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Effect of sodium nitroprusside, a nitric oxide donor, on the *in vitro* maturation of bovine oocytes

K.S. Viana, M.C. Caldas-Bussiere *, S.G.C. Matta, M.R. Faes, C.S. Paes de Carvalho, C.R. Quirino

Laboratório de Reprodução e Melhoramento Genético Animal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes 28013-602, RJ, Brazil

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Abstract

Nitric oxide (NO) is a highly reactive free radical involved in intra- and intercellular signaling in various stages of reproduction. The objective of the present study was to evaluate the effect of the addition of sodium nitroprusside (SNP), a NO donor, on nuclear and cytoplasmic *in vitro* maturation of bovine oocytes. Analysis of variance was conducted and the means were compared by *t* test at a level of 5%. Low (10^{-7} and 10^{-9} M) and intermediate (10^{-5} M) concentrations of SNP had no significant effect on nuclear maturation, however, when a greater concentration of SNP (10^{-3} M) was added, oocytes remained in metaphase I (MI) after 24 h culture ($P < 0.05$) and did not show cumulus expansion. To evaluate if this effect was reversible and if a retardation or inhibition had occurred in the progression from MI to MII, oocytes were cultured in presence of 10^{-3} M of SNP for 24 h followed by culture for an additional 24 h in medium with or without SNP. After 48 h, the oocytes remained in MI even when the medium was changed at 24 h with or without SNP. The kinetics of nuclear maturation was assessed to evaluate if there had been or not a retardation in the progression of meiosis with the concentration of 10^{-3} M SNP. This concentration delayed germinal vesicle breakdown (VGBD) at 8 h of culture ($P < 0.05$), and at 12 h there was no significant difference between the control and the treated group. The concentrations that did not induce alterations in nuclear maturation were evaluated for cytoplasmic maturation. The concentration of 10^{-5} M improved the percentage of peripheral cortical granules ($P < 0.05$), and significantly increased the percentage of blastocysts. These results demonstrate that SNP at greater concentrations (10^{-3} M) has a cytotoxic effect, but at intermediate (10^{-5} M) concentrations it

* Corresponding author. Tel.: +55 22 27261665; fax: +55 22 27261682.
E-mail address: claracal@uenf.br (M.C. Caldas-Bussiere).

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increases blastocyst rates. NO exhibits a dual effect on bovine oocytes, inhibits (10^{-3} M of SNP) nuclear and cytoplasmic maturation or stimulates (10^{-5} M of SNP) cytoplasmic maturation, depending on concentration in the culture medium.

Keywords: Sodium nitroprusside; Cortical granules; Bovine; Oocyte maturation; Nitric oxide; Embryo development

1. Introduction

Nitric oxide (NO), a highly reactive free radical, has been shown to be a molecule involved in intra- and intercellular signaling. It is generated in various cells by nitric oxide synthase (NOS) from L-arginine (Lamas et al., 1992; Herrero and Gagnon, 2001). NOS exists in three isoforms, which are dimers of identical subunits (Lamas et al., 1992; Xie et al., 1992). Constitutional isoforms of NOS; neuronal NOS (nNOS) and endothelial NOS (eNOS); are calcium- and calmodulin-dependent and produce small amounts of NO for a short period of time (Lamas et al., 1992; Nathan, 1992). An inducible isoform of NOS (iNOS) produces a stable amount of NO for a much longer period (Moncada et al., 1991; Nathan, 1992), and this NO-production is independent from calcium or calmodulin (Bian and Murad, 2003). Cells often contain more than one isoform of NOS (Mehta et al., 1995).

In rodents and pigs, several studies have demonstrated the presence of the three isoforms in the ovary by immunohistochemistry and western blotting, which are involved in several aspects of female reproduction such as: ovarian follicular development (Jablonka-Shariff and Olson, 2000), oocyte maturation (Jablonka-Shariff and Olson, 2000; Kazuo et al., 2001; Matta et al., 2002), ovulation (Jablonka-Shariff et al., 1999; Olson et al., 1999), and embryo development (Gouge et al., 1998). NO can induce cytotoxicity in different cell types, as well as increase the cytotoxicity of different agents, being able to cause cellular apoptosis (Messmer et al., 1995). However, it can also function by protecting the cell from oxidative stress, acting as an antioxidant (Kanner et al., 1991; Kuo et al., 1996).

Adequate concentrations of NO are required for normal nuclear and cytoplasmic *in vitro* maturation in bovine oocytes (Matta et al., 2002). A dose-response effect during *in vitro* maturation was observed in this previous study, when increasing concentrations of the NOS inhibitor *Nω*-nitro-L-arginine methyl ester (L-NAME) were added. From inhibition of cytoplasmic maturation (0.01 mM) to the inhibition of nuclear maturation followed or not by alterations in the metaphase plate (1 mM) were noted. These observations were also described in rodents (Jablonka-Shariff et al., 1999; Jablonka-Shariff and Olson, 2000) and pigs (Tao et al., 2004).

However, Sengoku et al. (2001) observed that nuclear maturation was stimulated in a dose-dependent manner when sodium nitroprusside (SNP), a NO donor, was added during *in vitro* maturation. Lesser concentrations of NO (10^{-5} to 10^{-7} M) enhanced meiotic maturation of mouse cumulus cell-enclosed oocytes (CEOs) arrested by dibutyryladenosine 3′,5′-cyclic AMP (dbcAMP) (Kazuo et al., 2001) or hypoxanthine (HX) (Bu et al., 2004). Accordingly, SNP significantly stimulated meiotic maturation to metaphase II stages in CEOs, whereas L-NAME promoted a significant suppression in meiosis resumption and this inhibition was reversed by the addition of SNP (Bu et al., 2003; Sengoku et al., 2001). Greater NO concentrations (0.5 and 1 mM) seemed, however, to exert opposite effects on spontaneous (Bu et al., 2003) and hCG-induced meiotic maturation (Yasuhiko et al., 2002). These observations suggest adequate concentrations in the medium are needed for normal oocyte maturation, fertilization and development.

Considering that the inhibition of NO synthesis reduced cattle blastocyst development rates *in vitro* (Matta et al., 2002) and that in other species NO addition stimulated IVM, the aim of the present study was to evaluate the dose-response effect of the addition of sodium nitroprusside (SNP) to the maturation medium. An understanding of the mechanisms of action of NO during nuclear and cytoplasmic maturation of bovine oocytes and the identification of the adequate NO concentration for maturation will allow greater embryo development rates, increasing the efficiency of *in vitro* embryo production.

2. Materials and methods

2.1. Oocyte collection and in vitro maturation

Bovine ovaries were collected at a local abattoir and brought to the laboratory soon after slaughter in sterile saline (0.9% NaCl) supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulphate) at 30 °C. Oocytes were aspirated from 2 to 6 mm follicles using a 19-gauge needle connected to a vacuum pump (90 mmHg). Follicular fluid was collected into centrifuge tubes containing TCM 199–HEPES with 5% FCS, antibiotics (100 IU/ml penicillin and 100 UI/ml streptomycin), and 3-isobutyl-1-methyl-xanthine (IBMX, 0.5 mM) to maintain meiotic arrest during manipulation of the oocytes (Eppig and Downs, 1984). Recovered oocytes with homogeneous cytoplasm and at least three layers of cumulus cells were washed four times in medium without IBMX before being placed in maturation medium. Cumulus oocyte complexes (COC) were cultured in TCM-199 supplemented with 10% FCS, 0.5 μg/ml FSH, 5.0 μg/ml LH, 1 μg/ml 17β-estradiol and antibiotics for 22 or 24 h under mineral oil, at 38.5 °C in 5% CO_2 in air with maximum humidity.

2.2. Evaluation of cumulus expansion and meiosis stage

At the end of the culture period, cumulus expansion was assessed using a subjective scoring method based on Tao et al. (2005) with few modifications: total expansion (all layers of cumulus cells), partial expansion (expansion of outer cumulus cells layers) or absence of expansion (no response observed).

To determine the stage of meiosis, the oocytes were mechanically denuded from cumulus cells by repeated pipetting in 0.3 ml saline solution + 1% FCS in a microtube and then placed between a slide and a coverslip. Fixation was in ethanol:acetic acid (3:1, Merck, Rio de Janeiro, Brazil) for 24 h and staining in 2% acetic orcein. Oocytes were observed under a differential interference contrast (DIC) system (400×, Eclipse TE300/TE200, Nikon) and classified as: germinal vesicle stage (GV, immature oocytes), metaphase I (MI) or metaphase II (MII, mature oocytes).

2.3. In vitro fertilization

For *in vitro* fertilization (IVF), frozen thawed semen from the same bull (Genética Avançada, São Carlos, Brazil) was prepared by Percoll (Pharmacia, Uppsala, Sweden) gradient technique. One milliliter 45% Percoll was placed over 1 ml 90% Percoll and thawed semen was added and centrifuged for 20 min at 700 × *g*. The supernatant was then removed and 5 ml TALP (Parrish et al., 1988) was added to the pellet and centrifuged again for another 2 min at 200 × *g*. Separated motile spermatozoa were added to the fertilization droplet at a final concentration of 2×10^6 sperm cells/ml. IVF medium was TALP supplemented with 2 μM penicillamine, 1 μM hypotaurine,

250 μM epinephrine and 20 μg/ml heparin. Oocytes and sperm were co-incubated for 18 h under the same temperature and atmospheric conditions used for IVM.

2.4. In vitro culture

After the 18 h sperm-oocyte co-culture, presumptive zygotes were washed and transferred to the *in vitro* culture (IVC) medium (TCM 199 + 10% FCS and antibiotics). Forty-eight hours after insemination, cumulus cells were removed by pipetting and cleavage rates recorded. Only cleaved oocytes were maintained in the droplet in co-culture with their own removed cumulus cells. Blastocyst development rates were recorded at day 8 of IVC. Temperature and gas atmosphere were the same as used for IVM and IVF.

2.5. Cortical granule staining

Cortical granules (CG) were stained as described previously (Yoshida et al., 1993). The zona pellucida was removed with 0.5% pronase in PBS for 5 min. The oocytes were fixed in 3% paraformaldehyde (Vetec-694, Brazil) in PBS for 30 min at room temperature (∼25 °C) and then incubated overnight at 4 °C in blocking solution (BS; PBS containing 1 mg/ml BSA and 100 mM glycine). The oocytes were permeabilized with 0.1% Triton X-100 (Sigma, Germany) in BS for 5 min at 38 °C and then incubated in 10 μg/mL fluorescein isothiocyanate conjugated *Lens culinaris* agglutinin (FITC-LCA) in BS for 30 min at 38.5 °C. Following three washes in BS (5 min each), the oocytes were mounted on slides and viewed under an epifluorescence microscope (400×, Eclipse TE300/TE200, Nikon). The oocytes were classified according to the distribution of the cortical granules (CG). Oocytes with CG arranged in clusters throughout the entire cytoplasm were classified as immature, with clusters and peripheral CG as partially mature and with peripheral CG as mature.

2.6. Experimental design

2.6.1. Experiment 1: effects of different SNP concentrations on nuclear maturation and cumulus expansion

The effect of different SNP concentrations (10^{-9}, 10^{-7}, 10^{-5}, 10^{-3} M) on nuclear maturation was determined by assessment of nuclear configuration. The degree of cumulus expansion was also observed. From the observation that oocytes matured with 10^{-3} M SNP remained in MI after 24 h maturation and minimal cumulus expansion, COC were cultured in three groups to determine whether there was a delay or a block in meiosis progression: (1) for 24 h without SNP (control); (2) for 48 h, with SNP during the first 24 h of culture and without SNP for the next 24 h; (3) for 48 h with SNP. Nuclear maturation kinetics were also observed in oocytes treated with 10^{-3} M SNP, to verify at which point the treatment interfered with meiosis progression. The oocytes were removed from the maturation medium after 0, 2, 4, 6, 8, 10 and 12 h of culture, denuded, fixed, stained and assessed for the presence of a GV. Thirty COC were placed in each culture droplet (150 μl–1 oocyte/5 μl medium), and each experiment was repeated six times.

2.6.2. Experiment 2: effects of different SNP concentrations on cytoplasmic maturation

In this experiment, the effect of addition of the concentrations that did not affect nuclear maturation (10^{-9}, 10^{-7} and 10^{-5} M SNP), were assessed for cytoplasmic maturation by observing cortical granule distribution, and embryo development (cleavage and blastocyst rates). Twenty to

Table 1
Addition of different SNP concentrations on oocyte nuclear maturation and cumulus cells expansion

Treatments	*n*	MI (%)	MII (%)	Cumulus expansion (%)	
				Total	Partial
Control	128	0 b	100 a	100 a	0 a
SNP					
10^{-9} M	100	0 b	100 a	100 a	0 a
10^{-7} M	161	0 b	100 a	100 a	0 a
10^{-5} M	165	0 b	100 a	100 a	0 a
10^{-3} M	160	97.5 ± 3.1 a	2.5 ± 3.1 b	0 b	100 b

Values with different alphabets (a, b) within columns are significantly different ($P < 0.05$). MI: metaphase I; MII: metaphase II. Data are presented as mean ± S.E.M. of six replicates.

thirty COC were used in each culture droplet (100 or 150 μl, 1 oocyte/5 μl medium) and each experiment was repeated six times. Controls consisted of COCs without SNP treatment.

2.6.3. Statistical analysis

Data were expressed as mean ± S.E.M. The results regarding the effect of addition of different SNP concentrations on oocyte nuclear maturation, cumulus cells expansion and the effect of addition of 10^{-3} M SNP on MI to MII progression in oocytes matured *in vitro* for 48 h were evaluated by χ^2-square test. Analysis of variance (ANOVA) was performed for the effect of different SNP concentrations on cytoplasmic maturation and the means were compared by *t* test at 5% level of significance.

3. Results

3.1. Experiment 1

3.1.1. Different SNP concentrations on nuclear maturation and cumulus expansion

Oocytes in controls and groups treated with 10^{-9}, 10^{-7} or 10^{-5} M SNP (Table 1), reached metaphase II stage (MII, 100%) and presented total cumulus expansion. Only oocytes treated with 10^{-3} M SNP remained in metaphase I (MI, 97.5 ± 3.1%) and had only partial cumulus expansion, differing from the other groups ($P < 0.05$) (Table 1).

Table 2
Addition of 10^{-3} M SNP on MI to MII progression in bovine oocytes matured *in vitro* for 48 h with change of medium with and without SNP at 24 h

Treatment (SNP)	*n*	Stage of nuclear maturation	
		MI (%)	MII (%)
Control (24 h)	72	0 a	100 a
10^{-3} M with change (48 h)	114	100 b	0 b
10^{-3} M without change (48 h)	110	100 b	0 b

Values with different alphabets (a, b) within columns are significantly different ($P < 0.05$). MI: metaphase I; MII: metaphase II. Data are presented as mean ± S.E.M. of six replicates.

Table 3
Effect of 10^{-3} M SNP on the proportion of GV oocytes during the first 12 h of *in vitro* maturation

Treatment	Maturation time (h)						
	0	2	4	6	8	10	12
Control	100[a] a	100 a	100 a	100 a	42.5 ± 17.4 a	22.9 ± 19.1 a	6.0 ± 6.1 a
SNP (10^{-3} M)	100 a	100 a	100 a	100 a	80.3 ± 12.8 b	35.9 ± 13.4 a	19.2 ± 8.5 a

Values with different alphabets (a, b) within columns are significantly different ($P < 0.05$). Data are presented as mean ± S.E.M. of six replicates.
[a] Numbers are percentages.

3.1.2. Reversibility of meiotic inhibition with SNP

Groups cultured for 24 h in SNP, remained arrested at MI whether they were cultured for an additional 24 h in the presence or absence of SNP ($P > 0.05$) (Table 2).

3.1.3. SNP delays germinal vesicle breakdown with 8 h culture

In the control group, 42.5 ± 17.4% of the oocytes were in GV stage at 8 h, while in the groups treated with 10^{-3} M SNP, 80.3 ± 12.8% of the oocytes remained in this phase ($P < 0.05$). At 0, 2, 4, 6, 10 and 12 h in culture, the incidence of GV was similar in both groups ($P > 0.05$) (Table 3).

3.2. Experiment 2

3.2.1. Effects of different SNP concentrations on cytoplasmic maturation

Oocytes treated with 10^{-5} M SNP (94.1 ± 6.8%), showed an increase in total cortical granule migration to the periphery of the ooplasm, when compared to the control group (78.1 ± 11.4%) ($P < 0.05$). However, the groups treated with 10^{-9} M (82.7 ± 12.5%) and 10^{-7} M (85.3 ± 11.2%) SNP were similar to the 10^{-5} M SNP group ($P > 0.05$) (Table 4). The effect of 10^{-3} M SNP on cortical granule migration was not assessed because the oocytes did not resist the initial steps of the technique when exposed to pronase.

Oocytes treated with 10^{-5} M SNP presented greater blastocyst rate (40.4 ± 15.9%) when compared with controls (27.2 ± 6.0%). However, the treatments did not differ between them ($P > 0.05$) (Table 4).

Table 4
Effect of addition of different SNP concentrations during *in vitro* maturation of bovine oocytes on total cortical granule migration to the peripheral region and embryo development (cleavage and blastocyst rates)

MIV treatment	*n*	Peripheral CG (%)	*n*	CL (%)	BL/T (%)
Control	87	78.1 ± 11.4 a	113	79.2 ± 10.1 a	27.2 ± 6.0 a
SNP					
10^{-9}	93	82.7 ± 12.5 a,b	92	75.8 ± 5.4 a	33.4 ± 11.0 a,b
10^{-7}	132	85.3 ± 11.2 a,b	93	84.9 ± 2.9 a	26.9 ± 7.7 a,b
10^{-5}	114	94.1 ± 6.8 b	98	80.3 ± 10.6 a	40.4 ± 15.9 b

Values with different alphabets (a, b) within columns are significantly different ($P < 0.05$). CG: cortical granule; Cl: cleavage; BL/T: blastocyst rate regarding total oocyte number. Data are presented as mean ± S.E.M. of six replicates.

4. Discussion

In the present study, the addition of high concentration of SNP (10^{-3} M) to the maturation medium blocked the progression from MI to MII after 24 h culture and also impaired total cumulus expansion. These results are in agreement with those obtained in mice (Bu et al., 2003), where first polar body extrusion was inhibited, when the same SNP concentration was used.

At the end of the maturation period (24 h), cumulus cell expansion was inhibited by the presence of the 10^{-3} M SNP. However, cumulus cells were easily removed from oocytes treated with this dose of SNP, compared to other treatments. Ingram et al. (2000) demonstrated that NO can cause alterations in the cytoskeleton, leading us to suggest that the inhibitory concentration (10^{-3} M) used in the present study might have caused changes in the conformation of the microtubule present in the transzonal projections of cumulus cells (Albertini et al., 2001), causing these projections to retract precociously, thus abolishing the gap junctions between oocytes and cumulus cells (Webb et al., 2002).

Another mechanism of action of NO on oocyte maturation is the inhibition of MAPK activity (Ingram et al., 2000), which is responsible for meiotic progression (Fan and Sun, 2004). The process of protein phosphorylation/dephosphorylation is mediated by protein kinases and phosphatases and has an important role in the meiotic cell cycle. MPF and MAPK are key molecules in the regulation of cell cycle progression during meiosis. MPF increases its concentration in the beginning of meiosis resumption and acts directly on GVBD. The MAPK is of an elevated concentration after GVBD, however, with a greater activity during the MI to MII transition (Fan and Sun, 2004). These data suggest that NO in greater concentrations could be inhibiting progression from MI to MII, by modulating the components required for normal meiotic progression, such as MAPK and MPF, inhibiting their actions and thus impeding these oocytes from completing meiosis. However, more experiments are necessary to advance this hypothesis in bovine oocytes. Bertagnolli et al. (2004) demonstrated that the action of PK-C in the beginning of MI is mediated by cumulus cells and that this protein regulates oocyte maturation coinciding with the periods described for MPF activation, suggesting that it might be necessary for MPF activation. These observations suggest that the precocious interruption of communication between the oocyte and cumulus cells observed in this study could be affecting the action of PK-C, hampering progression form MI to MII.

In the present study, when the same SNP concentration that inhibited MI–MII progression was added to the maturation medium, there was also an inhibition of total cumulus expansion in COC cultured for 24 h. It is well established that paracrine signaling from the oocyte to cumulus cells is essential for cumulus expansion to occur. Mouse oocytes secrete a soluble factor that enables cumulus cells to produce matrix molecules in response to FSH (Buccione et al., 1990; Dragovic et al., 2005). This demonstrates that mouse oocytes produce a cumulus expansion capacitating factor (CECF), which is required for expansion to occur. The first molecule to be identified as a candidate CECF was the growth and differentiation factor 9 (GDF-9). However, it has also been suggested that oocyte-produced GDF-9 is insufficient for promoting cumulus cell expansion and that another oocyte-specific factor may be the CEEF, demonstrating that the oocyte produces something in addition to GDF-9 that is required for cumulus cell expansion (Dragovic et al., 2005). Cumulus cells with partial expansion were easily removed from the oocytes, suggesting that there was an interruption in the communication between oocytes and cumulus cells, therefore impeding the exchange of factors between the oocyte and cumulus cells, and consequently impairing cumulus cells to respond to gonadotropins to produce hyaluronan for total expansion (Salustri et al., 1990; Dragovic et al., 2005).

Reactive oxygen species, including superoxide (O_2^-), which are produced as byproducts of normal metabolism, are known to cause cellular damage, leading to cell death (Dinara et al., 2001). NO causes a variety of intracellular effects depending on its concentration, redox state, quantity of metal ions, thiols, glutamine and other nucleophylic targets (Gross and Wolin, 1995). The present study has demonstrated that even after the COC remaining for 48 h in maturation medium, performing or not the medium change removing SNP at 24 h culture, the 10^{-3} M concentration did not allow meiosis progression to occur. Although apoptosis in COC treated with SNP was not assessed, the block of oocytes in MI after 48 h in culture, the easy dissociation of cumulus cells from oocytes and the sensitivity of the oocytes to pronase treatment (initial step of the cortical granule staining technique), suggest that the oocytes treated to result in greater SNP concentrations could be undergoing a process cell degeneration, confirming the hypothesis that NO concentration can be a critical factor for cellular survival and function (Moncada and Erusalimsky, 2002).

The first event in nuclear maturation is GVBD, which occurs in bovine oocytes between 7 and 12 h after the onset of *in vitro* culture (Vignola et al., 1994). During diakinesis, the nuclear membrane starts to undulate, the nuclear pores disappear and then breaks down and disappears (Szollosi et al., 1972). In the present study, during maturation kinetics at 8 h in culture, when GVBD began to be observed in the control group, the addition of 10^{-3} M SNP resulted in 80.3% of the oocytes in GV stage, differing from the observed for the control group (42.5% in VG). This difference was no longer observed at 10 and 12 h in culture, leading us to conclude that greater NO concentration retards the onset of GVBD, but does not affect further meiosis progression to MI.

Oocytes obtained from antral follicles, removed form the ovarian follicular environment and cultured *in vitro* in adequate medium, spontaneously resume meiosis (Milovanov and Sirard, 1994). During the growth process until the acquisition of competence for meiosis resumption, the oocyte undergoes several biochemical and structural changes important for maturation, fertilization and for supporting embryo development (Hurk and Zhao, 2005). However, the percentage of embryos developing from an oocyte matured and fertilized *in vitro* is inferior to that obtained *in vivo* due to problems during the process of cytoplasmic maturation (Bertagnolli et al., 2004).

In the present study, the assessment of cytoplasmic maturation *in vitro* was performed by observing cortical granule migration to the cortical region of the oocytes and by blastocyst production. The addition of 10^{-5} M SNP to maturation medium increased significantly total migration of cortical granules and blastocyst production when compared with the controls. These data demonstrate that the addition of this concentration caused a beneficial effect on cytoplasmic maturation due to an increase in cortical granule migration, improving the quality of matured oocytes, and consequently, the blastocyst production. Release of cortical granule contents into the perivitelline space after fertilization results in biochemical modifications of the zona pellucida that block polyspermy (Abeydeera, 2000). In addition, Hoodbhoy et al. (2001) demonstrated that proteins released by the cortical granules are also necessary for preimplantation embryo development. These data demonstrate that the addition of SNP to the maturation medium improved cytoplasmic maturation, but it remains unclear if improved blastocyst development was related to cortical granule migration or other aspects of cytoplasmic maturation that could be affected by NO.

Kanner et al. (1991) observed that NO can also act to protect the cell from oxidative stress, acting as an antioxidant. One of the mechanisms of action of NO as an antioxidant is by the control of transcription of γ-glutamil-cysteine synthetase, the rate-limiting enzyme for the synthesis of glutathione synthetase (GSH). The uptake of the amino acids cystine and methionine stimulated by NO is also an important step for GSH synthesis, one of the most important antioxidants in the cell (Kuo et al., 1996). Funahashi et al. (1996) observed that oocyte glutathione content is related with

microfilament organization at the end of maturation and early development following *in vitro* maturation and fertilization. Wessel et al. (2002) demonstrated that cortical granule migration is microfilament mediated. In the present study, 10^{-5} M SNP significantly increased cortical granules migration rate and *in vitro* blastocyst production when compared to controls, suggesting that NO at this concentration could be acting increasing oocyte glutathione content, promoting a better organization of microfilaments and therefore, improving cortical granules migration. More studies are necessary to understand the mechanism of action of NO during *in vitro* maturation and to define whether NO at this concentration acts or not increasing oocyte glutathione content.

It has been demonstrated that NO presents dual effects (inhibitory or stimulatory) in rodent (Bu et al., 2003; Sengoku et al., 2001) and pig (Tao et al., 2004) oocyte maturation depending on its concentration. NO has also been detected in the cytoplasm of bovine oocytes (Reyes et al., 2004) and it possesses an important role in events involved bovine oocyte maturation (Matta et al., 2002). Based on the results presented in the present study, SNP at greater concentrations (10^{-3} M) has a cytotoxic effect, but intermediate (10^{-5} M) concentration increase blastocyst rates, showing that NO also presents a dual effect in bovine oocytes, inhibiting (10^{-3} M) nuclear and cytoplasmic maturation or stimulating (10^{-5} M) cytoplasmic maturation, depending on the concentration in the maturation medium. The understanding of mechanisms of action of NO during *in vitro* maturation may result in the increase of the efficiency of biotechniques applied for assisted reproduction in livestock, as well as endangered animals and humans.

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