IN VITRO MEIOTIC MATURATION OF MOUSE OOCYTES: ROLE OF NITRIC OXIDE

F. Amidi[1]. M. Abbasi[1], M. Akbari[1], E. Sato[2], A. R. Dehpour[3], S. Ejtemaei-Mehr[3] and F. Abolhassani[1*]

1) Department Of Anatomy, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran
2) Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Japan
3) Department Of Pharmacology, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran

Abstract- In this experiment we used cultured mouse cumulus cell-enclosed oocytes (CEOs) and denuded oocytes (DOs) to study the function of nitric oxide (NO) in mouse oocyte meiotic maturation. CEOs and DOs were cultured in a medium containing 4 mM hypoxanthine (HX) to maintain meiotic arrest, in maturation medium (without HX) supplemented with different doses of sodium nitroprusside (SNP, a NO donor), and in *N*-omega-nitro-L-arginine methyl ester (L-NAME) (inhibitor of NO synthase). NOS inhibitor suppressed the formation of first polar body (PB1) of the oocytes in CEOs in a dose dependent manner, but no effect on germinal vesicle break down (GVBD) was observed. Treatments of low concentrations of SNP stimulated significantly the oocyte meiotic maturation of CEOs which were inhibited with HX, but had no effect on DOs in the same culture medium. The treatment with high concentrations of SNP (0.1-2 mM) during the CEOs cultured in maturation medium resulted in a lower percentage of oocytes at PB1 stage and a higher percentage of atypical oocytes. A dose of SNP at 1 mM exhibited significant inhibitory effect on the formation of PB1, and the number of atypical oocytes compared with control. The results showed that the treatment with 1 mM concentration of SNP could significantly delay GVBD during the first 5 h culture period. The concomitant addition of L-NAME with SNP did not reverse the inhibitory effect of SNP on CEOs. Pre-incubation use of SNP did not have any effect on oocyte maturation. These data support the idea that NO could act in mouse meiotic maturation depending on its concentration.
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Key words: Nitric oxide; NOS inhibitor; mouse oocyte maturation

INTRODUCTION

Mammalian oocytes are arrested in the prophase of the first meiotic cycle throughout much of their development until the ovulatory surge of gonadotropin. Both cumulus cell-enclosed GV-stage oocytes (CEOs) and denuded GV- stage oocytes (DOs) isolated from pregnant mare's serum gonadotrophin (PMSG)-primed immature mice undergo spontaneous hormone-independent maturation and progress to the metaphase II (MII) stage *in vitro* when cultured in a suitable medium, but the developmental competence of oocytes matured *in vitro* in absence of cumulus cells is compromised compared with IVM as CEOs, and especially compared with those matured *in vivo* (26, 28). This has led to the concept that meiotic arrest is maintained by some intrafollicular inhibitory substances (34). Two major *in vitro* models have

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*** Corresponding Author:**
F. Abolhassani, Department of Anatomy, School of Medicine, Medical Sciences/University of Tehran , Tehran, Iran
Tel: +98 21 66419072
Fax: +98 21 66419072
E-mail: abolhasf@sina.tums.ac.ir

been employed for investigating the control of meiotic maturation in mammalian oocytes. One model is spontaneous maturation model without any need for hormonal stimulation (33, 10). Another model indicates that oocytes is cultured in medium containing specific molecules that are thought to play a physiological role in the maintenance of meiotic arrest including cyclic adenosine 3, 5-monophpsphate (cAMP) analogs (39, 13), phosphor-diestrase (PDE) inhibitors or purines such as hypoxanthine or guanosine when released from their follicles (8, 12, 9).

Nitric oxide (NO) is a ubiquitous, endogenously produced free radical. It has been identified as a major biologic signal, exerting both inter- and intra-cellular effects and has been implicated in the modulation of platelet function and in the regulation of blood flow, macrophage cytotoxicity, and neurotransmission (35). NO is synthesized from L-arginine by the catalytic action of NO synthases (NOS) (29). Three isoforms of NOS have been identified, grouped in two subclasses based on cofactor requirements. They are inducible NOS (iNOS) (43) and constitutive NOS (cNOS) including neuron NOS (nNOS) (4) and endothelium NOS (eNOS) (25). The cNOS are calmodulin (Ca^{2+})-dependent isoforms that are involved in cellular signaling, while iNOS is an inducible isoform produced only in response to a stimulus and is not Ca^{2+} dependent. All three isoforms of NOS have been identified in the female reproductive system where NO plays an important role in a variety of reproductive functions (6). In the ovary, NOS has a well-established presence, and has been shown to participate in a variety of reproductive process in the ovary physiology, such as follicular development (19), ovulation (11, 36, 3, 18, 20, 21, 32), corpus luteum function (30), and oocyte meiotic maturation (18-20).

A few studies using L-NAME to inhibit endogenous NO and sodium nitroprusside (SNP) to give extrogenous NO demonstrated that NO is essential for optimal meiotic maturation of murine cumulus-oocyte complexes both *in vitro* and *in vivo* (20, 19, 24). But, the effects of NO on the morphologic defect of the oocytes remain a subject of debate. Jablonka-Shariff reported that eNOS deficiency has been shown to impair the morphology of oocytes significantly in the experiment using eNOS knock-out mice or NOS inhibitor. However, Kazuo reported that treatment of NOS inhibitor did not impair the morphology of oocytes. Perhaps NO's action depended on doses in oocyte maturation just as inhibiting or stimulating secretion of testosterone, production of cyclic GMP, and regressing of corpus luteum depending on the type of cell or duration and amount of NO exposure (40, 30).

So, utilizing oocytes isolated from PMSG-primed immature mice, combined with the *in vitro* serum-free system, we studied the effects of L-NAME, a pharmacological inhibitor of NOS, SNP, a commonly used NO donor, and HX-arrested maturation. The aim of the present study was to examine the function of NO on the oocyte meiotic maturation and thereby determine whether NO can overcome the inhibitory effect of HX, which is a physiological inhibitor of (PDE), whether NO play on the isolated mouse oocytes, and whether NO has toxic effect on the morphology of oocytes. The first operation was conducted in the presence of HX in order to mimic the natural inhibitory function. Then, the oocytes were exposed to different doses of above-mentioned medicines in a medium with or without HX for 24 h.

MATERIALS AND METHODS

Reagents

Hypoxanthine (HX), Sodium nitroprusside (SNP), N^{w}-nitro-L-arginine methyl ester (LNAME), PMSG, were obtained from Sigma Chemicals Co. M199 and bovine serum albumin (Sankyo Kagaku, Tokyo, Japan). Sodium nitroprusside (SNP), N^{w}-nitro-L-arginine methyl ester (L-NAME) were directly dissolved in culture media and used immediately.

Isolation and culture of oocytes

Oocytes were obtained from ICR mice were purchased from Japan SLC, inc. (Shizuoka, Japan), and bred in laboratory of animal reproduction, Tohoku University, Sendai, Japan. Immature 20- to 23-day –old mice were used for all experiments. In

conducting the research described in this study, the investigators adhered to the guide for the care and use of laboratory animals published by Tohoku University. The mice received an intra-peritoneal injection of 10 IU/0.1 ml PMSG. Forty-eight hours later, the animals were killed by cervical dislocation. The ovaries were dissected out and placed in Leibovitz's L15 medium (Invitrogen, Grand Island, NY) containing 4 mM HX, 10 mg/ml bovine serum albumin (Sankyo Kagaku, Tokyo, Japan), in order to maintain the oocytes at the GV- stage. For the culture of denuded oocytes (DOS) from cumulus cells, were obtained by flushing a portion of the CEOs (repeat pipetting) through a small fine-bore-mouth-controlled pipette. The culture medium used for spontaneous meiotic maturation experiments was Waymouth's MB752/1 medium (Invitrogen) containing 5% bovine serum albumin (Sankyo Kagaku, Tokyo, Japan), 0.23 mM pyretic acid (Nacalai Tesque, Kyoto, Japan), 75 mg/L penicillin G (Meiji Seika, Tokyo, Japan); 50 mg/L streptomycin sulfate (Meiji Seika). This medium was termed as maturation medium. A medium similar to the above but with HX was designated as medium+HX. The oocytes were isolated in L15+HX under a stereomicroscope by manual rupture of follicles using a pair of 26 gauge needle. The spherical oocytes with intact cumulus cells and germinal vesicle were categorized as CEOs. Prior to each experimental set-up, all oocytes were pooled before being distributed to the culture dish containing 200 micro liter of the HX-medium or HX-free medium. Each drop contained 20-30 oocytes and was cultured at 37 °C in 100% humidity air with 5% CO_2.

Experiment design

Test 1: Dose effects of NO donor (SNP) on oocyte maturation in the presence of HX: to test whether NO was involved in the mechanism of HX induced meiotic arrest, CEOs were cultured for 24 h in the medium + HX supplemented with different doses of SNP (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M). Moreover, DOs were cultured in the medium + HX with the optimal dose of SNP on CEOs for 24 h to evaluate the involvement of cumulus cells on oocyte maturation.

Test 2: Dose effects of NOS inhibitors on spontaneous oocyte maturation: to evaluate the function of endogenous NO on the spontaneous oocyte maturation, CEOs were cultured for 24 h in maturation medium with L-NAME in concentration range of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M. Moreover, DOs were also cultured for 24 h in maturation medium plus or minus the optimal dose of L-NAME which were proved to have a function on CEOs.

Test 3: Effects of NO donor (SNP) on L-NAME-inhibited oocyte maturation CEOs were cultured for 24 h in maturation medium with L-NAME (10^{-3} M) alone or with L-NAME (10^{-3} M) +SNP (10^{-5} M).

Test 4: Dose effects of NO donor (SNP) on spontaneous oocyte maturation: different doses of SNP (0.1–1 mM) were added to the maturation medium to culture CEOs for 24 h, respectively, to evaluate the function of exogenous NO on the spontaneous oocyte maturation. Moreover, DOs were incubated in maturation medium without (control) or with those doses for 24 h.

Test 5: Effects of NO donor (SNP) on the time course of germinal vesicle break down (GVBD) occurrence: To demonstrate the importance of exogenous NO during GVBD, CEOs were cultured in maturation medium plus or minus the optimal concentration of SNP on the formation of first polar body (PB1) in CEOs. During the first 5 h the number of oocytes in GV and GVBD were counted at 1-h intervals and again after 24 h.

Examination of oocytes

Twenty-four hour after in vitro culture of CEOs or DOs, oocytes were assessed for meiotic stage under an inverted microscope (phase contrast ulwcd 0.30) (Olympus, Japan) and classified as the following stages:

(1) GVBD, healthy oocytes showing GVBD (the outline of the GV had disappeared, but no polar body had been released); (2) PB1, healthy oocytes with a polar body; and (3) atypical oocytes showing degenerative changes or atypical morphology: cytoplasmic fragmentation, an irregular shape of the vitellus, etc.

The percentage of oocytes at each stage of meiosis was calculated by dividing the number of oocytes at each stage by the total number of oocytes.

Statistical analysis

All data are presented as the mean ± S.E.M. Each experiment was repeated at least four times. Significant differences between oocytes in different concentrations of chemicals were determined for various parameters using an independent *t* test.

RESULTS

SNP (10^{-6}, 10^{-5} M) significantly increased the percentage of GVBD and PB1 in a dose-dependent fashion in CEOs compared with control (Fig. 1), while the other concentrations of SNP exhibited stimulatory effects on GVBD but caused some atypical oocytes. The percentage of PB1 reached to the maximum in the dose of 10^{-7} M (47.05 ± 2.66%). Low concentrations of SNP promote oocyte maturation in the presence of HX in CEOs but not in DOs.

SNP, at a concentration of 10^{-5} that could effectively induce HX-arrested meiotic resumption of CEOs did not promote HX-arrested meiotic resumption of DOs (Fig. 2).

When CEOs were cultured in maturation medium plus different doses of SNP (0.1–1 mM) for 24 h, all oocytes had resumed meiosis. However, the extrusion of PB1 was inhibited significantly by SNP in a dose-dependent manner.

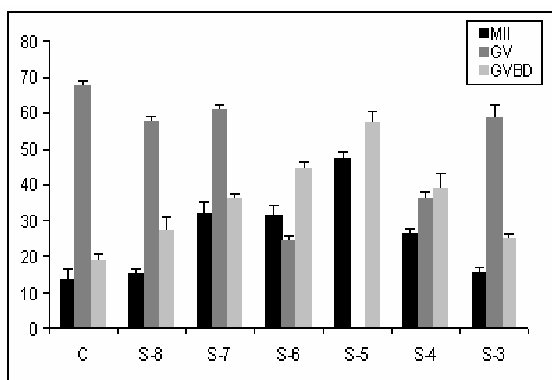

Fig. 1. The effect of SNP on the maturation of mouse oocytes in CEOs. CEOs were cultured in HX-medium with various doses of SNP for 24 h. The mean ± S.E.M. have been calculated in four experiments.

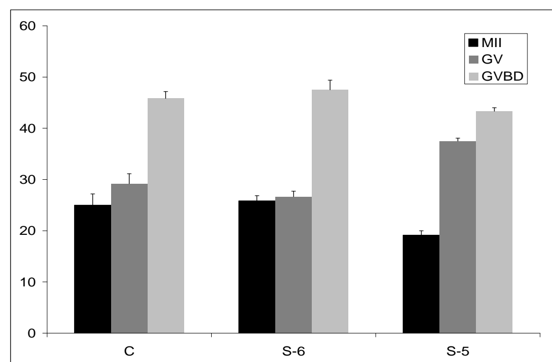

Fig. 2. The effect of SNP on the maturation of mouse oocytes in DOs. DOs were cultured in the HX-medium with the optimal doses of SNP on CEOs. The mean ± S.E.M. have been calculated in four experiments.

The inhibition of the highest concentration of SNP was reflected by the vast reduction in the percentage of oocytes at PB1 (9.52 ± 1.82%) and a large increase in the percentage of atypical oocytes (59.04 ± 3.46%) compared with oocytes cultured in SNP-free medium (control) (Fig. 3). The optimal concentration of SNP caused decrease in the percentage of PB1 in DOs, and increased the percentage of atypical oocytes (Fig. 4). High concentrations of SNP suppressed spontaneous oocyte maturation.

A complete prevention of GVBD in CEOs was obtained after exposure to SNP for 1–5 h. But at the end of 24-h culture period, almost all of them showed GVBD (Fig. 5).

When CEOs were cultured in maturation medium for 24 h, almost all the oocytes underwent GVBD spontaneously and about 69.19% of the oocytes extruded PB1, as shown in (Fig. 5).

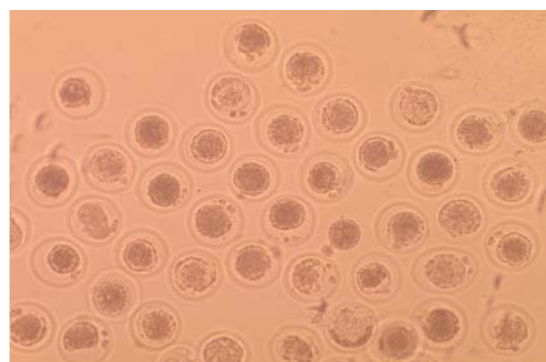

Fig. 3. High concentration of SNP causing atypical oocytes.

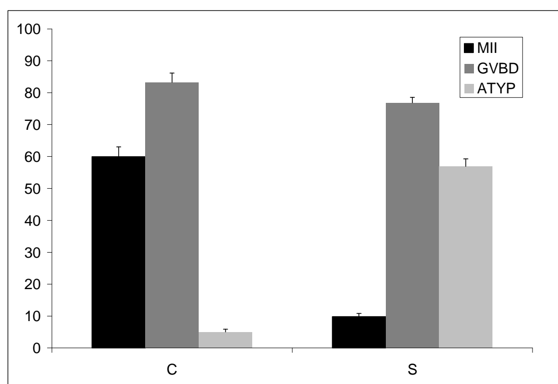

Fig. 4. Effects of high concentration of SNP on the spontaneous maturation of mouse oocytes in DOs. DOs were cultured in the maturation medium with the 1 mM concentration of SNP. The mean ± S.E.M. have been calculated in four experiments. (*P* <0.0001, compared with control).

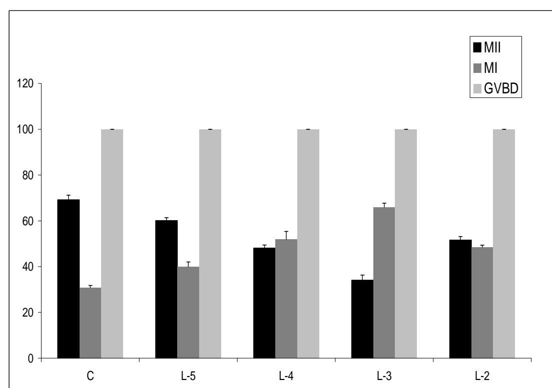

Fig. 6. The effect of L-NAME on the maturation of mouse oocytes in CEOs. CEOs were cultured in maturation medium with various doses of L-NAME for 24 h. The mean ± S.E.M. have been calculated in four experiments ($P < 0.0001$).

The addition of L-NAME concentration ranging from 10^{-5} to 10^{-2} M in medium significantly inhibited the formation of PB1 in dose dependent manner compared with controls in the CEOs group, but had no effect on GVBD (Fig. 6).

At an L-NAME concentration of 10^{-3} M, PB1 extrusion in CEOs decreased to its minimum (34.05 ± 2.213%). However, treatment of the optimal L-NAME concentration (10^{-3} M) caused significant reduction on CEOs oocytes but no significant effects on meiotic maturation in the DOs group (Fig. 7). Co-administration of L-NAME (10^{-3} M) with SNP (10^{-5} M) could abolish the inhibitory effect of L-NAME (Fig. 8).

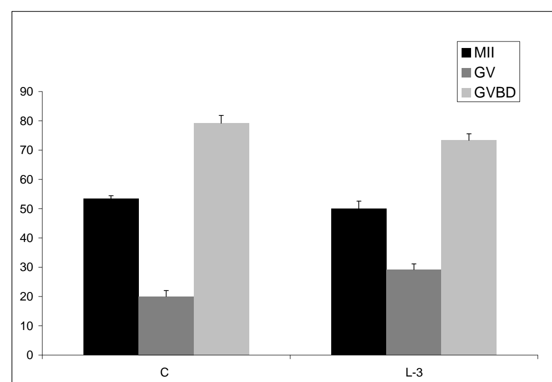

Fig. 7. The effect of L-NAME on the maturation of mouse oocytes in DOs. DOs were cultured in maturation medium with 1 mM L-NAME for 24 h. The mean ± S.E.M. have been calculated in four experiments.

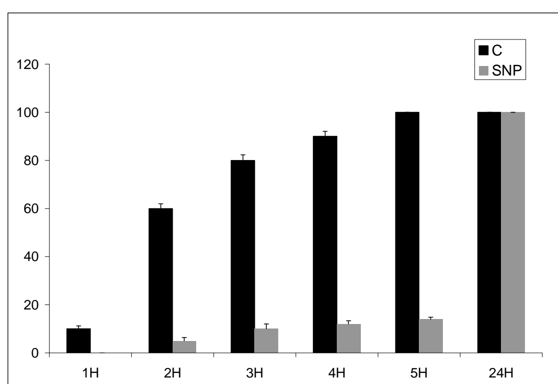

Fig. 5. Influence of SNP on the time-course of GVBD. CEOs were cultured in maturation medium plus or minus 1 mM SNP for 24 h and observed every hour during the first 5 h and again after 24 h. Oocytes were examined for GVBD at varying times after removing the cumulus cells. The mean ± S.E.M. were calculated on four experiments. *P*< 0001, when compared with control.

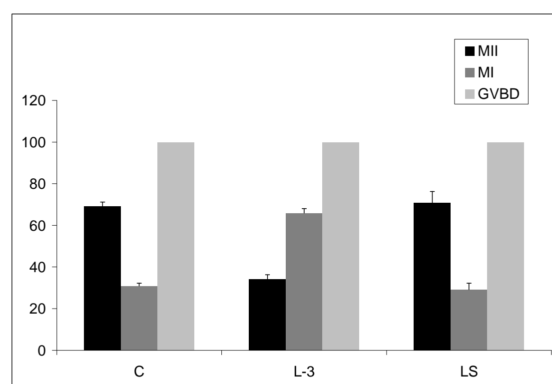

Fig. 8. The effect of SNP on L-NAME–inhibited oocyte maturation. CEOs were cultured in the maturation medium with L-NAME (1 mM) alone and L-NAME plus SNP (10^{-5} M). The mean ± S.E.M. have been calculated in four experiments. ($P < 0.01$ compared with control).

DISCUSSION

The present study provides that NO can exerts different action (inhibiting or stimulating) dose-dependently on the mouse oocyte maturation. On one hand, SNP in 1 mM concentration inhibited PB1 extrusion and caused atypical of both CEOs and DOs cultured in maturation medium and the inhibitory action on DOs was greater than that of CEOs. On the other hand, low concentrations of SNP (10^{-5} M) exhibited stimulatory effect on CEOs in the presence of HX, but had no effect on DOs. These paradox results led us to conclude that NO have opposing actions on oocyte maturation *in vitro* depending on its concentration and the presence of HX. In particular, the examination of nitrite/nitrate in the culture medium revealed that NO stimulated the meiotic maturation of mouse oocytes.

NO is a non polar molecule which freely diffuses through membranes. Unlike other carbon, oxygen, or nitrogen-centered radicals, NO does not have the tendency to dimerize at standard temperature and pressure. In the presence of biological tissues the half life of NO is 3–5 s (34). It can react with oxygen, super oxide anion and other oxygen derived radicals and oxyhemoproteins (17). The enzymes responsible for the synthesis of NO from L-arginine in the mammalian tissues are known as NOS. L-NAME is one of the competitive inhibitors of NOS. It selectively inhibits NO production by both eNOS and iNOS, but is more potent for eNOS (25, 29). In our studies, the L-NAME inhibited the formation of PB1 in CEOs cultured in maturation medium, but had no effect on DOs. This indicated that eNOS-derived NO probably produced by the cumulus cells might be one of the oocyte maturation stimulator. These findings were in good agreement with that of the previous report (19, 34), where their data from *in vivo* and *in vitro* studies using eNOS–KO mice provided evidence that eNOS/NO played a critical and direct role in oocyte meiotic maturation in mice. Of course both the cumulus cells and oocytes themselves expressed eNOS and iNOS determined by immunocytochemistry (47). We demonstrated that the inhibitory effects of the NOS inhibitor were reversed by the concomitant addition of SNP. Hence, a common factor in the inhibitory effect of L-NAME is NO.

These findings are partly consistent with the observations of (24, 34) who reported that a significant reduction in the number of oocytes reached PB1 stage after treated with L-NAME compared with controls. SNP, a well known NO donor, releases NO from its molecule by a mechanism not involving NOS activity (46) and this could explain why its stimulatory effect are not affected by NOS inhibitor (L-NAME) in our experiment.

Reactive oxygen species (ROS) including super oxide radical (O_2^-), which are produced as products of normal metabolism, are well known to cause cellular damage, leading to cell death and tissue injury (5). It is well documented that NO elicits a wide spectrum of intracellular effects depending on its concentration, the amount of metal ions, protein thiols, glutathione, and other nucleophilic targets present (15). The present results showed that neither NOS inhibitors nor low concentration of SNP has a harmful effect on oocyte morphology, but high concentration of SNP significantly increase the number of atypical oocytes compared with the control. These results showed that the concentration of intracellular NO could be critical factor in cell survival and function. At low concentrations, NO transmits extra cellular signals to its intracellular targets and regulates meiosis progression of oocytes just as in other eukaryotic cells; when at high concentrations it harms oocyte greatly by its derivates (34). Because it is reported that redundant NO in the cell might react with another free radical (O_2^-) and produce a more toxic radical (peroxynitrite, $ONOO^-$) (14).

There are many mechanisms through which NO acts either intracellularly or in a paracrine fashion, diffusing through cell membranes (29). In several somatic cell systems, the effects of NO are mediated via activation of soluble guanylyl cyclase (sGC) and induction of cGMP synthesis. This intracellular transduction pathway is known to mediate the effects of NO, for instance, in vascular smooth muscle cell relaxation, platelet aggregation and neurotrans-mission (2).

However, a growing amount of experimental date indicate that NO can induce its biological effects even via cGMP-dependent pathways (e.g. binding to heme-containing proteins other than sGC) (15). As about the exact mechanisms through which NO influences oocyte maturation have not been reported.

In the present study, we found that a complete prevention of GVBD was only obtained after exposure to high concentration of SNP for 1–5 h. This effect is very similar to that of forskolin, a stimulator of adenylate cyclase (AC), which can stimulate cumulus cells to produce a positive signal (44). It is documented that cGMP has an important role in maintaining the meiotic arrest of oocytes (22). The difference between cGMP and SNP suggested that the inhibitory effect of SNP on spontaneous oocyte maturation is not completely cGMP-dependent and other signaling pathway may present including Calcium–Calmodulin (23), protein kinas A (27). In fact, 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 (38). Interestingly, our present study also demonstrated that low concentration SNP (10^{-5} M) was able to reverse the inhibitory effect of hypoxanthine on CEOs, which is a physiology inhibitor of PDE (8) or cAMP-dependent inhibitor of PDE (42).

The present study demonstrated that although SNP-treated (high concentration) oocytes all eventually showed GVBD, they required a significantly longer time to resume meiosis. So slower rate of GVBD, in turn, may affect the quality and viability of oocytes observed during the later stage of maturation. NO is also known as a survival factor in ovarian follicles of rat, inhibiting apoptotic DNA fragmentation in granulose cells (37). NO is synthesized by the ovary and hypothesized to play a role in steroid genesis, ovulation and luteolysis (41, 45, 3). It is documented that NO has emerged as a potential regulator of ovarian function suppressing the production of estradiol and progesterone in the rat follicles and corpora lutea (31, 30).

Moreover, previous studies have shown that NO is important in ovulation and blocking NO synthesis not only prevents apoptosis but also prevents ovulation, suggesting that NO is an important mediator in ovulatory process (1, 16). It is well known that the mid-cycle surge of gonadotropin release the oocytes from the arrest of the first meiotic division and meiosis proceeds (26, 28).

In conclusion, our results in vitro combined with other studies in vivo, which show that ovarian NO synthesis is required for maximal ovulation and a lack of NO during the preovulatory period results in severe defects in oocyte maturation (16, 20, 21) provide support for the importance of NO as a regulator of resumption of meiosis.

Conflict of interests

The authors declare that they have no competing interests.

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