oocytes showed positive FDA staining (viable).

Nuclear patterns of GV oocytes were classified into 3 categories (Figure 1a-c). The proportions of type I, II, and III oocytes were 25.5, 58.8 and 15.7%, respectively (Table 1). The intensity of cytoskeletal distribution including microtubules and microfilaments was also arbitrarily classified into 3 levels as described in Materials and Methods. Approximately, 24% (12/51) oocytes had a very weak or no (+-) MT staining as shown in Table 1 and only 12% (6/51) had very weak MF staining (+-). In other words, more than 75% of the oocytes had clearly stained (+ and ++) MT and MF structures. There were 94% (48/51) of the oocytes had visible or intact vitelline ring (Table 1).

Chromatin configurations and nuclear progression during IVM

COCs derived from ovarian follicles were rinsed 6 times with medium M199 before being allocated into 5 treatment groups, i.e., T₁ (M199-based control group), T₂ (T₁+0.245% NAHCO₃+0.55% glucose+0.009% sodium pyruvate+1 IU/ml FSH), T₃ (T₂+1 µg/ml E2+5 µg/ml LH), T₄ (T₃+7.5% FBS), and T₅ (M199+1 IU/ml FSH+1 µg/ml E2+5 µg/ml LH+7.5% FBS) for IVM. Ten to twenty

Table 1. The distribution of the nuclear and cytoskeletal patterns of the germinal vesicle stage rabbit oocytes

Criteria	Number of oocytes (%; n=51)	
GV stage	Type I	13 (25.5)
	Type II	30 (58.8)
	Type III	8 (15.7)
Microtubule*	++	19 (37.3)
	+	20 (39.2)
	+ -	12 (23.5)
	++	25 (49.0)
Microfilament*	+	20 (39.2)
	+ -	6 (11.8)
	Intact vitelline ring	48 (94.1)

* The intensity of microtubule and microfilament staining is arbitrarily classified into three levels. The amount of microtubules and microfilaments are designated as “+”, “++”, and “+ -” to represent strong, medium, and weak or none, respectively, in fluorescent intensity.

oocytes were fixed at 0, 6, 16, and 20 h after onset of IVM for immunocytochemical observations.

As mentioned previously, percentages of types I, II and III oocytes were 25 (13/51), 59 (30/51), and 16% (8/51), respectively, in the GV oocytes (control group) (Tables 1 and 2). The percentages of oocytes underwent GVBD from all treatment groups were pooled in Table 2. Although various morphologies of nuclear chromatins appeared in all treatment groups, the GV still remained intact and no GVBD was observed when the COCs were IVM for 6 h. Regardless of the treatments, a total of 21% of the oocytes entered GVBD in which only a small proportion (8%, n=97, data not shown) reached MII stage 16 h post-IVM. Similar proportions of oocytes were in GVBD (18%, n=107) and MII (6%, data not shown) matured to MII stage 20 h post-IVM.

Effects of different culture media on developmental competence

Different IVM media were compared and maturation (MR) and development rates of the oocytes were examined in this experiment. Generally, there was very low MR in all treatment groups ranging from 10 to 32%. The lowest MR was observed in T₂ (10%) and the greatest were in T₄ (28%) and T₅ (32%) (p<0.05; Figure 3).

Due to the time required for oocyte collection from the ovaries, it usually took 3-4 h to finish processing all the ovaries before the onset of IVM. However, the MR or development might be compromised when the handling time were prolonged. Therefore, we arbitrarily grouped the oocytes into two batches based on the exposure time during oocyte collection, i.e., within (<) 2 h and ≥2 h, and the results are presented in Table 3. The overall MR showed that no significant differences were observed among T₁, T₃, T₄, and T₅ (p>0.05), but T₂ had the lowest MR (6%, p<0.05). Most treatments in the <2 h group had greater MR than those in the ≥2 h group with T₃ and T₅ significantly greater than that in the ≥2 h group (p<0.05; Table 3).

All MII oocytes were selected and activated by ethanol+6-DMAP combined treatments described in previously study (Liu et al., 2002). After activation, cleavage rates in all groups ranged from 60-92% with

Table 2. Nuclear progression of immature rabbit oocytes during different stages of *in vitro* maturation⁽¹⁾

Time post-IVM, h	Total no. of oocytes	No. of GV oocytes (%)			No. of GVBD ⁽²⁾ (%)
		Type I	Type II	Type III	
0	51	13 (25)	30 (59)	8 (16)	0 (0)
6	91	22 (24)	50 (55)	19 (21)	0 (0)
16	97	22 (23)	36 (37)	19 (20)	20 (21)
20	107	22 (21)	54 (50)	12 (11)	19 (18)

⁽¹⁾ The COCs were cultured in different IVM media. The data are pooled regardless of types of media used.

⁽²⁾ GVBD includes oocytes that progress to the MI, A+TI, or MII stages.

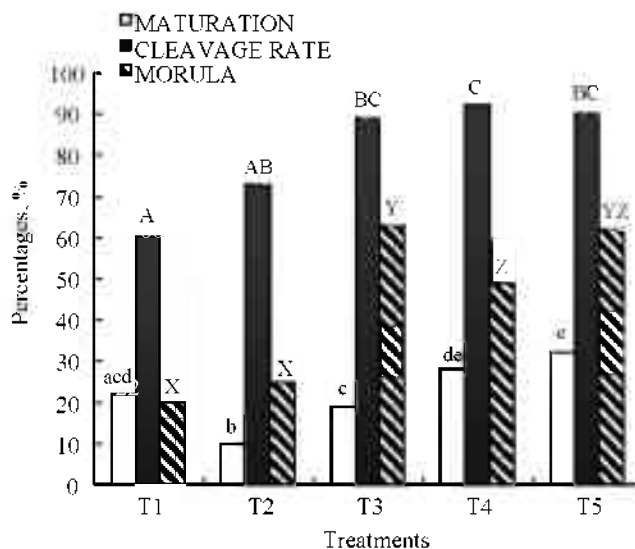


Figure 3. Effects of different maturation media on *in vitro* maturation and their subsequent development of rabbit oocytes (T₁: M199-based medium; T₂: M199+NaHCO₃+glucose+sodium pyruvate+FSH; T₃: M199+NaHCO₃+glucose+sodium pyruvate+FSH+E2+LH; T₄: M199+NaHCO₃+glucose+sodium pyruvate+FSH+E2+LH+FBS; T₅: M199+FSH+E2+LH+FBS). Total numbers of IVM oocytes for T₁, T₂, T₃, T₄ and T₅ are 115, 107, 93, 135, and 120, respectively. Calculations of development are as following: Maturation rate=No. matured oocytes/No. IVM; Cleavage rate=No. of cleavage/No. IVA; Morula= (No. of morula+blastocysts)/No. of cleavage. Means without the same alphabetic letters in the same parameter differ significantly (p<0.05).

Table 3. The effect of time during oocyte collection on the *in vitro* maturation of GV stage oocytes in rabbits

Time	Maturation rate, % (N)					Average
	T ₁	T ₂	T ₃	T ₄	T ₅	
<2 h	42 (25) ^{ab}	8 (31) ^d	42 (27) ^{ab}	37 (60) ^{bc}	65 (42) ^a	39 (185) ^a
≥2 h	17 (47) ^{abcd}	5 (53) ^d	6 (50) ^d	14 (50) ^{cd}	7 (74) ^d	10 (274) ^b
Overall	29 (72) ^a	6 (84) ^b	24 (77) ^a	26 (110) ^a	36 (116) ^a	24 (459)

T₁: M199-based medium;

T₂: M199-NaHCO₃-glucose+sodium pyruvate+FSH;

T₃: M199-NaHCO₃-glucose+sodium pyruvate+FSH+E2+LH;

T₄: M199-NaHCO₃-glucose+sodium pyruvate+FSH+E2+LH-FBS;

T₅: M199-FSH+E2+LH-FBS;

^{abcd} Means without common superscripts within or between treatments differ (p<0.05).

^{AB} Means without common superscripts in the same row differ (p<0.05).

^{xyz} Means without common superscripts in the same column differ (p<0.05).

(N): Numbers in the parentheses indicate the numbers of sample observed.

significantly better rates in T₃, T₄ and T₅ (89, 92, 90, respectively; p<0.05). Rates of the morula and/or blastocyst development ranged from 20 to 63% with significantly greater in T₃ (63%), T₄ (49%) and T₅ (62%) than those in T₂ (25%) and control groups (20%; p<0.05; Figure 3).

DISCUSSION

The microtubules in the GV stage oocytes

Cells in the interphase are characterized by a nucleus with intact nuclear membrane and microtubular network interacting with the nucleus. Normally, there is no cytoplasmic MT can be observed in the metaphase stage, in which the majority of the MT in the cell form the spindle before onset of mitosis. These phenomena are similar to those in embryos or blastomeres. However, previous studies reported that no microtubules were found in the GV stage oocytes, at least in the pig (Kim et al., 1996a; 1996b; 1997), cattle (Wu et al., 1998, unpublished data) and hamster (Plancha and Albertini, 1994). As mentioned previously, the GV stage is in its meiotic prophase, which is a G2-like (Leibfried-Rutledge et al., 1989) or an interphase-like stage. Interestingly, different amounts or intensity of MT network or cytoplasmic MT were observed during IVM in this study (Figure 2a, c, and e). It is also possible that distribution or visibility of the MT varies with the nature of species. Based on previous study, the amount of cytoplasmic MT along maturation process reduces as the oocyte approaching the metaphase (MI or MII; Liu et al., 1998), where no cytoplasmic MTs were observed in a normal MII oocyte.

Changes of cytoskeletal configurations during IVM

In Experiment 1, different patterns of MT and MF structures of the GV oocytes have been clearly observed. No significant changes were observed in MF patterns during maturation. The microfilament is the major component of the vitelline ring (VR) and membrane microvilli (MV). The MV pattern of the immature oocytes differs from a mature one in their shapes and distributions (Suzuki et al., 1994). An immature cattle oocyte has an irregular short MV whereas a more uniform MV was observed in a mature one. However, the VR was clearly observed throughout the maturation (GV to MII) without obvious changes in this experiment (Table 1). It suggested that immunocytochemical staining might not be sensitive enough to detect the changes of surface MV during IVM by subjective evaluation and an image analysis system may be required.

IVM and parthenogenetic development of rabbit oocytes

In this study, different IVM media were examined for capable of supporting maturation as well as development of the oocytes. However, maturation rates were not satisfactory in any one of the treatments with the greatest rates of 28 and 32% observed in the T₄ and T₃, respectively (Figure 3). Apparently, the IVM system has not been optimized in this study. Nevertheless, the reasons for the low MR are not completely clear yet. Possibly, some growth factors such as insulin-like growth factor (IGF-1)

and epidermal growth factors (EGF) maybe required. It was reported that these growth factors are beneficial for cumulus cell expansion or nuclear maturation during IVM of rabbit (Lorenzo et al., 1994; 1996; Lorenzo et al., 1997) and pig oocytes (Abeydeera et al., 1998; Reed et al., 1993), which may further affect blastocyst development (Happer and Brackett, 1993; Lalantha et al., 1998). Other possibility was that the influence of exposure time during oocyte retrieval. When the time for oocyte collection was restricted within 2 h, rates of *in vitro* maturation ranged from 8-65% depending on the treatments. When the time for oocyte collection was prolonged to more than 2 h, the MR significantly decreased to an average of 10% (5-17%) accordingly (Table 3). It suggested that rabbit ovarian oocytes are very sensitive or vulnerable to the environmental changes such as reduction of ambient temperatures.

Another interesting result observed from Experiment 2 was the proportion of oocytes undergoing GVBD. It has been known that spontaneous GVBD occurs in many species such as rats, pigs (Edwards, 1965) and humans (Cha et al., 1991). When the GV stage oocytes were released from their follicular environment, spontaneous GVBD occurs due to the removal or dilution of the regulatory elements such as oocyte maturation inhibitors (OMI; Hafez and Hafez, 2000). In the rat, more than 90% oocytes undergo GVBD when oocytes were removed from the follicles (Vanderhyden and Armstrong, 1990). In contrast, it appears that spontaneous GVBD was not prominent in rabbit oocytes. In Experiment 2, when rabbit oocytes were IVM in different media for 6 h, no GVBD was observed and, if any, only around 12-13% underwent GVBD (MII oocytes not included) when the culture last for 16-20 h (Table 2). This figure appeared much less than that in pigs and humans (30-50%). It is not possible that oocyte viability contributed completely to the low MR in this study, because oocytes were examined by FDA staining and, approximately, 90-92% of the oocytes were considered as viable in this experiment.

Electrical pulses and chemical procedures have been widely used for fusion of ooplasts and donor cells as well as activation of the reconstructed embryos in regular nuclear transfer procedures (Ozil, 1990; Yang et al., 1990; 1992; Collas et al., 1992; Du et al., 1995; Piotrowska et al., 2000). Mature oocytes were parthenogenetically activated based on previous reports (Liu et al., 2002) and then cultured in Ham's F-10 medium supplemented with 1.5% BSA and 10% RS as described by Cheng et al. (1988). The cleavage rates ranged from 60 to 92% and the morula/blastocyst development were from 20 to 63% (Figure 3). This information suggested that once the oocyte matured, it could develop morphologically normal in our current IVC system.

To our knowledge, we defined the nuclear patterns of rabbit GV oocytes and clearly identified microtubular structures in GV stage oocytes for the first time. However, optimization of the IVM system and/or IVC for IVM-derived rabbit oocytes requires more efforts.

ACKNOWLEDGMENTS

Authors wish to thank Tai-Yuan Pharmaceutical Co. and Whole Asia Biological Co. for providing rabbit ovaries. We also thank Dr. A. F. Parlow for his help in application of the highly purified NIDDK gonadotropins (oLH and oFSH) for IVM/IVC of the activated oocytes. This study was supported by a grant (# NSC89-44-B005) from the National Science Council, Executive Yuan, Taiwan, Republic of China.

REFERENCES

- Abeydeera, L. R., W. H. Wang, T. C. Cantley, A. Rieke, R. S. Prather and B. N. Day. 1998. Presence of epidermal growth factor during *in vitro* maturation of pig oocytes and embryo culture can modulate blastocyst development after *in vitro* fertilization. *Mol. Reprod. Dev.* 51:395-401.
- Albertini, D. F. and J. T. Clark. 1981. Visualization of assembled and disassembled microtubule protein by double fluorescence microscopy. *Cell. Biol. Intl. Reports* 5(4):387-397.
- Aman, R. B. and J. E. Parks. 1994. Effect of cooling and rewarming on the meiotic spindle and chromosomes of *in vitro*-matured bovine oocytes. *Biol. Reprod.* 50:103-110.
- Cha, K. Y., J. J. Koo, J. J. Ko, D. H. Choi, S. Y. Han and T. K. Yoon. 1991. Pregnancy after *in vitro* fertilization of human follicular oocytes collected from nonstimulated cycles, their culture *in vitro* and their transfer in a donor oocyte program. *Fertil. Steril.* 55:109-113.
- Cheng, S. P., Y. C. Chang, J. C. Ju and C. L. Young. 1988. *In vitro* development of intact and micromanipulated rabbit embryos. Proceedings of the three-way faculty exchange seminar on the application of biotechnology to agriculture. Seoul, Korea, pp. 235-244.
- Collas, P., J. J. Balise and J. M. Robl. 1992. Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. *Biol. Reprod.* 46: 492-500.
- Edwards, R. G. 1965. Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* 208: 349-351.
- Du, F., J. R. Giles, R. H. Foote, K. H. Graves, X. Yang and R. W. Moreadith. 1995. Nuclear transfer of putative rabbit embryonic stem cells leads to normal blastocyst development. *J. Reprod. Fertil.* 104, 219-223.
- Hahn, J. 1984. The value of laboratory animal models in embryo transfer research. *Theriogenology.* 21:45-59.
- Hafez, E. S. E. and B. Hafez. 2000. Folliculogenesis, egg, maturation and ovulation. In: "Reproduction in Farm Animals", (Ed. E. S. E. Hafez and B. Hafez). 7th edition, Lippincott Williams and Wilkins, Philadelphia, USA. pp. 68-81.
- Haper, K. M. and B. G. Brackette. 1993. Bovine blastocyst development after *in vitro* maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. *Biol. Reprod.* 48:409-416.
- Jelinkova, L., M. Kubelka, J. Motlik and P. Guerrier. 1994. Chromatin condensation and histone H1 kinase activity during growth and maturation of rabbit oocytes. *Mol. Reprod. Dev.* 37: 210-215.
- Ju, J. C., C. Tsay, C. W. Ruan and X. Yang. 2001. Reversibility in the cytoskeleton and development of pig oocytes treated with roscovitine. *Biol. Reprod.* 64:38(Abstr.).
- Ju, J. C., J. E. Parks and X. Yang. 1999. Thermotolerance of IVM-derived bovine oocytes and embryos after short-term heat shock. *Mol. Reprod. Dev.* 53:336-340.
- Kim, N. H., K. S. Chung and B. N. Day. 1997. The distribution and requirements of microtubules and microfilaments during fertilization and parthenogenesis in pig oocytes. *J. Reprod. Fertil.* 111:143-149.
- Kim, N. H., H. Funahashi, R. Prather, G. Schatten and B. N. Day. 1996a. Microtubule and microfilament dynamics in porcine oocytes during meiotic maturation. *Mol. Reprod. Dev.* 43:248-255.
- Kim, N. H., C. Simerly, H. Funahashi, G. Schatten, B. N. Day. 1996b. Microtubule organization in porcine oocytes during fertilization and parthenogenesis. *Biol. Reprod.* 54:1397-1404.
- Lalantha, R., L. R. Abeydeera, W. H. Wang, T. C. Cantley, A. Rieke, R. S. Prather and B. N. Day. 1998. Presence of epidermal growth factor during *in vitro* maturation of pig oocytes and embryo culture can modulate blastocyst development after *in vitro* fertilization. *Mol. Reprod.* 51(4): 395-401.
- Leibfried-Rutledge, M. L., H. M. Florman and N. L. First. 1989. The molecular biology of mammalian oocyte maturation. In: "The Molecular Biology of Fertilization", (Ed. H. Schatten and G. Schatten). Academic Press Inc., San Diego, CA 92101. pp. 259-301.
- Liu, L., J. C. Ju and X. Yang. 1998. Parthenogenetic development and protein pattern of newly matured bovine oocytes after chemical activation. *Mol. Reprod. Dev.* 49:298-307.
- Liu, C. T., C. H. Chen, S. P. Cheng and J. C. Ju. 2002. Parthenogenesis of rabbit oocytes activated by different stimuli. *Anim. Reprod. Sci.* 70:267-276.
- Lorenzo, P. L., M. J. Illera, J. C. Illera and M. Illera. 1994. Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I. *J. Reprod. Fertil.* 101:697-701.
- Lorenzo, P. L., P. G. Rebollar, M. J. Illera, J. C. Illera, M. Illera and J. M. R. Alvarino. 1996. Stimulatory effect of insulin-like growth factor I and epidermal growth factor on the maturation of rabbit oocytes *in vitro*. *J. Reprod. Fertil.* 107:109-117.
- Lorenzo, P. L., J. C. Illera, G. Silvan, C. J. Munro, M. J. Illera and M. Illera. 1997. Steroid level response to insulin-like growth factor-I in oocytes matured *in vitro*. *J. Reprod. Immunol.* 35: 11-29.
- McDonald, L. E. 1980. Introduction. In: "Veterinary

- Endocrinology and Reproduction", 3rd ed., (Ed. L. E. McDonald, Lea and Febiger). Philadelphia, pp.1-12.
- McGaughey, R. W., D. H. Montgomery and J. D. Richter. 1979. Germinal vesicle configurations and patterns of polypeptide synthesis of porcine oocytes from antral follicles of different size as related to their competency for spontaneous maturation. *J. Exp. Zool.* 209:239-254.
- Motlik, J. and J. Fulka. 1986. Factors affecting meiotic competence in pig oocytes. *Theriogenology* 25:87-96.
- Motlik, J., J. Fulka Jr, R. Prochazka, Z. Rimkevicova, M. Kubelka and J. Fulka. 1989. RNA and protein synthesis requirements for the resumption of meiosis in rabbit oocytes: The role of cumulus cells. *Reprod. Nutr. Dev.* 29:601-609.
- Ocampo, M. B., T. C. Lerma and H. Kanagawa. 1991. Timing of sequential changes in chromosome configurations during the 1st meiotic division of pig oocytes cultured *in vitro*. *Jpn. J. Vet. Res.* 38:127-137.
- Ozil, J. P. 1990. The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* 109:117-128.
- Piotrowska, K., J. A. Modlinski, M. Korwin-Kossakowski and J. Karasiewicz. 2000. Effects of preactivation of ooplasts or synchronization of blastomere nuclei in G1 on preimplantation development of rabbit serial nuclear transfer embryos. *Biol. Reprod.* 63:677-682.
- Plancha, C. E. and D. F. Albertini. 1994. Hormonal regulation of meiotic maturation in the hamster oocyte involves a cytoskeleton-mediated process. *Biol. Reprod.* 51:852-864.
- Reed, M. L., J. L. Estrada, M. J. Illera and R. M. Petters. 1993. Effects of epidermal growth factor, insulin-like growth factor-1, and dialyzed porcine follicular fluid on porcine oocyte nuclear maturation *in vitro*. *J. Exp. Zool.* 266:74-78.
- Sutovsky, P., L. Jelinkova, L. Antalikova and J. Motlik. 1993. Ultrastructural cytochemistry of the nucleus and nucleolus in growing rabbit oocytes. *Biol. Cell* 77:173-180.
- Suzuki, H., X. Yang and R. H. Foote. 1994. Surface alterations of the bovine oocyte and its investments during and after maturation and fertilization *in vitro*. *Mol. Reprod. Dev.* 38:421-430.
- Vanderhyden, B. C. and D. T. Armstrong. 1990. Effect of gonadotropins and granulosa cells secretions on the maturation and fertilization of rat oocytes *in vitro*. *Mol. Reprod. Dev.* 26:337-346.
- Wu, B., J. Tong and S. P. Leibo. 1998. Effect of chilling bovine germinal vesicle-stage oocytes on formation of microtubules and the meiotic spindle. *Theriogenology* 49:177.
- Yang, X., Y. Chen and R. H. Foote. 1990. Potential of hypertonic medium treatment for embryo micromanipulation: I. Survival of rabbit embryos *in vitro* and *in vivo* following sucrose treatment. *Mol. Reprod. Dev.* 27:110-117.
- Yang, X., S. Jiang, A. Kovacs and R. H. Foote. 1992. Nuclear totipotency of cultured rabbit morulae to support full-term development following nuclear transfer. *Biol. Reprod.* 47:636-643.