

Effects of inhibiting nitric oxide synthase on cumulus expansion and nuclear maturation of sheep oocytes

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ABSTRACT: Nitric oxide (NO) is a biological signaling molecule that plays a crucial role in oocyte maturation of mammals. It is generated by the nitric oxide synthase (NOS) enzyme from L-arginine. Although the effect of NO has been shown in oocyte maturation of some species, there is no report about its effect on the *in vitro* maturation of sheep oocyte. So, this study aimed to investigate the importance of NO/NOS system in the *in vitro* maturation of ovine oocytes. Different concentrations of L-NAME (a NOS inhibitor) (0.1, 1 and 10mM) were added to maturation medium to evaluate the effect of inhibiting NOS on cumulus expansion and meiotic resumption of sheep oocytes. After 26 h culture, low and medium concentrations of L-NAME (0.1 and 1mM) had no significant effect on cumulus expansion, however, its higher concentration (10mM) decreased percentage of oocytes with total cumulus expansion as compared to control ($P < 0.05$). The extrusion of the first polar body was also suppressed in a dose-dependent manner, so that the addition of 10mM L-NAME to maturation medium significantly stopped oocytes in GV stage ($P < 0.05$). Moreover, to confirm the results and to evaluate if this effect is reversible, 0.1mM sodium nitroprusside (SNP, a NO donor) was added only to the maturation medium which had the highest concentration of L-NAME (10mM). The concomitant addition of NOS inhibitor with NO donor reversed the inhibitory effect of L-NAME on cumulus expansion and meiotic maturation. These results indicated that NO/NOS system is involved in the maturation of sheep oocytes.

Keywords: sheep; oocyte maturation; nitric oxide; L-NAME

Nitric oxide (NO) is a highly reactive free radical which is involved in inter- and intracellular signaling pathways in various aspects of reproduction such as sexual behavior, steroidogenesis, follicle survival, ovulation and even embryo implantation in several species (Rosselli et al., 1998; Thaler and Epel, 2003). NO is generated from molecular oxygen and L-arginine by nitric oxide synthase (NOS) (Moncada et al., 1991). NOS exists in three isoforms that have been classified depending on tissue of origin as well as functional properties (Dixit

and Parvizi, 2001). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are calcium and calmodulin dependent and both of them synthesize a small amount of NO. Inducible NOS (iNOS), the third isoform, is calcium independent and produces a higher amount of NO (Nathan, 1992).

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 (Dixit and Parvizi, 2001). Previous literatures have shown that NO is involved in oocyte meiotic matu-

ration of pig (Tao et al., 2005b; Chmelíková et al., 2009), mouse (Bu et al., 2003, 2004), cattle (Matta et al., 2009) and rat (Sela-Abramovich et al., 2008). It is well known that mammalian ovaries express eNOS, iNOS and nNOS (Van Voorhis et al., 1995; Jablonka-Shariff and Olson, 1998; Klein et al., 1998; Nakamura et al., 2002; Tao et al., 2004). The presence of mRNA for all three isoforms were recorded in cattle (Tesfaye et al., 2006) and porcine oocytes (Chmelíková et al., 2009). Findings about the effect of NO/NOS system on the oocyte maturation are different as some researchers (Jablonka-Shariff and Olson, 1998; Jablonka-Shariff et al., 1999) shown that NO inhibited or others have reported that it stimulated the nuclear maturation (Sengoku et al., 2001; Bu et al., 2004; Huo et al., 2005; Tao et al., 2005a). NO has different functional roles such as the antioxidant agent, saving cells from oxidative stress (Kenner et al., 1991), however, it could induce cytotoxicity in some type of cells (Messmer et al., 1995). The necessity of an appropriate concentration of NO for normal maturation of bovine oocytes were found out by Matta et al., (2002). Viana et al. (2007) approved that an intermediate dose of NO stimulated cytoplasmic maturation of bovine oocytes while its high dose inhibited their nuclear and cytoplasmic maturation.

Thus, based on these established effects of NO/NOS system on the maturation of mammalian oocytes and the absence of any report about its effect on the ovine oocyte *in vitro* maturation, the aim of the present study was to investigate the effects of inhibiting NOS on the *in vitro* maturation of ovine oocytes through the addition of different concentrations of a NOS inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME), to the maturation medium.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals and reagents used in this study were obtained from Sigma-Aldrich, Inc., Iran (<http://www.sigmaaldrich.com/>), unless otherwise indicated.

Ovary collection and oocytes selection

Ovaries were collected from slaughtered ewes in a local slaughterhouse during the non-breed-

ing season (February–March) and transported to the laboratory in a thermos flask containing sterile saline (0.9% NaCl, 30–35°C) supplemented with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco/invitrogen)) within 1–2 h following collection. After washing ovaries with freshly prepared sterile saline containing antibiotics, all visible follicles with a diameter of 2–5 mm were aspirated with a vacuume pump (with pressure of 10 kPa) equipped with a 20 gauge needle and a 50 ml plastic falcon tube. Follicular fluid containing cumulus-oocyte complexes (COCs) was collected in centrifuge tubes containing TCM-199-HEPES with 5% FBS (Gibco/invitrogen), 0.2mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 IU/ml heparin and 3-isobutyl-1-methyl-xanthin (IBMX, 0.5mM). IBMX inhibited enzyme synthesis that degraded phosphodiesterase cAMP (PD-cAMP), that as a result, maintained meiotic arrest during manipulation of the oocytes (Eppig and Downs, 1984). After deposition, COCs with homogenous cytoplasm and with three or more layers of cumulus cells were selected into plastic dishes 35 mm in diameter by a narrow-bore pipette for the experiments.

In vitro maturation

Before culture, selected COCs were collected under the stereomicroscope and washed three times in the same medium used for collection without IBMX and two times in the maturation medium used according to the experimental treatments described as follows. Oocytes were matured under mineral oil in 50 µl droplets (8–10 oocytes per droplet) of TCM-199 medium (Gibco/invitrogen) supplemented with 0.5 µg/ml FSH, 5 µg/ml LH (Sioux Biochemical, Sioux Center, USA), 1 µg per ml estradiol, 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (as a control medium) at 38.5°C in 5% CO₂ in air with maximum humidity for 26 h.

Evaluation of cumulus expansion

At the end of the culture period, cumulus expansion was assessed using a subjective scoring method described by Tao et al. (2005b) with few modifications. Cumulus expansions were classified to three classes: (1) total expansion (expansion of

all layers of cumulus cells), (2) partial expansion (expansion of outer layers of cumulus cells), or (3) without expansion (no response observed).

Evaluation of nuclear maturation

After evaluation of cumulus expansion, COCs were mechanically denuded from cumulus cells by repeated pipetting. Denuded oocytes were then mounted on glass slides and fixed with ethanol/acetic acid with rate of 3:1 for 24–48 h and stained in 2% acetic orcein. The oocytes were examined by a phase contrast microscope (400× Olympus, CKX41 Japan) and classified according to Viana et al. (2007) as germinal vesicle (GV), metaphase I (MI), or metaphase II (MII) stages.

Experimental design

Experiment 1: Effects of different L-NAME concentrations on cumulus expansion and nuclear maturation. Increasing concentrations of L-NAME (0.1, 1, 10mM) were used to evaluate cumulus expansion of COCs and the nuclear maturation of oocytes during maturation period (26 h). Such concentrations were chosen according to Bu et al. (2003). COCs were cultured in 4 groups to determine whether there was a delay or a block in cumulus expansion and/or meiosis progression: (1) without L-NAME (Control); (2) with 0.1mM L-NAME; (3) with 1mM L-NAME; (4) with 10mM L-NAME. The most effective concentration (10mM) was used in further experiment.

Experiment 2: Effect of SNP (a NO donor) on L-NAME-inhibited oocyte maturation. This experiment was aimed to observe if the inhibitory effect of L-NAME could be reversed by co-administration with a NO donor. So, only in the treatment with the highest L-NAME concentration, 0.1mM Sodium nitroprusside (SNP), a NO donor, was added. Then, the COCs were cultured in maturation medium with L-NAME (10mM) + SNP (0.1mM) for 26 h. Such concentration of SNP was chosen according to Matta et al. (2009).

Statistical analysis

Statistical analysis was performed using SAS software version 9.12. All data are presented as mean \pm SEM. the results regarding the effect of addition of different concentrations of L-NAME on meiotic maturation and cumulus expansion were evaluated by chi-square test and $P < 0.05$ were considered significant.

RESULTS

Effects of different L-NAME concentrations on cumulus expansion

After 26 h of culture, cumulus cells expansion was affected by L-NAME. The percentage of oocytes which showed that total expansion was the lowest at the highest concentration of L-NAME (10mM) when compared to the control group ($P < 0.05$). However, other treatments (0.1mM and 1mM)

Table 1. Effect of addition of different L-NAME concentrations on cumulus cells expansion of sheep oocytes

Treatments	<i>n</i>	Degree of cumulus expansion (%)		
		total	partial	without
Control	196	85.21 \pm 2.01 ^a	6.12 \pm 2.98 ^a	8.67 \pm 2.53 ^a
L-NAME				
0.1mM	187	81.28 \pm 1.88 ^{ab}	10.70 \pm 2.36 ^a	8.02 \pm 2.70 ^a
1mM	191	83.24 \pm 1.62 ^{ab}	8.38 \pm 2.22 ^a	8.38 \pm 2.22 ^a
10mM	176	76.13 \pm 1.76 ^b	10.22 \pm 2.48 ^a	13.64 \pm 2.2 ^a
10mM + SNP	157	87.90 \pm 2.44 ^a	9.55 \pm 2.71 ^a	2.55 \pm 0.50 ^b

^{a,b}values with different superscripts within the same column are significantly different ($P < 0.05$)

Data are presented as mean \pm SEM of four replicates

L-NAME = N ω -nitro-L-arginine methyl ester, SNP = sodium nitroprusside

Table 2. Effect addition of different L-NAME concentrations on nuclear maturation of sheep oocytes

Treatments	n	Stage of nuclear maturation		
		GV (%)	MI (%)	MII (%)
Control	157	1.91 ± 0.58 ^b	22.93 ± 1.90 ^c	75.16 ± 1.84 ^a
L-NAME				
0.1mM	127	1.57 ± 0.71 ^{ab}	34.64 ± 1.86 ^{ab}	63.78 ± 1.84 ^b
1mM	140	4.28 ± 0.41 ^{ab}	55.00 ± 1.70 ^a	40.72 ± 1.72 ^c
10mM	136	7.35 ± 0.32 ^a	66.16 ± 1.81 ^a	26.47 ± 1.94 ^d
10mM +SNP	140	2.86 ± 0.50 ^{ab}	24.29 ± 1.97 ^{bc}	72.86 ± 1.90 ^{ab}

^{a,b,c,d}values with different superscripts within the same column are significantly different ($P < 0.05$)

Data are presented as mean ± SEM of four replicates

L-NAME = N ω -nitro-L-arginine methyl ester, SNP = Sodium nitroprusside

were similar to the control group ($P > 0.05$). In experiment 2, when it was examined whether the inhibitory effect of L-NAME (10mM) on cumulus expansion could be reversed by application of 0.1mM SNP in combination with L-NAME, the results approved that the addition of SNP to culture medium reversed the inhibitory effect of L-NAME on cumulus expansion and even decreased percentage of oocytes without cumulus expansion when compared with the control group (Table 1).

Effects of different L-NAME concentrations on nuclear maturation

The addition of different concentrations of L-NAME to the maturation medium inhibited the formation of the first polar body (as complete nuclear maturation) in a dose-dependent manner (Table 2) and the percentage of MII reached to the minimum level in the concentration of 10mM L-NAME (26.47 ± 1.94%). L-NAME in the concentration of 10mM significantly increased the percentage of oocytes which were remained at GV stage, while 0.1 and 1mM concentrations of L-NAME exhibited no inhibitory effect on GV compared to the control group. Nonetheless, low and intermediate concentrations of L-NAME (0.1 and 1mM) stopped more oocytes in MI (34.64 ± 1.86% and 55 ± 1.7%, respectively) when compared to the control group (22.93 ± 1.9% and $P < 0.05$). The addition of SNP (0.1mM) to 10mM L-NAME could reverse the inhibitory effect of L-NAME on meiotic resumption.

DISCUSSION

This study demonstrated that *in vitro* maturation of ovine oocytes was affected clearly by different concentrations of L-NAME, NOS inhibitor, (0.1, 1, 10mM). Cumulus expansion was inhibited only by the highest concentration of L-NAME (10mM). However, the meiotic progression was suppressed by all concentrations in a dose-dependent manner. Moreover, the concomitant addition of SNP, NO donor, reversed inhibitory effects of L-NAME on both cumulus expansion and meiotic maturation. Similar results were obtained in cattle (Schwarz et al., 2008; Matta et al., 2009), mice (Bu et al., 2003), porcine (Tao et al., 2005a; Chmelíková et al., 2010) and rat (Jablonka-Shariff et al., 1999).

At the end of the maturation period (26 h), expansion of cumulus cells was inhibited by the presence of the 10mM L-NAME and the concomitant addition of the inhibitor with SNP (0.1mM) could reverse the inhibitory effect of L-NAME on cumulus expansion and it even decreased the percentage of oocytes without cumulus expansion as compared to the control group.

Nitric oxide is a free radical gas with critical physiological functions (Moncada et al., 1991). There are many mechanisms such as activating soluble guanylate cyclase (sGC), inhibition of adenylyl cyclase (AC), alteration of phosphodiesterase (PDE) and activating G_i through which NO may affect mammalian cells (Tranguch et al., 2003; Bu et al., 2004), but the exact mechanism of the influence of NO/NOS system on oocyte maturation has not been fully clarified up to date. L-NAME is

a non-selective inhibitor of NOS that can reduce production of NO by inhibiting the expression of both eNOS and iNOS. It is reported that iNOS-derived NO is necessary for cumulus expansion and meiotic maturation by mediating the function of the surrounding cumulus cells, and eNOS-derived NO is also involved in porcine meiotic maturation (Tao et al., 2005b). Ovarian nitric oxide synthesis is required for maximal ovulation, and a lack of nitric oxide during the periovulatory period results in severe defects in oocyte maturation (Jablonka-Shariff et al., 1999).

The transient synthesis and accumulation of hyaluronan bring about expansion of cumulus cells. Hyaluronan accumulates among the cumulus cells and embeds them in a gelatinous matrix, a process that is termed cumulus expansion (Kimura et al., 2002). Eppig (1981) suggested that prostaglandin E_2 (PGE_2) might play a role in the indirect stimulation of cumulus expansion of mice. Moreover, Yamauchi et al. (1997) reported that nitric oxide stimulated the production of PGE_2 in rabbit. They indicated that ovarian production of PGE_2 and $PGF_{2\alpha}$ in response to hCG was significantly blocked by L-NAME, and exogenous administration of NP (sodium nitroprusside) stimulated the production of PGs. Considering these results, we guess that the effects of L-NAME on cumulus expansion may be related to the suppressing effect on the production of PGE_2 by decline in NO production. Also, it has been shown that low concentration of SNP ($10^{-5}M$), a NO donor, has had a stimulatory effect on oocyte maturation while its high concentration ($10^{-3}M$) has had a cytotoxic effect and inhibits cumulus cell expansion (Viana et al., 2007). Thus, in this trial, the inhibitory effect of high concentration of L-NAME on cumulus expansion presents that NO concentration has been declined to a critical level in the culture medium.

Regarding assessment of nuclear maturation in the present study, L-NAME inhibited nuclear maturation in a dose-dependent manner, and the most inhibition rate on nuclear maturation was obtained in 10mM L-NAME. In the other word, low and intermediate levels of L-NAME (0.1 and 1mM) could suppress the progression of meiotic maturation when compared to the control group, while these concentrations had no significant effect on cumulus expansion. It shows that the required NO concentration for nuclear maturation is more than that of cumulus expansion. These findings agree with previous studies which reported that NOS

inhibition by the L-NAME reduced meiosis progression of oocytes to MII stage (Jablonka-Shariff and Olson, 2000; Sengoku et al., 2001; Tao et al., 2004; Schwarz et al., 2008). In addition to MI-MII transition, the inhibition of NOS with L-NAME (at 10mM) enhanced the percentage of oocytes that remained in GV stage after maturation period. Our results were similar to those from mouse (Bu et al., 2003) and porcine oocytes (Tao et al., 2005a; Chmelíková et al., 2010). However, other literatures (Bu et al., 2004; Schwarz et al., 2008) reported that L-NAME had not any effect on GVBD.

In mammals, meiosis arrest and resumption are modulated by numerous messengers including cyclic adenosine mono-phosphate (cAMP), cAMP-dependent protein kinase (PKA) and calcium ions (Hurk and Zhao, 2005; Tosti, 2006). Moreover, it is well known that meiotic maturation is regulated by maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Lu et al., 2002). It seems that deficiency in NO production by using NOS inhibition may be affected meiotic maturation by one of these mechanisms. It is well established that intraoocyte cAMP levels play an important role in the maintenance of meiotic arrest at diplotene (GV) stage in mouse oocyte (Mehlmann, 2005). But results obtained in cattle (Sirard et al., 1992) or sheep (Moor and Heslop, 1981) indicated that cAMP accumulation in the oocyte might not be the principal physiological way to maintain meiotic arrest. Therefore, we suggested that other mechanisms were important for involvement of NOS inhibition on meiotic arrest of sheep oocytes.

Independently of differences between animal species, MPF and MAPK activation are both important in oocyte maturation. Interference in any of these processes will inhibited MPF and MAPK activation, preventing resumption of meiosis and arresting the oocyte at GV stage.

At the protein level, mitogen-activated protein was present in constant amounts throughout the first meiotic division. It was shown that MAPK activation occurred after GVBD, indicating that MAPK was not required for GVBD but was involved in regulating post-GVBD events (Lu et al., 2002). Unlike MAPK, MPF activity increased just before GVBD and during MI. It was reported that MPF played a key role in maintenance of GV arrest (Tripathi et al., 2010). Lander et al. (1996) suggested that NO and reactive oxygen intermediates activated the MAPK family in human cells through upstream activation. Huo et al. (2005) also reported

that the phosphorylation of MAPK was inhibited by aminoguanidine (AG, a specific inhibitor for iNOS) in mouse oocyte. Oocyte maturation was accompanied by important structural changes, such as reorganization organelles upon GVBD (Albertini et al., 1987), formation of meiotic spindle (Albertini, 1992), and chromosome segregation (Longo and Chen, 1985). Gordo et al. (2001) demonstrated that in bovine oocytes MAPK activity was required for spindle organization. Also Lu et al. (2002) reported that active MAPK was required for normal meiotic spindle formation and chromosome condensation, and inhibition of MAPK activation resulted in compromised microtubule polymerization, no spindle formation and loosely condensed chromosome. These data suggest that L-NAME may cause inhibition in phosphorylation of MAPK and MPF activity and subsequently inhibition of MI-MII transition and it is stopped at GV stage. However, more experiments are necessary to advance this hypothesis in ovine oocytes.

In conclusion, our results demonstrate that NO/NOS system involves in cumulus expansion and meiotic maturation of sheep oocytes. Although NO is needed for nuclear maturation and cumulus expansion of ovine oocytes, NO requirement for nuclear maturation is higher than that for cumulus expansion.

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