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Full Length Research Paper

Effect of cumulus-oocyte complexes (COCs) culture duration on *in vitro* maturation and parthenogenetic development of pig oocyte

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We investigated and optimized the cumulus-oocyte complexes (COCs) culture duration for pig oocyte *in vitro* maturation and produced a number of high-quality metaphase-II (M-II) oocytes for generation of parthenotes. The present study graded the COCs into levels A, B and C according to layers of cumulus cells, which were cultured *in vitro* for 24, 32, 38, 44, 48 and 54 h, respectively. Subsequently, the oocytes with extruded polar body at different time point were electrically activated. The rates of cleavage and blastocyst formation were assessed on day 2 and 7, respectively. The maturation rates of COCs of both level A and B arrived at the highest point at 44 to 48 h, which were statistically insignificant (P > 0.05), while level C COCs needed longer maturation time (54 h), for most of the oocytes to become mature. COCs of different levels were cultured *in vitro* for the same term, the maturation rates of levels A and B were not significantly different (P > 0.05), but both were significantly higher (P < 0.05) than that of level C. After parthenogenetic activation, there were no significant differences between the cleavage rates in the groups of 38 to 54 h maturation duration, whereas the developmental rate to blastocysts after 44 h (16.1%) and 48 h (16.5%) maturation duration were significantly higher than those from the other term groups (0, 2.2, 9.8 and 12.4% for 24, 32, 38 and 54 h, respectively, P < 0.05). COCs of levels A and B were more suitable for oocytes preparation *in vitro*, which led to a high maturation rate: 44 to 48 h duration was beneficial for maturation, cleavage and blastocyst formation. As for 38 h or less maturation duration, rates of blastocyst were extremely low although some oocytes could be seen with the polar body and cleaved to some extent (64.5% for 3 8h IVM), indicating that oocytes were not really matured. Therefore, the developmental maturation of oocytes could not be judged only by excluding the first polar body.

Key words: Cumulus-oocyte complexes (COCs), IVM, duration, polar body, activation.

INTRODUCTION

Since the cloned sheep “Dolly” was produced by the transplantation of nucleus of sheep mammary gland cell into an enucleated oocyte (Wilmut et al., 1997), the development of somatic cell nuclear transfer (SCNT) has made great progress and success in generating cloned offspring in mouse, cattle and goat (Cibelli et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Galli et al., 1999; Wells et al., 1999). However, SCNT in pigs has little progress and has been proven to be more difficult than in other livestock, so the somatic cell nuclear transfer offspring could not be produced successfully until 2000 (Betthauser et al., 2000; Onishi et al., 2000;

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Abbreviations: COCs, Cumulus-oocyte complexes; **M-II,** metaphase-II; **SCNT,** somatic cell nuclear transfer; **NT,** nuclear transfer; **pFF,** pig follicular fluid; **BSA,** bovine serum albumin.

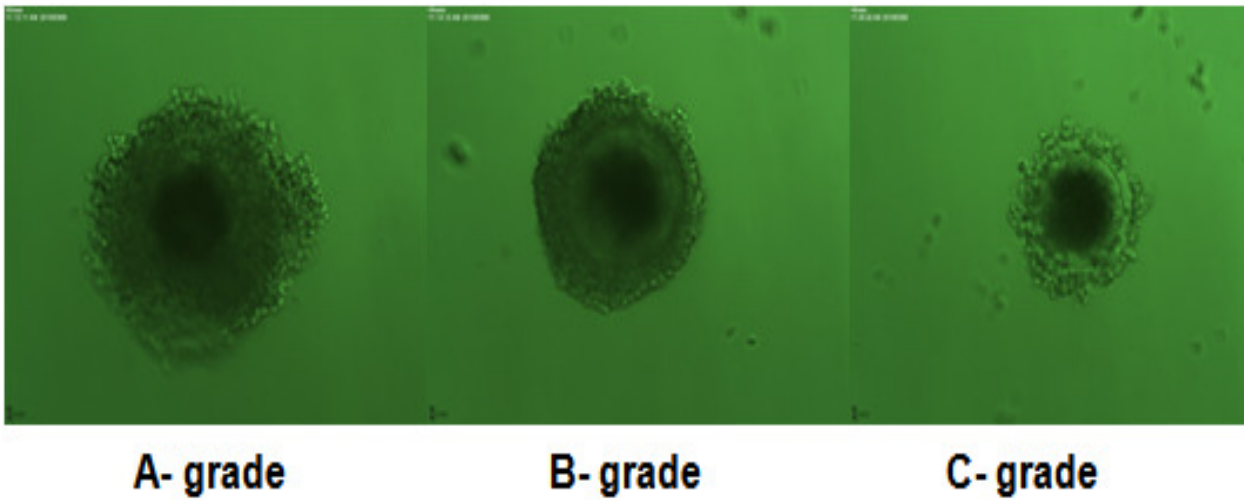

Figure 1. Pig oocytes graded.

Polejaeva et al., 2000). Animal cloning by nuclear transfer (NT) was dependent upon a range of factors, oocyte maturation has been seen as the most important step in SCNT procedure, which directly impact the development of cloned embryos and the efficiency of SCNT (Wolf et al., 2001). Therefore, the maturation conditions, especially the duration of culturing *in vitro* should be optimized. Without enough maturation culturing may result in cytoplasm and nucleus premature and cleavage abnormalities. In turn, too long duration is likely to lead to age and apoptosis of the oocytes.

The aim of this study was to investigate whether cumulus cells improved developmental ability of pig oocytes and screen the best lasting time for oocytes maturation *in vitro* for production of parthenotes. The oocytes with extruded polar body at different term were electrically activated using an Electro Cell Manipulator 2001 (BTX Inc., San Diego). The best term of oocytes maturation *in vitro* was assessed based on developmental ability to the cleavage and blastocyst stage.

MATERIALS AND METHODS

Chemicals

DPBS was purchased from Gibco Company. PMSG and hCG were from Hormone Products Factory in Ningbo, China and other chemical reagents used for oocyte maturation, activation and embryo culture were purchased from Sigma Aldrich Chemical Co. (Budapest, Hungary) unless otherwise noted.

Preparation of pig follicular fluid

The pig follicular fluid (pFF) was collected from follicles (3 to 8 mm diameter) of pig ovaries using a 10-ml disposable syringe with a 16-gauge needle. After centrifugation at 1600 rpm for 20 min at 4°C, the suspension was filtered through 0.22 µm syringe filters and stored at -20°C until use.

Cumulus-oocyte complexes (COCs) collection and classification

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in a thermal container at 28 to 37°C in 0.9% NaCl solution supplemented with penicillin (200 IU/ml) and streptomycin (150 IU/ml). The ovaries were washed 3 to 5 times with saline solution (28 to 37°C) until they were aspiration. Pig follicular fluid was aspirated from follicles (3 to 8 mm diameter) using a 18-gauge needle fixed a 10 ml disposable syringe.

COCs were washed three times in DPBS and graded into level A, B and C according to cumulus cells (A: cumulus cells of 5 or more layers, B: cumulus cells of 3 to 5 layers, C: cumulus cells of 1 to 2 layers) (Figure 1).

In vitro culture of pig oocytes

After COCs were divided into level A, B and C, they were washed three times with a specified maturation medium-mTCM199 or NCSU-23 with pFF and hormone, and transferred to a 500 µl drop of the same medium which had been previously covered with warm paraffin oil in a polystyrene culture dish and equilibrated at 39°C in atmosphere of 5% CO_2 in air overnight, and cultured for 24, 32, 38, 44, 48 and 54 h, respectively. During the first 22 h of maturation, the medium contained 10 IU/ml PMSG and 10 IU/ml hCG. Culturing for the subsequent 22 h was performed in the same medium without hormone supplementation. The oocytes cultured in different terms were observed under stereo microscope at 4 × 10 times, and expanded cumulus cells were completely removed by treatment with 0.1% hyaluronidase and pipetted. The oocytes with uniform cytoplasm and emission of the first polar body (Figure 2) were used for parthenogenetic activation. As *in vitro* culturing lasted longer, cumulus cells fell off on their own, the perivitelline space of oocyte was obviously widened, and some oocytes excluded the second polar body (Figure 3).

Activation and embryos culture

Oocytes with the first polar body were washed three times with activation fluid (0.3 M mannitol, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$ and 0.05 mg/ml bovine serum albumin, BSA), and transferred to a

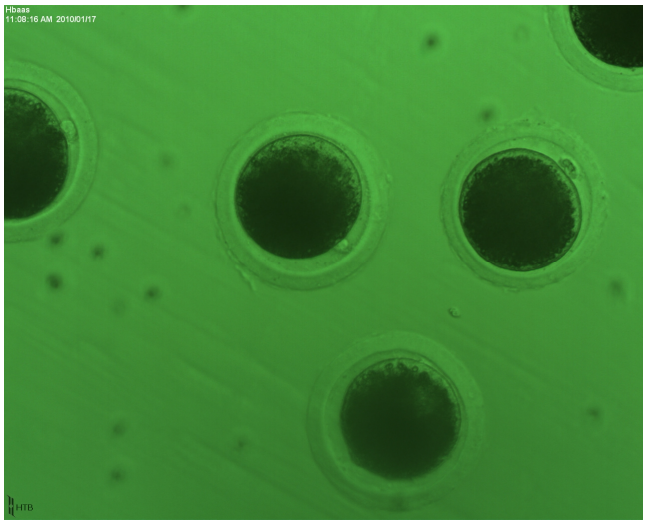

Figure 2. COCs after 44 h of IVM.

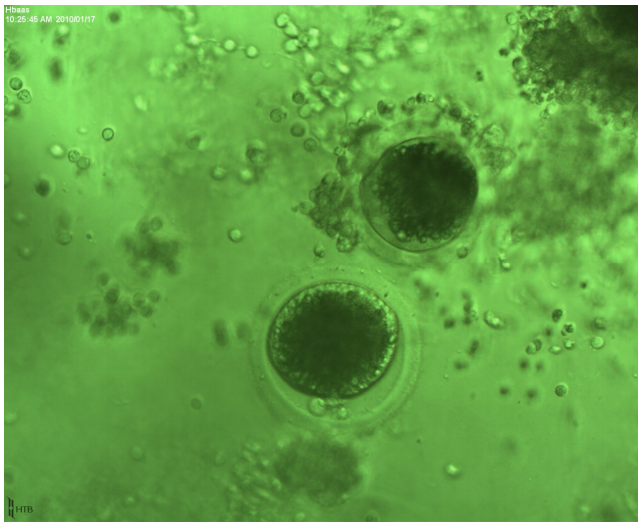

Figure 3. COCs after 54 h of IVM.

chamber containing the same fluid. Activation were induced by application of an AC pulse of 10 V for 5 s followed by a single DC pulses of 1.6 kV/cm for 60 μs using an Electro Cell Manipulator 2001 (BTX Inc., San Diego). After each activation treatment, the embryos were washed five times with NCSU-23 containing 4 mg/ml BSA, and were then cultured in the same medium which had been previously covered with paraffin oil in a polystyrene culture dish and equilibrated at 38.5°C in an atmosphere of 5% CO_2. The rates of cleavage and blastocyst formation were assessed on day 2 and 7, respectively (Figure 4).

Statistical analysis

All data were obtained from five replicates. Percentage data were analyzed by chi-square tests. A probability of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of *in vitro* culture duration on maturation of COCs of level A

Cumulus-oocyte complexes (COCs) of level A were cultured *in vitro* for 24, 32, 38, 44, 48 and 54 h, respectively, oocytes with extruded polar body were counted. The results showed that the maturation rates of oocytes cultured for 44 (79.4%), 48 (83.2%) and 54 h (74.2%) were significantly higher than those of those cultured for 38, 32 and 24 h (32.6, 15.0 and 4.0%, respectively, $P < 0.05$) (Table 1).

Effect of *in vitro* culture duration on maturation of COCs of level B

Only oocytes with extruded polar body were selected. From Table 2, the *in vitro* maturation duration of 44, 48 and 54 h led to more matured oocytes (the rates were 61.4, 65.7 and 63.9%, respectively) than those led to by duration of 24 ~ 38 h (3.1, 13.9 and 30.0, respectively for rates, $P < 0.05$) (Table 2).

Effect of *in vitro* culture duration on maturation of COCs of level C

After culturing cumulus-oocyte complexes of level C for 24 to 60 h, the number of oocytes with extruded first polar body was very small. Clearly, under the same conditions, the maturation rates of level A and B were significantly higher than that of level C. Meantime, the time that the maturation rates reached maximum (30.3%) extended to 54 h (Table 3).

The maturation rate of different levels of COCs

In the same culture conditions, the maturation rates of levels A and B were not significantly different at the same time point, respectively ($P > 0.05$), but both were significantly higher when compared with level C ($P < 0.05$) (Table 4).

Also, from Figure 1, we can clearly see that COCs of levels A and B had better maturation ability than the COCs of level C.

Effect of maturation duration of COCs on parthenogenetic development

Oocytes with extruded polar body were activated after maturation culture of COCs for 24, 32, 38, 44, 48 and 54 h, respectively. The rates of cleavage and blastocyst formation were assessed after activation on day 2 and 7,

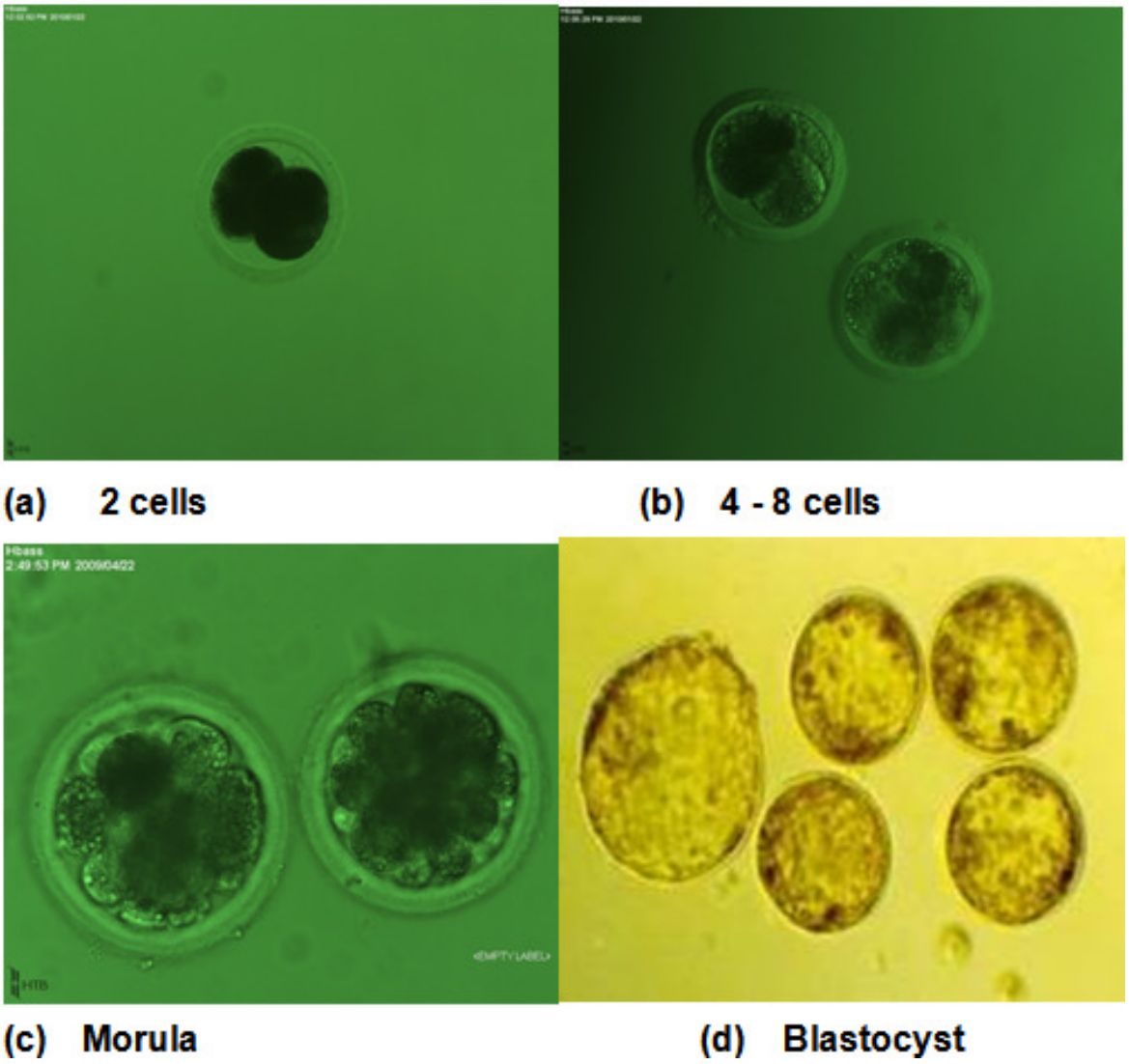

Figure 4. Embryos of parthenogenetic activation.

Table 1. *In vitro* maturation of COCs of level A.

IVM duration (h)	Number of COCs of level A	Number of matured oocytes (%)
24	600	24 (4.0)[a]
32	640	96 (15.0)[b]
38	614	200 (32.6)[c]
44	985	782 (79.4)[d]
48	856	712 (83.2)[d]
54	620	460 (74.2)[d]

respectively (Table 5).

The results showed that the cleavage rates of oocytes after IVM for 38 to 54 h were significantly higher than for 24 and 32 h. Although there were no significant differences in the cleavage rates within 38 and 54 h IVM groups, the developmental rate to blastocysts in 44 (16.1%), 48 (16.5%) and 54 h (12.4%) groups were significantly higher than at 38 h groups, as well as at 24 and 32 h groups (9.8, 0 and 2.2%, respectively, $P < 0.05$). From Figure 2, we can clearly see the trend that the parthenogenetic development went and it was better with the IVM duration extending.

Table 2. *In vitro* maturation of COCs of level B.

IVM duration (h)	No. of COCs of level B	No. of matured oocytes (%)
24	860	27 (3.1)[a]
32	920	128 (13.9)[b]
38	875	262 (30.0)[c]
44	914	586 (64.1)[d]
48	880	578 (65.7)[d]
54	906	579 (63.9)[d]

No. = Number.
Within the same column, values with same superscripts are not significantly different (P > 0.05). Values with different superscripts are significantly different (P < 0.05).

Table 3. *In vitro* maturation of COCs of level C.

IVM duration(h)	No. of COCs of level C	No. of matured oocytes (%)
24	420	7 (1.7)[a]
32	480	45 (9.4)[b]
38	498	92 (18.5)[c]
44	590	138 (23.4)[c]
48	504	142 (28.2)[d]
54	479	145 (30.3)[d]
60	405	106 (26.2)[d]

No. = Number.
Within the same column, values with same superscripts are not significantly different (P > 0.05). Values with different superscripts are significantly different (P < 0.05).

Table 4. The maturation rate of different levels of COCs.

COCs level	Percent of matured oocyte (%)					
	24 h	32 h	38 h	44 h	48 h	54 h
A	4.0[a]	15.0[a]	32.6[a]	79.4[a]	83.2[a]	74.2[a]
B	3.1[a]	13.9[a]	30.0[a]	64.1[a]	65.7[a]	63.9[a]
C	1.7[b]	9.4[b]	18.5[b]	23.4[b]	28.2[b]	30.3[b]

Table 5. Effect of maturation duration of COCs on parthenogenetic development.

IVM duration (h)	No. of embryos treated	No. of embryos cleaved (%)	No. of blastocyst (%)
24	58	15(25.9)[a]	0(0)[a]
32	272	104(38.2)[b]	6(2.2)[a]
38	572	370(64.5)[c]	56(9.8)[b]
44	1595	1247(78.2)[c]	256(16.1)[c]
48	1506	1230(81.7)[c]	248(16.5)[c]
54	1253	968(77.3)[c]	155(12.4)[b]

No. = Number.

DISCUSSION

It had been documented that *in vitro* pig oocyte maturation was accompanied by cumulus cell proliferation, which could regulate the transporation of a variety of chemical substances through oocyte membranes, and inhibit oocyte degradation by discharging adverse factors (Chen et al., 2003; Shirazi et al., 2007). In turn, the

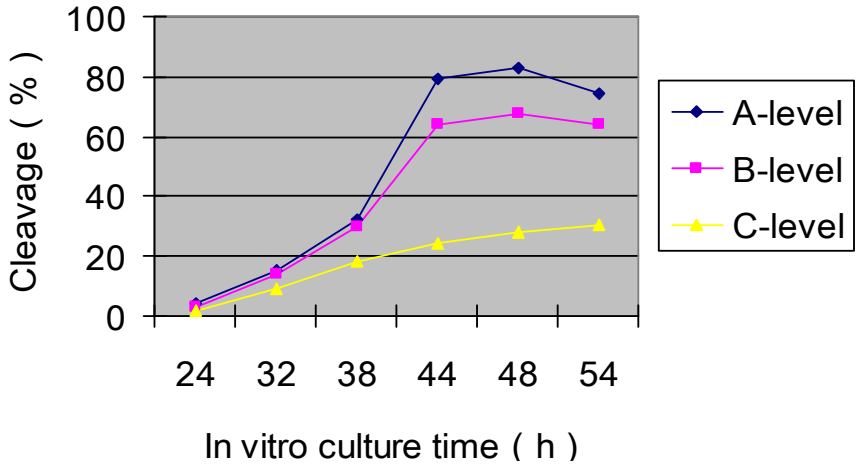

Figure 5. The maturation rate of COCs level A, B and C.

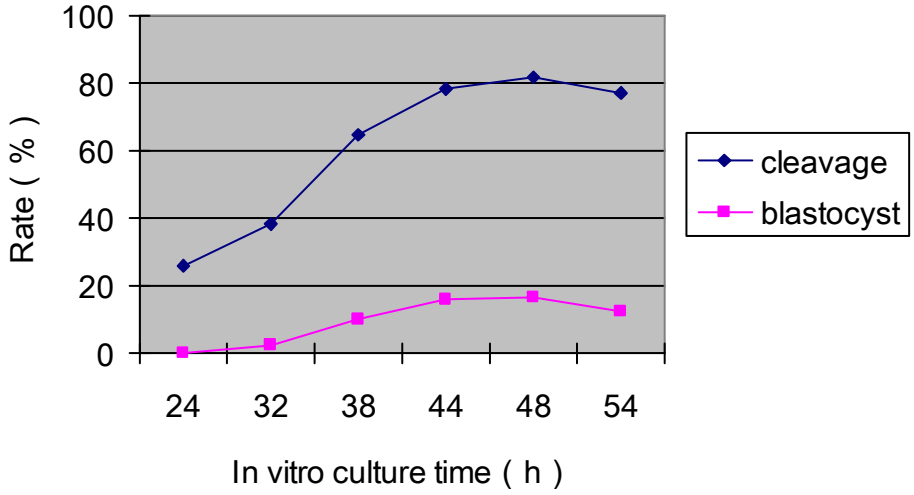

Figure 6. The rates of embryos cleavage and the blastocysts.

growth factors released by pig oocytes can promote the development of cumulus cells (Krisher et al., 2007; Ge et al., 2008; Zhang et al., 2010). However, details underlying this mutual *in vitro* process is yet to be discovered. Here, we aimed to investigate the effect of granule cells upon *in vitro* maturation of cumulus-oocyte complexes (COCs) by grading COCs into A, B and C levels based on layers of cumulus cells. Our results suggested that cumulus cells affected the maturation of pig oocyte *in vitro.*

In the process of producing embryos by SCNT, the first polar body (PBI) presentation was used as a sign of blind suction enucleation. Under this kind of protocol, it was usually considered that oocytes developmental maturation can be judged only by excluding the first polar body. In our experiments, some oocytes could be seen with the polar body when IVM was cultured for 24 to 32 h, but the rates of cleavage and blastocyst were extremely low. Even 38 h IVM duration was not enough which led to significant less blastocyst than 44 h or more, although the cleavage percent for 38 h group was substantial, these indicated that oocytes cultured for less than 38 h were not really matured. Unmaturation of oocytes may significantly affect subsequent embryo development by reducing both the developmental rate and total cell number in blastocysts. Yajuan et al. (2004) found that among the different ages of pig, eggs matured oocytes parthenogenetically developed with a great difference in rate, 48 h eggs obtained a high rate of development.

In our study, oocytes with extruded first polar body were activated after IVM for 24, 32, 38, 44, 48 and 54 h, respectively. The results showed that although there were no significant differences in the cleavage rates for COCs culture *in vitro* for 38 to 54 h, the developmental rate to blastocysts of 44 and 48 h oocytes were significantly higher than of the other term groups ($P < 0.05$). So, oocytes cultured *in vitro* for 44 to 48 h were the best maturation duration and provided a number of high-quality M-II oocytes for production of parthenotes. If PBI

emission was delayed, in other words, COCs were cultured longer *in vitro*, and PBI and metaphase chromosome location would be deviated which could lead to decrease of enucleation rate (Kono et al., 1991, 1992) in SCNT. On the one hand, this kind of aged oocytes may also compromise further development by inducing egg's free Ca^{2+} increase, which leads to a lower level of maturation promoting factor MPF, and then starting oocytes' uncompleted pre-activation (Mtango et al., 2002; Somfai et al., 2007).

Conclusion

Layers of cumulus cells have some influence on oocyte maturation; COCs of level A and B were more suitable for oocytes preparation *in vitro*, obtained a high maturation and cleavage rate. cumulus-oocyte complexes were cultured i*n vitro* for less than 44 h and the rates of cleavage and blastocyst were extremely low, except for the cleavage rate for 38 h, although some oocytes could be seen with the polar body, indicating that oocytes were not really matured. At the same time, oocytes of the developmental maturation could not be judged only by excluding the first polar body. Oocytes cultured *in vitro* for 44 to 48 h had the best maturation time and provided a number of high-quality metaphase-II (M-II) oocytes for production of parthenotes.

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