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Morphological Changes on Nuclear Phase of Germinal Vesicles in Porcine Follicular Oocytes

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돼지 난포난자에서 난핵포 핵상의 형태학적 변화

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ABSTRACT

The morphological changes on nuclear phase of the germinal vesicle of porcine follicular oocytes during in vitro culture were examined. The high rates (75~77%) of the oocytes collected from follicles of 1~2mm or 6~10mm in diameter were at the GV-I to GV-II stages. When oocytes with or without cumulus cells after collection from follicles of 2~6mm in diameter were cultured for 5 h, the rates of oocytes at GV-IV to GV-VI stages were higher in oocytes with (52%) than in oocytes without (30%) cumulus cells. After 1 h of oocyte culture, there was no differences in the distribution of GV-IV to GV-VI stages in the media with or without catalase, xanthine and catalase+xanthine. After 5 h of culture, however, the distribution of GV-IV to GV-VI stages were 46, 69, 69 and 70% for medium with none, catalase, xanthine and catalase+xanthine. The highest rate of GVBD was also observed in the medium with catalase+xanthine (6%). These results indicate that exposure of porcine follicular oocytes to catalase+xanthine excels maturation to GV stage and enhances oocyte nuclear maturation.

(Key words: Nuclear phase, Germinal vesicles, Porcine)

Ⅰ. INTRODUCTION

Methods for the long-term culture of follicles were developed in mice, but are becoming more widely used in other species including rats, cattle, pigs and humans (Roy and Treacy, 1993; Hirao et al., 1994; Smyth et al., 1994; Abir et al., 1995; Cain et al., 1995; Ralph et al., 1995). The follicle architecture is broadly similar in these species, but the eventual diameter of mature intact follicles varies widely. The size that follicles can achieve *in vitro* has physical constraints, including limited diffusion gradients and the absence of the rich blood supply to the theca; however, the interior of follicles is calculated to be quite hypoxic, even *in vivo* (Boland et al., 1994), so follicular oocytes may be quite tolerant of *in vitro* conditions.

It was reported that the morphology of follicular cells attached to oocytes before culture affects *in*

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vitro maturation of oocytes (Nagai et al., 1993). Therefore, for the successful maturation of oocytes, it would be important to observe details of cumulus-oocyte complexes before culture and collect morphologically intact cumulus-oocyte complexes. To do so Ding et al. (1992) and Miyano et al. (1995) collected cumulus-oocyte complexes from only non-atretic intact follicles that had been dissected. *In vivo*, the oocytes retain the germinal vesicle until they degenerate, or enter the final stages of oocyte maturation inside a pre-ovulatory follicle. The germinal vesicle stage is not static but includes functional changes which relate to subsequent development (Chouinard, 1975). However, there have been few reports on nuclear status, or germinal vesicle stages, of oocytes obtained from various sizes of follicles before culture.

Toxic metabolites of oxygen, including the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), are important mediators of inflammatory tissue injury (Weiss, 1986). Moreover, these highly toxic oxygen metabolites have been found to be the final common mediator of tissue damage in a large number of disparate processes, including inflammation and post-ischaemic re-perfusion injury (Bulkley, 1987). There are therefore striking similarities between many known actions of oxygen-derived free radicals and the events leading to oocyte maturation.

The present study was undertaken to determine whether exposure of cumulus-oocyte complexes to catalase and/or xanthine in the maturation culture *in vitro* would affect nuclear morphology of porcine oocytes of various follicle sizes.

Ⅱ. MATERIALS AND METHODS

1. Oocyte Preparation

Porcine ovaries were collected at a local slaughter house and were kept in saline solution(NaCl, 0.9% W/V ; penicillin 100,000 IU/l ; streptomycin 100mg/l and amphotericin B 250㎍/l ; Sigma) at 30 ~32℃. Cumulus-oocyte complexes were aspirated from 2 to 6mm follicles with a 10ml syringe with 18-G needle. The collected oocytes were washed three time in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Ten oocytes with a compact and complete cumulus cells were introduced to droplets (50㎕) of maturation medium, and covered with mineral oil and cultured under the atmosphere of 5% CO_2 in air at 39℃. The maturation medium consisted of TCM-199 with Earle's salt (Gibco, Lab., NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes, 10% FCS, 0.2 mM Na-pyruvate, 50㎍ /ml gentamycin, 1㎍/ml FSH, 5㎍/ml LH, 1㎍/ml estradiol 17β and 10% (v/v) porcine follicular fluid (PFF).

2. Experimental Design

In Experiment 1, oocytes were collected from follicles of 1~2mm, 2~6mm and 6~10mm in diameter. All the oocytes were fixed and stained and examined under a phase-contrast microscope. After collection of oocytes from follicles of 2~6mm in diameter, the oocytes with or without cumulus cells were cultured for 5 h and examined for the assessment of GV stages.

In Experiment 2, oocytes collected from follicles 2~6mm in diameter were cultured for 0, 1, 3 and 5 h for maturation. For examination of changes in GV stages, the oocytes with or without cumulus cells were cultured in the media containing catalase (0.1mg/ml), xanthine (0.7mg/ml) or catalase+xanthine. After culture, the oocytes were fixed and stained, and examined under a phase-contrast microscope for the assessment of GV stages.

3. Classification of Nuclear Morphology of Oocytes at the Germinal Vesicle Stage

The nuclear morphology of bovine oocytes at the GV stage was classified to six stages based on the appearance of both chromatin and nucleolus stages. In the GV-I stage, germinal vesicle have one clear nucleolus. In the GV-Ⅱ stage, nuclear membrane and nucleolus were distinctly visible, condensed filamentous chromatin clumps were observed around the nucleolus and a few were situated near the nuclear membrane. In the GV-Ⅲ stage, filamentous chromatin clumps were localized mainly around the nucleus. In the GV-Ⅳ stage, filamentous chromatin clumps were evenly distributed in the nucleus, and the nucleolus had disappeared completely. In the GV-Ⅴ stage, chromatin was condensed into thick clumps and in the GV-Ⅵ stage, chromatin was condensed in to a single clump and the nuclear membrane was still visible.

4. Assessment of Meiotic Maturation of Oocytes

At the end of experiment, oocytes were freed from cumulus cells by vortexing for 2 min in TCM 199 (No. 31100-035, Gibco) supplemented with 10 mM Hepes, 2 mM $NaHCO_3$, 0.3% BSA (A-4378; Sigma) and 0.2% hyaluronidase from bovine testis (Sigma). Denuded oocytes were then mounted, fixed in 25% (v:v) acetic alcohol for 48 to 72 h at room temperature, and stained with 1% (w/v) orcein in 45% (v/v) acetic acid. The maturation stages of oocytes were examined under a phase-contrast microscope at a magnification of ×400.

Ⅲ. RESULTS

The distribution of nuclear morphology of oocytes when collected from various sizes of follicles is shown in Table 1. The high rate (54%) of the oocytes aspirated from follicles of 1~2mm in diameter were at the GV-II stage followed by the GV-I (23%), GV-III (9%), GV-IV (13%), GV-V (1%) and GV-VI (0%) stages. In oocytes collected from follicles of 2~6mm in diameter, the distribution of GV stages were observed equally at GV-I (28%), GV-II (24%) and GV-III (25%) stages. However, most of oocytes aspirated from follicles of 6~10mm in diameter were distributed at GV-I (42%) and GV-II (33%) stages. On the

Table 1. Nuclear phase of germinal vesicles in oocytes collected from various sizes of porcine follicles

Diameter of follicle (mm)	No. of oocytes examined	No. (%) of oocytes at stage					
		GV-Ⅰ	GV-Ⅱ	GV-Ⅲ	GV-Ⅳ	GV-Ⅴ	GV-Ⅵ
1 ~ 2	252	57(23)[a]	137(54)[a]	22(9)[a]	33(13)	3(1)	0(0)
2 ~ 6	254	70(28)[a]	61(24)[b]	63(25)[b]	36(14)	8(3)	16(6)
6 ~10	152	64(42)[b]	51(33)[b]	23(15)[ab]	7(5)	0(0)	7(5)

[a,b] Within each column, values with different superscripts differ significantly, $P<0.05$.

Table 2. Effect of cumulus cells on morphological change of nuclear phase of germinal vesicle at 5 h of culture of porcine follicular oocytes

Presence of cumulus cells	No. of oocytes examined	No. (%) of oocytes					
		GV-Ⅰ	GV-Ⅱ	GV-Ⅲ	GV-Ⅳ	GV-Ⅴ	GV-Ⅵ
+	71	14(20)	0(0)	20(28)	32(45)[a]	3(4)	2(3)
-	69	18(26)	1(1)	29(42)	18(26)[b]	1(1)	2(3)

[a,b] Values with different superscripts are different ($P<0.05$).

other hand, when oocytes with or without cumulus cells were cultured for 5 h after collection from follicles of 2~6mm in diameter, the rate of oocytes at GV-IV to GV-VI stages were higher in oocytes with cumulus cells (52%) than without (30%) them (Table 2).

In Experiment 2, as shown Table 3, the effect of catalase and/or xanthine on meiotic progression were examined. After 1 h of culture, there was no differences in the frequency of GV-IV to GV-VI stages in medium with none (32%), catalase (34%), xanthine (39%) and catalase+xanthine (36%). At 3 h after culture, the proportions of GV-IV to GV-VI stages were higher in medium with none (61%), catalase (53%) and catalase+ xanthine (53%) than in medium with xanthine (42%). After 5 h of culture, the distribution of GV-IV to GV-VI stages were 46, 69, 69 and 70% for medium with none, catalase, xanthine and catalase+xanthine. The highest rate of GVBD was observed in the medium with catalase+ xanthine (6%).

Ⅳ. DISCUSSION

The present study demonstrates a clear relationship between follicle size and changes in germinal vesicle stages during culture of porcine oocytes. While oocytes for *in vitro* maturation are usually obtained from follicles about 2~6mm in size (Sirard et al., 1992). Oocytes resuming meiosis *in vivo* originate from dominant follicles of about 15mm in size (Pavlok et al., 1992). In pigs, the majority of oocytes collected from follicles 2~6mm in diameter are known to be at very early GV stages (Funahashi and Day, 1993). In this study, oocytes from follicles of various sizes were cultured for 48 h, GVBD rates were not different among follicle sizes. Within ovary, hormones might be

Table 3. Effects of catalase and/or xanthine on nuclear phase of germinal vesicles at various times of culture in porcine oocytes

Periods of culture(h)	Conditions of culture*	No. of oocytes examined	No. (%) of oocytes						
			GV-Ⅰ	GV-Ⅱ	GV-Ⅲ	GV-Ⅳ	GV-Ⅴ	GV-Ⅵ	GVBD
0	Control	117	36(31)	27(23)	28(24)	17(15)	3(3)	6(5)	0(0)
1	None	110	35(32)	25(23)	15(14)	28(25)	4(4)	3(3)	0(0)
	C	124	36(29)	16(13)	29(23)	34(27)	6(5)	3(2)	0(0)
	X	110	26(24)	16(15)	24(22)	37(34)	3(2)	4(3)	0(0)
	C + X	118	24(20)	18(15)	34(29)	29(25)	5(4)	8(7)	0(0)
3	None	145	26(18)	14(10)	29(20)	50(34)	16(11)	10(17)	0(0)
	C	137	28(20)	8(6)	28(20)	41(30)	14(10)	18(13)	0(0)
	X	142	33(23)	11(8)	39(27)	34(24)	15(11)	10(7)	0(0)
	C + X	140	21(15)	16(11)	29(21)	43(31)	17(12)	14(10)	0(0)
5	None	79	9(11)	10(13)	14(18)	17(12)	13(16)	14(18)	2(3)
	C	84	7(8)	11(13)	5(6)	16(19)	21(25)	21(25)	3(4)
	X	81	8(10)	8(10)	8(10)	19(23)	22(27)	15(19)	1(1)
	C + X	81	12(15)	2(3)	5(6)	23(28)	16(20)	18(22)	5(6)

* C : catalase (0.1mg/ml), X : xanthine (0.7mg/ml), C+X: catalase + xanthine,
GV: germinal vesicle, GVBD: germinal vesicle breakdown

important to augment oocyte maturation and follicular development. Although a minority of porcine follicles appeared to undergo atresia throughout oocyte maturation, the number of atretic follicles decreased as ovulation approached. This is consistent with hCG-treated (Ainsworth et al., 1980) and hemiovariectomized (Clark et al., 1982) gilts in which medium and large antral follicles were lost throughout follicular development. Atretic follicles usually contained oocytes classified at the GVBD stage. A some critical event might have occurred which coincident with reinitiation of meiosis. In this study, the FSH, LH and estradiol were supplemented in the media for follicle culture. We suppose that these hormones and oocyte maturation during follicle culture are not correlated.

Motlik and Fulka (1993) observed the breakdown of the GV of oocytes maturing *in vitro* and *in vivo*, and then divided the process into four well-defined stages, based on the chromatin changes, and on nucleolus and nuclear membrane disappearance according to time after hCG injection or *in-vitro* culture. In this study, however, GV stages were divided into six stages based on chromatin changes. Nagai et al. (1997) found that many oocytes collected not only from follicles of prepubertal gilts but also from follicles of 5~8mm in diameter in ovaries of cycling gilts on the 18th~19th day of the cycle were already at GV-Ⅱ to GV-Ⅳ. The results of this study showed that more oocytes from various sizes of follicle were at stages of GV-Ⅰ to GV-Ⅱ (77, 52 and 77%) before culture. Although there is no obvious reason for this difference, different sizes of follicle may be one explanation, as found in this study where oocytes with (52%) than without (29%) cumulus cells showed higher percentages of GV-Ⅳ to GV-Ⅵ stages at 5 h after culture. The beneficial effects of cumulus cells on oocyte maturation have been previously documented. Thus, the selection of oocytes with compact and multiple cumulus layers seems to be essential for successful *in vitro* maturation.

This study was undertaken to further elucidate the effect of catalase and/or xanthine on changes of GV stages during culture. In mammals, all cells are exposed to the risk of injury by active oxygen, which is formed when molecular oxygen is utilized as an electron acceptor during oxidoreductive reactions in the cells. Superoxide anion is major species of active oxygen and is formed by enzymes such as monomine oxidase, xanthine oxidase, and aldehyde oxidase and auto-oxidation reactions such as those of ubiquinone, catechols, ferredoxins and hemoproteins (Noda et al., 1991). The enzyme has been found to be ubiquitous among oxygen-metabolizing organisms (McCord et. al., 1970) and is the major radical scavenger in the defence against oxygen toxicity. Thus, it was expected that the addition of catalase to maturation medium is effective on advance of GV stages during culture. However, the results of this study show that the supplementation of catalase and/or xanthine for *in vitro* maturation of porcine oocytes not effects advance of GV stages at various times after culture. It is difficult to explain at present exactly why there was no different among various conditions with catalase and/or xanthine. It has become increasingly important to achieve a better understanding of oogenesis in porcine. Disparity in GVBD and oocyte maturation is directly related to embryonic development and survival.

In summary, the maturation of oocytes within the follicle cultured *in vitro* were arrested at anaphase-I stage despite of follicle size. When oocytes with or without cumulus cells after collection from follicles of 2~6mm in diameter were cultured for 5 h, the distributions of oocytes at GV-IV to GV-VI stages were higher in oocytes with than without cumulus cells. However, there was no differences in the distribution of GV-IV to GV-VI stages in medium

with none, catalase, xanthine and catalase+xanthine.

V. 요 약

본 연구는 돼지난소로 부터 회수한 난포난자에서 난핵포기 핵상의 형태적 변화에 대하여 검토하였다. 그 결과, 난소에서 직경 1~2mm 또는 6~10mm의 난포로부터 회수한 난자의 대부분이 GV-I 또는 GV-II단계에서 발달이 정지되었다. 직경 2~6mm의 난포로부터 회수한 난자에서 난구세포를 부착 또는 제거하여 5시간 배양했을 때 GV-IV에서 GV-VI단계까지의 비율은 난구세포제거(30%) 보다는 부착(52%)된 난자에서 유의적으로 높게 나타났다 ($P<0.05$). 한편, 난포난자를 catalase, xanthine 및 catalase+xanthine이 첨가된 배양액내에서 1시간 배양했을 때 핵상의 변화에는 차이가 없었으나, 5시간 배양 후 GV-IV에서 GV-VI단계까지의 비율은 대조구, catalase, xanthine 및 catalase+xanthine첨가시 46, 69, 69 및 70%였으며, GVBD의 비율은 catalase와 xanthine이 동시에 첨가된 배양액내에서 가장 높게 나타났다. 이와 같은 결과로부터 난포란의 체외성숙시 catalase와 xanthine의 동시첨가는 GV단계의 핵성숙을 유도하고 돼지난자의 체외성숙에 효과적인 작용을 할 것으로 사료된다.

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