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# Effect of Catalase and/or Xanthine on *In Vitro* Maturation of Porcine Follicular Oocytes

**Sa, S. J., C. K. Park†, H. T. Cheong, B. K. Yang and C. I. Kim**
College of Animal Resources Sciences, Kangwon National University, Chunchon, 200-701, Korea

# 돼지 난포난자의 체외성숙에 있어서 Catalase와 Xanthine의 영향

사수진 · 박춘근† · 정희태 · 양부근 · 김정익
강원대학교 동물자원과학대학

## ABSTRACT

The objectives of the present study were to examine the relationship between catalase (0.1 mg/ml) and xanthine (5 mM) on *in vitro* maturation of porcine follicular oocytes. At 48 h after maturation, the proportions of oocytes matured to metaphase-Ⅱ stage were significantly higher ($P<0.05$) in the medium with control (72%), catalase (73%) or catalase plus xanthine (70%) than of oocytes cultured with xanthine (54%). On the other hand, oocytes cultured in medium with catalase and/or xanthine for 30 h were not significantly different in maturation rates (6~14%). At 36, 42 and 48 h after culture, however, the maturation rates were significantly ($P<0.05$) higher in medium with (49~70%) that than without (29~50%) catalase regardless of presence of xanthine. When the oocytes were cultured with periods prolonged in medium with and without xanthine, the maturation rates did increase with high proportions at 72 h of culture. No significant differences, however, were observed in maturation rates between groups with and without catalase. On the other hand, degenerated oocytes were increased with culture periods, the proportions was significantly ($P<0.05$) lower in medium with (28%) than without (47%) catalase at 120 h of culture. However, there were no significant differences between with and without catalase in medium added xanthine. The parthenogenetic oocytes were observed from 72 h after culture in medium with xanthine, but were no significant differences between with and without catalase. From these results, it is indicated that porcine oocytes may respond to maturation stimulus by 72 h of culture in medium with catalase and xanthine and that parthenogenesis can be obtained with prolonged culture periods.

(Key words : Catalase, *In vitro* maturation, Porcine oocytes, Parthenogenesis, Xanthine)

## Ⅰ. INTRODUCTION

Catalase and superoxide dismutase are the presently known antioxidant enzymes that protect cells

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† Corresponding author : Division of Animal Resources, College of Animal Resource Science, Kangwon University, Chunchon, 200-701, E-mail : parkck@kangwon.ac.kr

from damage produced by various forms of reactive oxygen species (ROS) such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($OH^-$). The ROS have been implicated in numerous pathological states such as aging, inflammation, cancer and heart disease (Marklund, 1986; McCord, 1993; Mruk et al., 1998). The mechanism by which superoxide dismutase protects cells from oxygen radicals is to scavenge $O_2^-$ by converting it into $H_2O_2$, which in turn is broken down into $H_2O$ (Flores et al., 1993) in the cytoplasm by glutathione peroxidase (Jones et al., 1979) and in peroxisomes by catalase (Alvarez et al., 1987). 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 (Bulkely, 1987). There are therefore striking similarities between many known actions of oxygen -derived free radicals and the events leading to oocyte maturation.

Because of their highly reactive nature, free radicals have extremely short half-lives and consequently do not accumulate in tissues at levels that can be readily detected. Many investigators have therefore used indirect means, at least in initial studies, to characterize free radical mechanism in physiological processes (Bulkely, 1987). An indirect approach frequently employed is the use of the highly specific inhibitor of free radicals, superoxide dismutase and catalase. Catalase, which serve as radical scavengers in culture medium, has been found to have beneficial effects on embryonic development *in vitro* in mice (Noda et al., 1989 ; Dumoulin et al., 1992). However, it has also been reported that superoxide dismutase in culture medium did not benefit on embryonic development in mice (Legge and Sellens, 1991) and sheep (Walker et al., 1992). On the other hand, the evidence that ROS can have beneficial effects on sperm functions came from experiments in which spermatozoa that incubated xanthine plus xanthine oxidase in the presence of catalase demonstrated levels of hyperactivation and capacitation in human (Lamirande and Gagnon, 1995). Although correlations have been reported between the effectiveness of ROS and spermatozoa capacitation, the actions of catalase and ROS on *in vitro* maturation of porcine oocytes has not been elucidated.

The objectives of the present study were to examine the relationship between catalase and xanthine during the *in vitro* maturation of porcine follicular oocytes. Reactive oxygen species were produced using a xanthine, which catalyses the reduction of ground state oxygen $O_2$ and $H_2O_2$ by spontaneous dismutation of superoxide.

## Ⅱ. MATERIALS AND METHODS

### 1. Oocyte Preparation

Porcine ovaries were collected from a local slaughterhouse and kept in saline (NaCl, 0.9% w/v ; penicillin (100,000 IU/L); streptomycin (100 mg/L); and amphotericin B (250 μg/L; Sigma Chemical, St-Louis, MO, USA) at 32 to 37℃. Cumulus -oocytes complexes were aspirated from 1- to 5-mm follicles with a 10-ml syringe attached with an 18-G needle. Oocytes were washed 3 times in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Oocytes with a compact and complete cumulus were introduced into droplets of maturation medium (10 oocytes/50-μl droplet), covered with mineral oil and cultured under an 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 (Sigma), 10% fetal calf serum (FCS), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamycin (Sigma), 1 μg/ml FSH (sigma), 5 μg /ml LH (Sig-

ma, 1 $\mu$g/ml estradiol 17$\beta$ (Sigma) and 10% (v/v) porcine follicular fluid.

**2. Experimental Design**

In the first experiment, to evaluate the effect of catalase and/or xanthine on *in vitro* maturation, the follicular oocytes were cultured in maturation medium with catalase (C-6665, Sigma: 0.1 mg/ml), xanthine (X-7375, Sigma: 5 mM) and catalase + xanthine. Oocytes were examined for *in vitro* maturation at 48 h of culture.

In the second experiment, to evaluate changes of *in vitro* maturation at various times of culture, oocytes were examined at 30, 36, 42 and 48 h of culture in maturation medium containing non, catalase, xanthine and catalase + xanthine.

In the final experiment, oocytes were cultured in medium with or without catalase for 48, 72, 96 and 120 h. During the culture, hormones were removed from maturation medium at 24 h of culture and the medium was changed every 2 day. In another experiment, effect of catalase during oocyte culture in the medium with xanthine was examined.

**3. Evaluation of Oocyte Maturation**

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 (acetic acid : ethanol = 1:3) for 2 to 3 days and stained with 1% (w/v) aceto-orcein in 40% (v/v) acetic acid water solution. The maturation stages of oocytes were examined under a phase-contrast microscope at a magnification of × 400. Chi-square analysis with the Yates correction was used to test the significance of individual comparisons for the rates of oocytes maturated, degenerated and parthenogenetic oocytes.

## Ⅲ. RESULTS

To evaluate effect of reactive oxygen species during *in vitro* maturation, oocytes were cultured for 48 h in medium with catalase and/or xanthine. As shown in Table 1, the proportions of oocytes matured to M-Ⅱ stage were significantly ($P<0.05$) higher in medium with control (72%), catalase (73%) and catalase+xanthine (70%) than in oocytes cultured with xanthine (54%).

The effect of catalase and/or xanthine at various time of *in vitro* maturation were examined. When oocytes were cultured for 30 h, the maturation rates (6～14%) were not significantly different among the culture groups (Table 2). However, the proportions of M-II oocytes matured for 36 h were significantly ($P<0.05$) higher in medium with catalase (46%) and catalase+xanthine (49%) than in medium with xanthine (29%). At 42 and 48 h of culture, on the other hand, the maturation rates in medium with control (63 and 66%), catalase (64 and 69%) and catalase+xanthine (65 and 70%) were significantly ($P<0.05$) higher in medium with xanthine (34 and 50%).

Table 3 shows effect of catalase during the cul-

**Table 1. Effects of catalase and/or xanthine on *in vitro* maturation of porcine oocytes**

| Culutre conditions | No. of oocytes examined | No.(%) of oocytes with | | |
|---|---|---|---|---|
| | | GV | P-I～T-I | M-Ⅱ |
| Control | 134 | 8 ( 6) | 30 (22) | 96 (72)[a] |
| Catalase (C) | 132 | 12 ( 9) | 23 (17) | 97 (73)[a] |
| Xanthine (X) | 114 | 15 (13) | 38 (33) | 61 (54)[b] |
| C + X | 115 | 6 ( 5) | 29 (25) | 80 (70)[a] |

GV: germinal vesicle, P-I: prophase-I, T-I: telophase -I, M-Ⅱ: metaphase-Ⅱ.

[a,b] Different letters are significantly different ($P<0.05$).

**Table 2. Effects of catalase and/or xanthine during the various periods of culture on *in vitro* maturation in porcine oocytes**

| Culture conditions | No. (%) of oocytes matured after culture for: | | | |
|---|---|---|---|---|
| | 30 | 36 | 42 | 48 h |
| Control | 4/63 ( 6) | 26/66 (39)[ab] | 40/63 (63)[a] | 40/61 (66)[a] |
| Catalase (C) | 9/66 (14) | 31/67 (46)[a] | 41/64 (64)[a] | 43/62 (69)[a] |
| Xanthine (X) | 5/66 ( 8) | 15/52 (29)[b] | 21/61 (34)[b] | 33/66 (50)[b] |
| C + X | 5/65 ( 8) | 34/70 (49)[a] | 41/64 (65)[a] | 43/61 (70)[a] |

[a,b] $P<0.05$, within the same column.

**Table 3. Effect of catalase on *in vitro* maturation of porcine oocytes during the various periods**

| Periods of culture (h) | Presence of catalase | No. of oocytes examined | No. of oocytes matured | No. of oocytes degenerated | No. of parthenogenetic oocytes |
|---|---|---|---|---|---|
| 48 | + | 93 | 62 (67) | 4 ( 4) | 0 ( 0) |
| | − | 95 | 63 (66) | 7 ( 7) | 0 ( 0) |
| 72 | + | 101 | 80 (79) | 13( 13) | 0 ( 0) |
| | − | 91 | 72 (79) | 8 ( 9) | 0 ( 0) |
| 96 | + | 97 | 56 (58) | 18 (19) | 23 (24) |
| | − | 94 | 61 (65) | 21 (22) | 12 (13) |
| 120 | + | 100 | 37 (37) | 28 (28)[†] | 35 (35) |
| | − | 95 | 29 (31) | 45 (47) | 21 (22) |

[†] $P<0.05$, differences between with and without catalase.

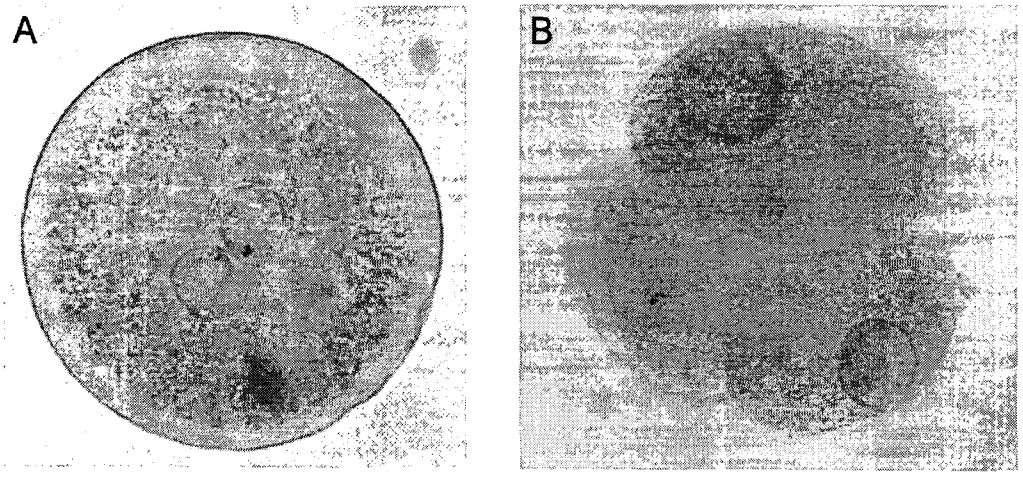


**Fig. 1. Parthenogenetic oocytes obtained at 120 of culture in porcine follicular oocytes. a: Two pronuclei were seen. b: Blastomeres can be seen.**

**Table 4. Effect of catalase on oocyte maturation during the culture of porcine follicular oocytes in medium with xanthine**

| Periods of culture (h) | Presence of catalase | No. of oocytes examined | No. of oocytes matured | No. of oocytes degenerated | No. of parthenogenetic oocytes |
|---|---|---|---|---|---|
| 48 | + | 101 | 76 (75)† | 5 ( 5) | 0 ( 0) |
| | − | 102 | 52 (51) | 7 ( 7) | 0 ( 0) |
| 72 | + | 95 | 58 (61) | 7 ( 7) | 1 ( 1) |
| | − | 92 | 50 (54) | 9 (10) | 4 ( 4) |
| 96 | + | 95 | 55 (58)† | 11 (12) | 15 (16) |
| | − | 103 | 45 (44) | 19 (18) | 23 (22) |
| 120 | + | 100 | 49 (49)† | 14 (14) | 30 (30) |
| | − | 94 | 32 (34) | 25 (27) | 29 (31) |

† $P<0.05$, difference between with and without catalase.

ture with periods prolonged in porcine follicular oocytes. The proportions of oocytes matured to M-Ⅱ stage were not significantly different between with and without catalase despite of different culture periods. At 72 h after culture, the maturation rates increased in medium with (79%) and without (79%) catalase. However, degenerated oocytes were increased with culture periods to 120 h, the proportion was significantly ($P<0.05$) lower in medium with (28%) than without (47%) catalase. On the other hand, the parthenogenetic oocytes were higher in medium with (24 and 35%) than without (13 and 22%) catalase at 96 and 120 h of culture, but catalase did not cause any significant changes on parthenogenesis.

In Table 4, effect of catalase during in vitro maturation of porcine oocytes in medium with xanthine were examined. The maturation rates were decreased with culture periods prolonged and were higher in medium with than without catalase, but were not significantly differences. The rates of degenerated oocytes were also increased with culture periods, but were higher in medium without than with catalase. At 72 h of culture, the parthenogenetic oocytes were observed (Fig. 1) and increased with culture periods prolonged, but there were no significantly differences between medium with and without catalase.

## Ⅳ. DISCUSSION

It is important to be able to culture oocytes and preimplantation embryos *in vitro* with minimal adverse consequences for long-term development and, if this is not possible, to understand the nature of and the reasons for failure. Although the culture of porcine oocytes for *in vitro* maturation has been achieved with increased results, its activation and the consequent resumption of meiosis seem to be regulated differently even among culture systems.

The xanthine is known to produce reactive oxygen species that are involved in cellular degradation in several cell types. The use of specific scavengers such as catalase allowed us to show that $H_2O_2$ and $O_2^-$ play a role in the alterations induced by xanthine. In fact, all the effect of the xanthine on the enzymatic antioxidant defence systems can be inhibited by the direct addition of catalase. The results of this study show that supplementation of xanthine during the culture of porcine follicular

oocytes inhibits the ability of oocyte maturation *in vitro*, and these toxic effect can be inhibited by catalase (Table 1). These results are similar to those of previous studies with spermatozoa in rabbit (Alvarez and Storey, 1983), rat (Fornes et al., 1993), and human (Hong et al., 1991).

Porcine oocytes from antral follicles were used for *in vitro* maturation experiments (Moor et al., 1990), in which duration of culture was less than 48 h. In this study, the proportions of oocytes matured to M-Ⅱ stage were maintained by addition of catalase and xanthine during *in vitro* maturation with various culture periods (Table 2). In culture of immature porcine oocytes, the time required for nuclear maturation among oocytes (Yoshida et al., 1989) and the occurrence of chromosomal abnormalities such as diploid nucleus is high (McGaughey and Polge, 1971). In the present study, it was not examined for *in vitro* fertilization using oocytes cultured in medium with catalase and/or xanthine for 30 to 48 h. However, oocytes obtained from these culture conditions showed normal status morphologically.

In this study, the nuclear maturation of porcine follicular oocytes was observed at 30 h of culture, and show that porcine oocytes may respond to maturation stimulus by 72 h of culture. However, there were not different by addition of catalase during *in vitro* maturation with culture periods prolonged. On the other hand, when oocytes were cultured in medium with xanthine, the maturation rates were higher in medium with than without catalase. It is suggested that oocyte maturation is protected from the oxidative stress under conditions with catalase in medium with xanthine. Mouatassim et al. (1999) reported that catalase transcripts were not detected either in mouse or in human oocytes, regardless of the stage of maturation and low levels of catalase mRNA were detected in the mouse blastocyst. This confirms that these transcripts are rather detected in embryos after genomic activation (Harvey et al., 1995). However, it is unknown whether catalase is synthesized directly in oocyte or is transferred into the oocyte after synthesis in cumulus cells via the oocyte-cumulus junction.

In oocytes matured *in vivo* and *in vitro*, development is arrested at metaphase-Ⅱ until the oocyte is penetrated by a spermatozoon. During meiotic arrest, nuclear status does not changed. However, cytoplasmic changes, for example, viability, ability to be fertilized and ability for parthenogenetic activation of an oocyte, occur by prolonged culture period. This phenomenon is called 'ageing' in a mammalian oocyte. In this study, parthenogenetic oocytes were increased by catalase during the culture with prolonged periods. In oocytes cultured in medium with xanthine, the proportions of parthenogenetic oocytes were higher than in medium without than with catalase, but there were not significantly differences between in medium with and without catalase. Kikuchi et al. (1995) reported that when pig oocytes were electrically stimulated, parthenogenetic activation was low during the early stages of maturation and increased after 60 h of culture. A similar result was observed in the parthenogenetic activation of mouse (Kubiak, 1989) and cattle (Nagai, 1987) oocytes aged *in vitro*.

A combination of catalase and xanthine, an enzymatic free radical-generating system, primarily generates the superoxide anion, which is immediately dismuted into hydrogen peroxide (Ikeda et al., 1999). In this study, when oocytes were cultured for up to 96 to 120 h and then treated with catalase and xanthine, high rates of cultured oocytes appeared to be activated (Table 4). These seemed to result from a normal oocyte activation process as when fertilization occurs, since the female pronucleus was well developed. However, abnormal activation, resulting in fragmentation, after culture

without catalase increased when the duration of maturation was prolonged.

In conclusion, these results indicate that culture with catalase and xanthine has possible system on maturation and development of porcine oocytes *in vitro*. However, further studies are necessary to clarify oocyte ability after *in vitro* fertilization and cytoplasmic changes during ageing and parthenogenesis.

## V. 요 약

본 연구는 미성숙 돼지 난포 난자의 체외성숙에 있어서 catalase (0.1 mg/ml)와 xanthine (5 mM)의 역할에 대하여 검토하였다. 그 결과, 체외에서 성숙배양 48시간후 metaphase-II 단계로 발육한 난자의 비율은 xanthine (54%) 첨가 보다는 대조구 (72%), catalase (73%) 및 catalase+xanthine (70%) 첨가구에서 유의적으로 높은 성숙율을 나타냈다 ($P<0.05$). 한편, 체외에서 30시간 동안 성숙배양한 경우 모든 실험구에서 성숙율의 유의적인 차이는 인정되지 않았으나, 성숙배양 36, 42 및 48시간후 xanthine의 첨가 여부에 관계없이 catalase 무첨가 (29~50%) 보다는 첨가시 (49~70%)에 유의적으로 높은 성숙율을 나타냈다 ($P<0.05$). 체외에서 xanthine의 첨가 또는 무첨가시 배양시간의 연장이 난자의 성숙에 미치는 영향을 검토한 결과 배양 72시간에서 높은 성숙율을 나타냈으며, 퇴행난자의 비율은 배양 120시간에서 catalase 무첨가(47%)에 비하여 첨가시 (28%) 유의적으로 낮게 나타났으나 xanthine이 첨가된 배양액내에서 catalase첨가 유무에 의한 차이는 인정되지 않았다. 또한 xanthine을 첨가하여 72시간 배양한 경우 단위발생란이 처음으로 관찰되었지만 catalase의 첨가 유무에 의한 차이는 인정되지 않았지만 배양시간이 길어짐에 따라 발생비율이 증가하였다. 이와 같은 결과에서 돼지 난포난자는 catalase와 xanthine을 첨가한 배양액내에서 배양 72시간까지 성숙율이 증가할 수 있으며, 배양기간의 연장시 catalase에 의하여 난자의 퇴행을 억제하며 단위발생란의 증가를 가져오는 것으로 생각된다.

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