Nitric Oxide Exerts Different Functions on Porcine Oocytes Cultured in Different Models, Which is Affected by Beta-mercaptoethanol*

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ABSTRACT : The present study was conducted to investigate the involvement of nitric oxide (NO) in cumulus expansion, oocyte mortality and meiotic maturation of porcine cumulus enclosed oocytes (CEOs) cultured in two different models when gonadotropins, including follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) were presented or not. And the interaction between NO and β-mercaptoethanol (β-ME), a free radical scavenger was also investigated. Two models refer to spontaneous maturation model and hypoxanthine (HX) medium model. All the 3,433 eligible CEOs were incubated at 39°C and the cumulus expansion, oocyte morphology and nuclear phase were evaluated 44 h after incubation. (1) In spontaneous maturation model, NO stimulates the cumulus expansion and β-ME delayed it. NO doesn't affect the oocyte meiotic resumption but inhibits the oocytes to develop to metaphase II. (2) In HX medium model, NO or β-ME doesn't affect the expansion in the absence of gonadotropins, but in the presence of gonadotropins, NO or β-ME inhibits the expansion. In the presence of gonadotropins, NO inhibits the oocyte meiotic resumption and it especially inhibits the oocyte to develop to metaphase II, and β-ME reverses such inhibitory effects. The cooperation of gonadotropins and β-ME stimulates the meiotic resumption and especially, promotes the CEOs to develop to metaphase II in both models. Moreover, HX might contribute to the fragility of oocyte zona pellucida and gonadotropins, nitric oxide and β-ME could alleviate it separately, and cooperatively. It is concluded that NO exerts different functions in two models and β-ME affected the functions of NO in different models. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 3 : 317-324)***

Key Words : Nitric Oxide, β-mercaptoethanol, Gonadotropins, Pig, Oocyte, Meiosis

INTRODUCTION

Since Palmer found in 1987 that nitric oxide (NO), a free radical, was just the endothelium-derived relaxing factor (Palmer et al., 1987), there have been increasingly reports on it. By now, it has been proven that NO is involved in a variety of intracellular signaling mechanisms, such as in neutron, circulatory and reproductive systems. Previous studies have shown that NO is involves in the oocyte meiotic maturation in mouse (Jablonka-Shariff and Olson 2000; Bo et al., 2002; Bu et al., 2003), rat (Shukovski and Tsafriri, 1994), human (Mehta et al., 1995), rabbit (Hayashi et al., 1992), Cattle (Honaramooz et al., 1999) and pig (Hattori et al., 2000; Grasselli et al., 2001). However, its functions under various circumstances are not well consistent (Hattori et al., 2000; Nishida et al., 2000; Masuda et al., 2001; Takesue et al., 2001; Grasselli et al., 2001; Sengoku et al., 2001; Nakamura et al., 2002; Bo et al., 2002). On the other hand, nearly all the researches were based on spontaneous maturation culture model in pig and few on physiological maturation model.

It has been reported that sodium nitroprusside was commonly used as an exogenous NO donor to investigate the involvement and mechanism of NO (Yousif et al., 1998; Chen et al., 2001; Sengoku et al., 2001; Bo et al., 2002; Dobashi et al., 2002). The β-mercaptoethanol (β-ME), a low molecular weight thiol tripeptide and a free radical scavenger, can react with free radicals. It can provide cells with a reducing environment and protect against the toxic effect of oxidative damage. It has been reported that β-ME promoted cell viability and enhanced various cell reactions in lymphocytes (Ishii et al., 1981; Abeydeera et al., 1998) and protected bovine embryos from high concentration of oxygen when they were cultured *in vitro* (Takahashi et al., 1993; Hamano et al., 1994; Lim et al., 1996; Caamano et al., 1996; Lim et al., 1996; Kikuchi et al., 1997; Caamano et al., 1998; Geshi et al., 1999; de Matos and Furnus, 2000; Takahashi et al., 2002). Based on these previous reports, 1 mM sodium nitroprusside and 50 μM β-ME were accepted in the present study.

Hypoxanthine (HX), a low molecular weight compound, has been identified as the major inhibitory component existing in the follicular fluid (Miyano et al., 1995). The concentration of HX in several species has been examined and it ranged at 2-4 mM, which seemed enough to exert the inhibitory action on oocyte spontaneous meiotic resumption

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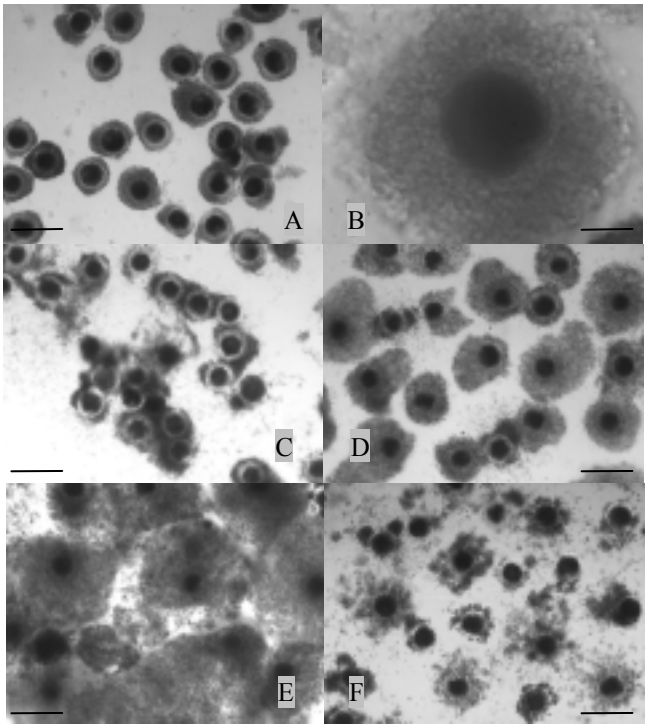

Figure 1. Criteria of ranking the cumulus expansion. Cumulus expansion was assessed 44 h after the onset of cumulus using a subjective scoring method. Briefly, no response was scored as 0 (A, B), minimum observable response was scored as 1 (C), expansion of outer cumulus-enclosed oocytes layers was scored as 2 (D), expansion of all cumulus-enclosed oocytes layers except the corona radiata as scored as 3 (E) and expansion of all cumulus-enclosed oocytes layers was scored as 4 (F). A, C, D, E and F, Bar =200 μm; B, Bar=50 μm.

(Xia et al., 1994; Miyano et al., 1995; Su et al., 1998; Su et al., 1999; Xia et al., 2000; Lu et al., 2000; Lu et al., 2001). Therefore, HX was often used to mimic the physiological situation in follicles.

The research on the development of porcine oocytes might allow many biomedical applications including xenotransplantation in human beings and the creation of bioreactors for industry (Lee and Moran, 2001; Okere and Nelson, 2002). The objective of the present study was undertaken to evaluate how the NO released by sodium nitroprusside affects cumulus expansion and meiotic maturation of porcine CEOs cultured in different maturation models and if β-ME affected the function of NO.

MATERIALS AND METHODS

All reagents and chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Collection of porcine oocytes

Ovaries were collected from prepubertal gilts at local slaughterhouse and transported to the laboratory in a thermal container containing sterile saline at 38°C within 3 h post-slaughter. The ovaries were rinsed two times with the 75% ethanol and immediately with the same saline for four times. The contents of follicles of 2 to 6 mm in diameter on the ovarian surface were aspired with a 10 ml syringe equipped with 18 gauge needle. The mixture was collected to a 15 ml centrifuge tube. After deposition, the supernatant was centrifuged for porcine follicular fluid (pFF) and the sediment was the pellet of oocytes. Only oocytes with a uniform ooplasm and a compact cumulus cell mass was picked up into plastic dishes of 35 mm in diameter by a narrow-bore pipette. The oocytes were washed 3 times with Tyrode's lactate-Hepes medium with polyvinyl (PVA) (TL-Hepes-PVA) and then washed three times in maturation medium warmed up.

Collection of pFF

The pFF was harvested simultaneously when the oocytes were collected. The porcine follicular fluids were pooled from antral follicles (2-6 mm diameter) described as above. After the CEOs and any other oocytes are removed, the fluid was centrifuged at 1,000×g at 4°C for 15 min (Tatemoto et al., 2001). The supernatant was collected, supplemented with antibiotic, filtered with membrane filters with 0.22 μm pores and stored at -20°C.

IVM of porcine oocytes

The basic maturation medium was North Carolina State University 37 (NCSU37) (Funahashi et al., 1994) supplemented with 5.55 mM Glucose, 1.00 mM Glutamine, 0.6 mM L-cysteine, 100 iu/ml penicillin G potassium, 50 ug/ml streptomycin sulfate and 10% porcine follicular fluid (pFF, v/v). About 50 CEOs were incubated in each 500 μl medium in a 4 well dish (Nunc, Roskilde, Denmark). The culture was carried out at 39°C in an atmosphere with 5% CO_2 in air for 44 h.

Assessment of cumulus expansion

Cumulus expansion was assessed 48 h after the onset of cumulus using a subjective scoring method (Downs, 1989; Vanderhyden, 1993). Briefly, no response was scored as 0, minimum observable response was scored as 1, expansion of outer CEOs layers was scored as 2, expansion of all CEOs layers except the corona radiata as scored as 3 and expansion of all CEOs layers was scored as 4 (Figure 1). The value of cumulus expansion in a well was recorded as the value of the majority since most of CEOs in a well had a same level of expansion.

Examination of morphology and nuclear phase

The CEOs were free from cumulus cells by flushing 30 times mildly with 200 μl tip (yellow) 44 h after incubation.

Table 1. Porcine cumulus expansion 44 h after *in vitro* culture

Treatment		N	Cumulus expansion
Spontaneous maturation model	Control	162	1
	Gonadotropins	171	3
	Sodium nitroprusside	187	3
	β-ME	205	2
	Gonadotropins+sodium nitroprusside	203	4
	Gonadotropins+β-ME	251	3
	Sodium nitroprusside+β-ME	244	2
	Gonadotropins+sodium nitroprusside+β-ME	269	4
Hypoxanthine medium model	Control	183	1
	Gonadotropins	186	4
	Sodium nitroprusside	190	1
	β-ME	220	1
	Gonadotropins+sodium nitroprusside	238	3
	Gonadotropins+β-ME	245	3
	Sodium nitroprusside+β-ME	249	1
	Gonadotropins+sodium nitroprusside+β-ME	230	2

Four trails were conducted. β-ME=β-mercaptoethanol. The cumulus expansion was scored as one value according to the majority since most of cumulus-enclosed oocytes in a well had a same level of expansion.

The morphology of the denuded oocytes was examined under stereomicroscope and then the normal oocytes were selected and then mounted on the glass slides after rinsing with saline. They were fixed for 48 to 72 h in acet-ethanol (acetic:ethanol=1:3, v/v), and then stained with 1% acetic-orcein for 5-10 min. Finally, the nuclear phase of oocytes on glass slides were observed under the phase-contrast microscope. The oocytes with germinal vesicle (GV), germinal vesicle breakdown (GVBD) and polar body 1 (PB1) were evaluated respectively (Wu et al., 2002).

Experimental design

Experiment 1 : This experiment aimed to examine the effects of NO in the presence or absence of gonadotropins in spontaneous maturation model. Therefore, 1 mM sodium nitroprusside, 50 μM β-ME (Merck KGaA, Darmstadt, Germany), 0.05 IU/ml FSH and 0.05 IU/ml hCG (Lovens Kemiske Fabrik Ballerup, Denmark) were supplemented separately or concomitantly to the medium at the very beginning of incubation. Cumulus expansion, oocyte morphology and oocyte nuclear phase were observed 44 h after incubation.

Experiment 2 : This experiment aimed to examine the effects of NO in the presence or absence of gonadotropins in HX medium model. Bases on Experiment 1, 4 mM HX was supplemented to the medium.

Statistical analysis

Data were expressed as means±SEM. Differences among the treatments in each experiment were analyzed by ANOVA using the Duncan multiple range test of Statistical analysis system (SAS).

RESULTS

Effects of sodium nitroprusside and β-ME on cumulus expansion

Cumulus expansion was evaluated 44 h after incubation (Table 1). In both models, gonadotropins strongly stimulated the cumulus expansion.

In spontaneous maturation model, sodium nitroprusside stimulated the cumulus expansion in the absence, which was delayed by β-ME. In the presence of gonadotropins, sodium nitroprusside also stimulated the expansion, which was not delayed by β-ME.

In the HX medium model, sodium nitroprusside did not facilitate the cumulus expansion and β-ME did not affect it in the absence of gonadotropins. In the presence of gonadotropins, sodium nitroprusside delayed the expansion.

Effects of sodium nitroprusside and β-ME on oocytes ZP fragility

In order to evaluate the fragility of oocyte zona pellucida (ZP), the cumulus cells were stripped from oocytes by flushing with 200 μl tip 44 h after incubation, and then the oocytes were observed under stereomicroscope (Figure 2). The effects of sodium nitroprusside and β-ME on the proportion of oocytes with only empty ZP were shown in Figure 3 with statistical analysis.

In the spontaneous maturation model, the proportion of empty ZP oocytes was significantly lower than other treatments or control when sodium nitroprusside and β-ME were supplemented simultaneously ($p<0.05$).

In the HX medium model, the control has significantly higher proportion of empty ZP oocytes than any treatments ($p<0.05$). Among the treatments, co-existence of sodium

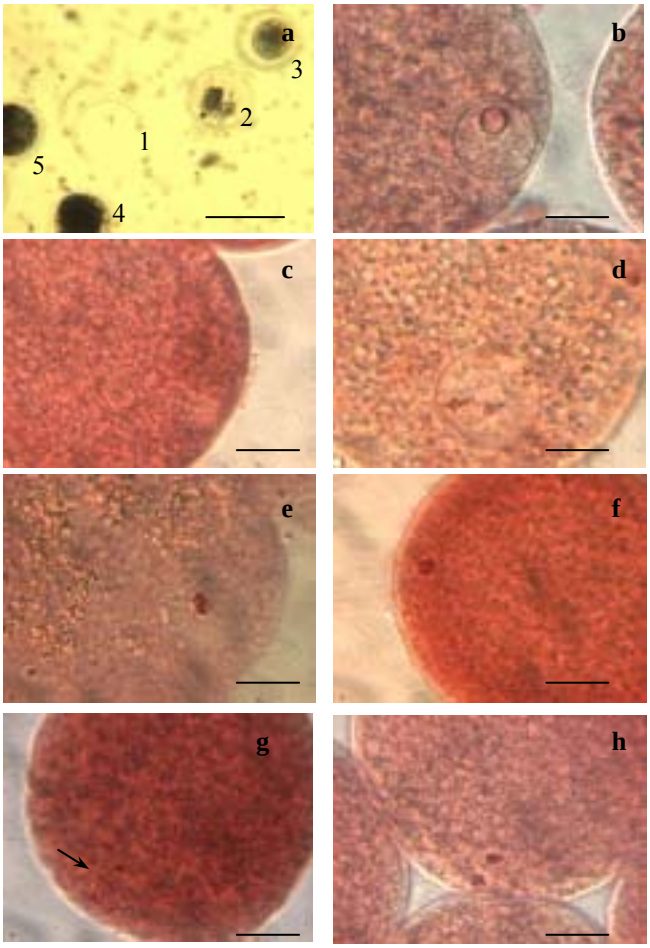

Figure 2. Representative morphology of porcine oocytes. (a) Degenerative porcine oocytes, including those with empty zona pellucida (1), little ooplasm (2), abnormal morphology (3) and yellow ooplasm (4) and morphologically normal oocyte (5), Bar=200 μM. Germinal vesicle (GV) oocytes including (b) GVI, a clear nucleolus has a condensed chromatin ring, (c) GVII, a few orcein-positive structures are visible on the nuclear membranes, (d) GVIII, clumps of chromatin are visible especially around the nucleolus and (f) GVIV, the nuclear membrane is less distinct and the nucleolus has disappeared completely. (g) Germinal vesicle breakdown (GVBD) oocyte with Spindle (spindle, arrow) visible. (h) PB1 is visible nearby the nuclear chromatin; Bar=50 μM.

nitroprusside and β-ME again caused lowest percentage of empty ZP oocytes.

Effects of sodium nitroprusside and β-ME on meiotic resumption

Effects of sodium nitroprusside on the proportion of oocyte meiotic resumption were shown in Figure 4 with statistical analysis. Compared to the control, gonadotropins treatment significantly lowered the proportion of oocytes arrested at GV stage either in spontaneous maturation model (16 vs. 30%, $p<0.05$) or in HX medium model (27 vs. 47%, $p<0.05$).

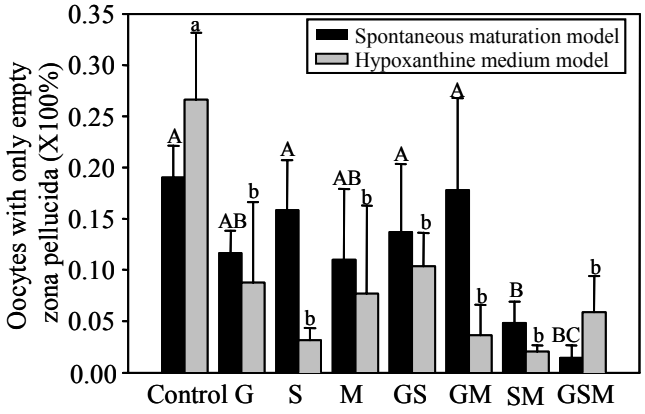

Figure 3. Effects of sodium nitroprusside and β-mercaptoethanol on the proportion of oocytes with only empty zona pellucide. The height of the bars indicates the mean % and the standard error of the mean of four separate experiments. Different letters on the bars lacking common ones in the same model denote significant differences ($p<0.05$). G=gonadotopins, S=sodium nitroprusside, M=β-mercaptoethanol. GS=gonadotopins+sodium nitroprusside, GM=gonadotopins+β-mercaptoethanol, MS=β-mercaptoethanol+ sodium nitroprusside, GSM=gonadotopins+sodium nitroprusside +β-mercaptoethanol.

In spontaneous maturation model, sodium nitroprusside did not affected the meiotic resumption in the absence of gonadotropins (23 vs. 30%, $p>0.05$). Concomitant addition of β-ME caused significantly higher percentage of GV oocytes (38 vs. 30%, $p<0.05$). β-ME alone had no effect on the percentage of GV oocytes. In the presence of gonadotropins, however, sodium nitroprusside significantly increased the proportion of GV oocytes (31 vs. 16%, $p<0.05$), which was not affected by addition of β-ME.

In the HX medium model, the treatment of sodium nitroprusside did not significantly change the proportion of GV oocytes compared to the control group (42 vs. 47%, $p>0.05$). Concomitant addition of β-ME caused significantly higher percentage of GV oocytes than sodium nitroprusside treatment (57 vs. 42%, $p<0.05$). In the presence of gonadotropins, sodium nitroprusside caused significantly higher proportion of GV oocytes (45 vs. 27%, $p<0.05$), and this could be reversed by β-ME. The β-ME alone did not change the meiotic resumption, but in the presence of gonadotropins, it significantly ($p<0.05$) reduced the proportion of GV oocytes (12 vs. 27%, $p<0.05$).

Effects of sodium nitroprusside and β-ME on the proportion of metaphase II oocytes

Effects of sodium nitroprusside on the proportion of oocyte with PB1 were shown in Figure 5 with statistical analysis. Gonadotropins significantly increased the proportion of oocytes with PB1 either in spontaneous maturation model (49 vs. 38%, $p<0.05$) or in HX medium model (35 vs. 25%, $p<0.05$).

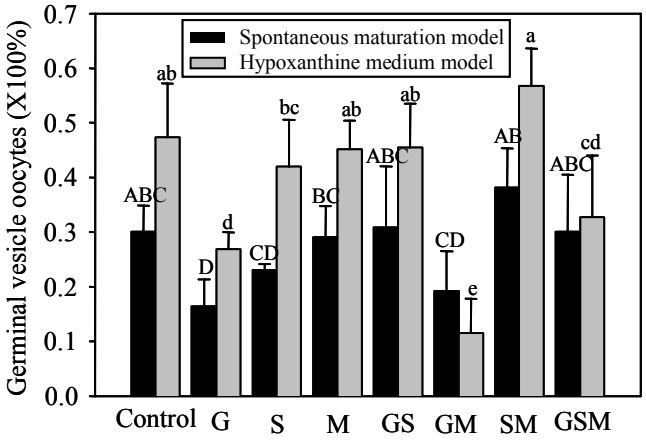

Figure 4. Effects of sodium nitroprusside and β-mercaptoethanol on the proportion of germinal vesicle oocytes. The height of the bars indicates the mean % and the standard error of the mean of four separate experiments. Different letters on the bars lacking common ones in the same model denote significant differences ($p<0.05$). G=gonadotopins, S=sodium nitroprusside, M=β-mercaptoethanol. GS=gonadotopins+sodium nitroprusside, GM= gonadotopins β-mercaptoethanol, MS=β-mercaptoethanol+ sodium nitroprusside, GSM=gonadotopins+sodium nitroprusside +β-mercaptoethanol.

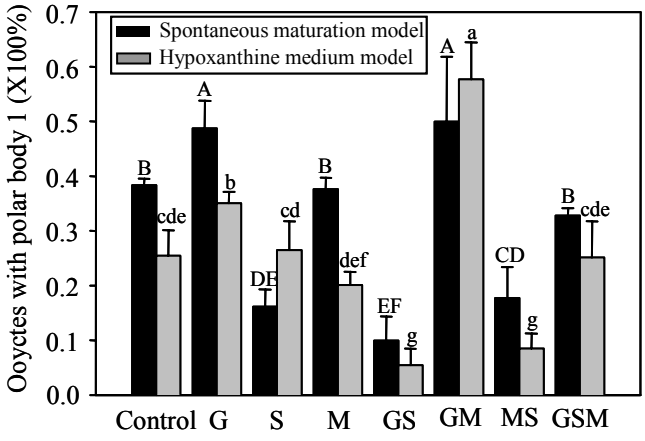

Figure 5. Effects of sodium nitroprusside and β-mercaptoethaneol on porcine oocytes with polar body 1. The height of the bars indicates the mean % and the standard error of the mean of four separate experiments. Different letters on the bars lacking common ones in the same model denote significant differences ($p<0.05$). G=gonadotopins, S=sodium nitroprusside, M=β-mercaptoethanol. GS=gonadotopins+sodium nitroprusside, GM= gonadotopins+β-mercaptoethanol, MS=β-mercaptoethanol+ sodium nitroprusside, GSM=gonadotopins+sodium nitroprusside+ β-mercaptoethanol.

In spontaneous maturation model, sodium nitroprusside significantly reduced the proportion of PB1 oocytes (16 vs. 38%, $p<0.05$). Such effect could not be affected by β-ME. In the presence of gonadotropins, sodium nitroprusside also significantly inhibited the extrusion of oocytes (10 vs. 38%, $p<0.05$), which was reversed by β-ME.

In the HX medium mode, sodium nitroprusside had no effect on the proportion of PB1 oocytes compared to the control, and concomitant addition of β-ME significantly reduced the percentage of PB1 oocytes (8 vs. 25%, $p<0.05$). In the presence of gonadotropins, sodium nitroprusside also caused significantly lower proportion of PB1 oocytes (5 vs. 25%, $p<0.05$), which was completely reversed by β-ME.

In both models, β-ME itself had no effects on the proportion of PB1 oocytes compared to the respective control.

DISSCUSION

We find in the present study that NO and β-ME exerts different functions in two models at three aspects. Meanwhile, their functions are affected by gonadotropins. Briefly, (1) in spontaneous maturation model, NO stimulates the cumulus expansion and β-ME delayed it. NO doesn't affect the oocyte meiotic resumption but inhibits the oocytes to develop to metaphase II. (2) In HX medium model, NO or β-ME doesn't affect the expansion in the absence of gonadotropins, but in the presence of gonadotropins, NO or β-ME inhibits the expansion. In the presence of gonadotropins, NO inhibits the oocyte meiotic resumption, and it especially inhibits the oocyte to develop to metaphase II, and β-ME reverses such inhibitory effects.

Among the factors investigated in the present study, the cooperation of gonadotropins and β-ME stimulates the meiotic resumption, and, especially, promotes the CEOs to develop to metaphase II in both models. HX might contribute to the fragility of oocyte ZP and gonadotropins, nitric oxide and β-ME could alleviate it separately and cooperatively.

As previous studies, NO affected the cumulus cells activity, such as synthesis of cGMP and hyaluronic acid (Jablonka-Shariff and Olson, 2000; Grasselli et al., 2001; Nakamura et al., 2002). We find that in spontaneous maturation model, NO stimulated the cumulus expansion and β-ME delayed it, but in HX medium model, NO did not affect the expansion in the absence of gonadotropins while inhibited the expansion in the presence of gonadotropins. This indicates that the effect of NO on cumulus expansion was also affected by HX and gonadotropins. The latter was strongly responsible for promoting expansion while HX inhibited expansion (Downs, 1989; Su et al., 1998; Su et al., 1999). The growth of cumulus cells was affected and then the expansion of cumuli oophori was facilitated (Hattori et al., 2000). As a free radical scavenger, β-ME understandably affected the functions of NO and these effects were inevitably affected by HX and gonadotropins. We provided the evidence that β-ME could scavenge, at least partly, the free radical released by sodium

nitroprusside. As for the mechanism, previous reports were not consistent. Some presumed that the possible mechanism was that cumulus expansion in porcine oocytes were stimulated to secrete oocyte factor, which stimulated glycosaminoglycan (GAG) synthesis in cumulus cells and then GAGs interacted with serum factor and accumulated in pericellular space. Some others demonstrated that porcine cumulus expansion was independent of the oocyte and it was due to a substance(s), which was 6.5 kDa, resistant to high temperature, freezing, thawing and proteinase K digestion (Sato et al., 2001).

Our present study shows that HX caused the ZP fragility of oocytes after *in vitro* maturation. We speculate that HX could compromise the ZP tenacity of oocytes and make it flimsy, so the ooplasm enclosed in it spilled out easily. In the late stage of follicular development, the follicles who failed to ovulate might tend to be atresia and the follicle-enclosed oocytes might tend to be degenerative. As our speculation, HX might be involved in the process. The injury could be alleviated by supplementation with any of gonadotropins, sodium nitroprusside and β-ME, which suggested the protective properties of them. It appears that gonadotropins and NO existed in the follicles might also exert the protection.

It seems that β-ME has double function. In the presence of sodium nitroprusside, β-ME can react with NO. On the other hand, β-ME can provide the oocytes with a reducing surrounding to protect the ooctyes from oxidative damage. It has been convinced that in the maturation medium β-ME can increase the level of glutathione (GSH), which directly scavenges the oxidative reagents (Takahashi et al., 1993; Kikuchi et al., 1997; Abeydeera et al., 1998; de Matos and Furnus, 2000).

It has been reported that gonadotropins stimulated oocyte meiotic resumption while HX inhibited the process (Downs, 1989; Funahashi et al., 1994; Xia et al., 1994; Su et al., 1998; Su et al., 1999; Xia et al., 2000; Lu et al., 2000; Lu et al., 2001; Bo et al., 2002). Based on these reports, we established two models. And we find that NO exerted different functions in different models. Even in the same model, it functioned differently in the absence or in the presence of gonadotropins.

It was reported that NO inhibited oocyte meiotic maturation in rat (Nakamura et al., 2002) but promotes the meiotic resumption in mouse (Bo et al., 2002). NO might play a biphasic role in reproduction. A narrow range of NO concentrations, usually low, could stimulate meiotic maturation, but either a lack of NO or too much NO has negative consequences (Zackrisson et al., 1996). Sodium nitroprusside is a strong NO releasing reagent and 1 mM used in the present study was considerable concentration. We find that in HX medium model, NO didn't affect the meiotic resumption in the absence of Gonadotropins, but inhibited the oocyte meiotic resumption in the presence of Gonadotropins, and it especially inhibited the oocyte to develop to metaphase II. In contrast, in spontaneous maturation model, NO doesn't affect the oocyte meiotic resumption but inhibits the oocytes to develop to metaphase II. The different effects indicate that the functions of NO are closely related to the surrounding conditions. For instance, NO can interact with gonadotropins. The evidence is that FSH suppressed the synthesis of nitric oxide in porcine oocytes (Hattori et al., 2000). NO might function differently in the follicles before ovulation by a subtle mechanism(s) to regulate the oocyte meiotic maturation.

As previously reported, NO stimulates the genylate cyclase (GC) in granule cells and produces a high cyclic guanosine monophosphate (cGMP) concentration which plays an important role in maintaining the meiotic arrest of oocytes (Hattori et al., 2000; Nishida et al., 2000; Nakamura et al., 2002; Bo et al., 2002). The level of both cGMP and cyclic adenosine monophosphate (cAMP) decreased in oocytes parallel to spontaneous meiosis and that the microinjection of these substances into oocytes caused a delay in oocyte maturation (Nakamura et al., 2002). The cGMP maintains the meiotic arrest of preovulatory oocytes via two pathways: one involving sustenance in cAMP level by inhibition of oocyte cAMP phosphodiesterase and the other involving activation of cGMP-dependent protein kinase in oocytes (Nakamura et al., 2002). But the exact mechanism is not clear and needs further study.

For the first time, β-ME was introduced to investigate the interaction with NO. We find that in the spontaneous maturation model, β-ME inhibited the effect of NO on meiotic resumption in the absence of gonadotropins but not in the presence of gonadotropins. Interestingly, in the HX medium model, β-ME suppressed the effect of NO on oocyte meiotic resumption in the presence of gonadotropins but not in the absence of gonadotropins. It suggests that the interaction of β-ME and NO could be affected by the circumstances. In both models, β-ME itself had no effects on the meiotic resumption and development to metaphase II. This indicates that β-ME dose not participate the oocyte meiotic maturation directly.

The β-ME also interacted with gonadotropins. We find that the concomitant treatment of gonadotropins and β-ME stimulated the meiotic resumption and especially, promotes the CEOs to develop to metaphase II in both models used in the present study. Furthermore, the cooperation of gonadotropins and β-ME promotes the CEOs to develop to metaphase II in both models. This might be due to the fact that β-ME changed the oxidative conditions and create reducing surroundings to facilitate the meiotic maturation (Caamano et al., 1996; Kikuchi et al., 1997; Abeydeera et al., 1998; Geshi et al., 1999; de Matos DG and Furnus CC,

2000; Takahashi et al., 2002).

In conclusion, HX might contribute to the fragility of porcine zona pellucida, and nitric oxide is involved in the cumulus expansion, meiotic resumption and zona pellucida fragility of porcine cumulus-enclosed oocytes but its functions are affected by HX and gonadotropins. The β-ME affects the nitric oxide function depending on the different circumstances.

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