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Animal Reproduction Science 111 (2009) 189–201

ANIMAL REPRODUCTION SCIENCE

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Effect of inhibition of synthesis of inducible nitric oxide synthase-derived nitric oxide by aminoguanidine on the *in vitro* maturation of oocyte–cumulus complexes of cattle

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Received 7 August 2007; received in revised form 8 February 2008; accepted 3 March 2008
Available online 26 March 2008

Abstract

The aim of the present study was to investigate the effects of inhibition of the enzyme inducible nitric oxide synthase (iNOS) by aminoguanidine (AG) on the *in vitro* maturation of oocyte–cumulus cell complex(es) (COC) of cattle. COC were cultured with different concentrations of AG (0, 1, 10, and 100 mM) for 24 h. In Experiment 1, the extent of cumulus complex expansion, nuclear maturation status and plasma membrane integrity of oocytes and cumulus cells from each treatment were assessed. Nitrate/nitrite (NO_3^-/NO_2^-) concentrations were determined in culture medium by the Griess method. Addition of different concentrations of AG to maturation medium promoted a dose–response inhibitory effect on cumulus expansion ($P<0.05$). Addition of 1 and 10 mM AG to IVM medium did not affect plasma membrane integrity of oocytes or nuclear maturation rates ($P>0.05$), but it did reduce plasma membrane integrity in cumulus cells. One hundred millimolar inhibited pre-metaphase I (pre-MI) to metaphase II (MII) transition, promoted plasma membrane damage in oocytes ($P<0.05$), and increased NO_3^-/NO_2^- concentration when compared to controls ($P<0.05$). To evaluate if this effect was reversible, 10^{-5} M sodium nitroprusside (SNP, NO donor) was added, only in the treatment with 100 mM AG that inhibited the nuclear maturation. However, association of 10^{-5} M SNP to 100 mM AG did not reverse the effects of AG, but increased NO_3^-/NO_2^-concentration ($P<0.05$). In Experiment 2, the effect of different AG concentrations on cytoplasmic maturation *in vitro* was assessed based on cortical granule migration, and embryonic development. There was a dose effect on cortical granule migration rate, in which 1 mM AG ($83.9 \pm 6.2\%$) did not differ from control oocytes

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doi:10.1016/j.anireprosci.2008.03.002

(83.6 ± 8.2%; $P > 0.05$), but 10 mM partially inhibited migration (3.8 ± 6.4%) and 100 mM totally inhibited migration ($P < 0.05$). SNP (10^{-5} M) did not revert this inhibitory effect on cortical granules migration in oocytes treated with 100 mM AG. Only those concentrations that did not inhibit IVM were used to assess cleavage and blastocyst development. Addition of 10 mM AG to IVM medium reduced (73.0 ± 8.1%, 15.0 ± 8.9%; $P < 0.05$) cleavage and blastocyst development, respectively when compared with controls (89.1 ± 3.4%, 37.6 ± 7.3%, respectively), but did not differ, ($P > 0.05$), from the group treated with 1 mM AG (80.9 ± 8.4%, 41.5 ± 10.5%, respectively). The results from the present study demonstrate that NO derived from iNOS affects the *in vitro* maturation of bovine COC, modulating the viability of cumulus cells and of oocyte, the progression of meiosis after GVBD, the migration of cortical granules, and cleavage and blastocyst development.
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Keywords: Cattle, Nitric oxide; Aminoguanidine; Nuclear maturation; Cortical granules; Early embryo development; Bovine

1. Introduction

A comprehension of the processes involved in oocyte–cumulus complex (COC) maturation is important for the improvement of biotechniques applied to assisted reproduction (Fissore et al., 2002). Yet, cellular changes do occur in COC during maturation, and the related mechanisms are not fully known. The involvement of nitric oxide (NO) in the process of *in vitro* maturation of cattle COC occurs (Matta et al., 2002; Schwarz et al., 2006; Tesfaye et al., 2006; Viana et al., 2007). NO is formed from L-arginine in a reaction catalyzed by isoforms of NO synthases (NOS) (Moncada et al., 1991). Three of the isoforms are constitutive (neuronal [nNOS], endothelial [eNOS] and mitochondrial [mtNOS]); the fourth is inducible (iNOS) (Moncada et al., 1991; Elfering et al., 2002). Notwithstanding, there are also some reports describing constitutive expression of iNOS in certain cells (Gath et al., 1996; Park et al., 1996).

In the oocyte maturation process, NO inhibits (mice, Jablonka-Shariff and Olson, 1998; rats, Jablonka-Shariff et al., 1999; Viana et al., 2007), or stimulates (mice, Sengoku et al., 2001; Bu et al., 2004; Huo et al., 2005; Tao et al., 2004, 2005) the nuclear maturation, depending on its concentration.

The iNOS can be detected in cattle (Tesfaye et al., 2006) and mice oocytes (Michell et al., 2004; Huo et al., 2005) as well as in the follicular fluid of mice (Michell et al., 2004) and rat follicles (van Voorhis et al., 1995; Nakamura et al., 2002). The iNOS-derived NO is required for nuclear maturation of oocyte, including the germinal vesicle breakdown (GVBD) and first polar body emission in mice (Blashkiv et al., 2001; Huo et al., 2005), rats (Bu et al., 2004), and pigs (Tao et al., 2004, 2005). Huo et al. (2005) demonstrated the subcellular localization of iNOS at different stages of mouse oocyte meiotic maturation. Thus, these data show that iNOS-derived NO affects the meiotic maturation of oocytes. However, currently there are no reports on the role of iNOS-derived NO *in vitro* maturation of bovine oocyte–cumulus complex.

Several drugs that inhibit NO synthesis are being used in studies on the ovary physiology, as, for example, the aminoguanidine (AG), which selectively inhibits NO production by inhibiting iNOS activity (Nakamura et al., 2002; Tao et al., 2004, 2005; Huo et al., 2005). Such NO synthesis inhibitor is structurally similar to L-arginine in that this compound contains two chemically equivalent guanidine nitrogen groups that competitively inhibit NO synthase (Corbett et al., 1992).

In the present study, we investigated the inhibitory action on iNOS during *in vitro* maturation of the bovine oocyte–cumulus cell complex, through the addition of AG in the means of maturation.

2. Materials and methods

2.1. Ovary collection and oocytes selection

Ovaries for cattle that had initiated estrous cycles before collection of tissues were obtained at local slaughterhouses and transported to the laboratory in a thermal container containing sterile saline (0.9% NaCl) supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulphate) at room temperature. Oocytes were aspirated from follicles 2 to 6 mm using a 19-gauge connected to a vacuum pump (100 mm/Hg). Time between puncture and start of oocyte maturation was approximately 2 h. COC were liberated by washing (TCM 199 – HEPES) supplemented with 5% FCS, antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulphate) and 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), to maintain meiotic arrest during manipulation of the oocytes. IBMX inhibits enzyme synthesis that degrades cAMP (phosphodiesterase cAMP, cAMP-PDE) (Eppig and Downs, 1984). The COC were selected, and only those in categories 1 and 2 (which presented compact cumulus cells with at least four layers of cells and uniform ooplasm) (de Loos et al., 1989) were used in this experiment.

2.2. In vitro maturation

The selected COC were then washed four times with the same medium without IBMX, and thereafter transferred to plastic dishes (35 mm in diameter) containing 150 μl drops of maturation medium under mineral oil, at 38.5° C in 5% CO_2 in air with maximum humidity. The maturation medium used was TCM-199 supplemented with 10% FCS, 0.5 μg/ml FSH (Folltropin-V, Bioniche, Beleville, Canada), 5.0 μg/ml LH (Lutropin-V, Bioniche, Beleville, Canada) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulphate – Merck, Darmstadt, Germany). After 24 h, the oocytes were removed and the medium collected (130 μl) and stored at −20 °C until the day of NO_3^-/NO_2 measuring.

2.3. Evaluation of cumulus expansion

Cumulus expansion was assessed 24 h after the start of *in vitro* maturation, using a subjective scoring method (Vanderhyden, 1993; Tao et al., 2005): 0 – no response; 1 – minimum observable response; 2 – expansion of outer COC layers; 3 – expansion of all COCs layers except the corona radiate, and 4 – expansion of all cumulus-enclosed oocyte layers.

2.4. Evaluation of nuclear maturation

After the maturation period, the oocytes were mechanically denuded from cumulus cells (CC) in a solution containing PBS and 10% FCS. Thereafter, the oocytes were mounted between a slide and a coverslip, there remaining for 24–48 h in ethanol/acetic acid (3:1, Merck S.A. Indústrias, Rio de Janeiro, Brazil), stained in 2% acetic orcein, washed with ethanol, and were observed under a differential interference contrast (DIC) system (400×, Eclipse TE300/TE200, Nikon) for determining the stages of nuclear maturation: germinal vesicle (VG), pre-metaphase I (pre-MI –

GVBD, chromosomes condensed and no formation of the metaphasic plate), metaphase I (MI), and metaphase II (MII) (Brunet and Maro, 2005).

2.5. Evaluation of cell membrane integrity

A double staining method was used to assess the integrity of cell membrane. Two fluorescent stains were used: ethidium bromide, which stains only the nucleus of cells that present an altered membrane (red color), characterizing those as necrotic or in late stage of apoptosis; and acridine orange, which stains DNA of live cells (green color). Both stains were diluted in a concentration of 1 mg/ml. For each 100 μl PBS supplemented with 10% FCS, 10 μl of the solution containing the two stains (1:1) diluted in PBS was added. The oocyte and CC were exposed to this double staining process, separately, at room temperature. Subsequently, they were each mounted between a slide and a coverslip for visualization. Following this procedure, the oocytes and CC underwent fluorescence microscopy for assessment of the cell membrane integrity. In each of the treatments, 200–250 CC were counted.

2.6. Cortical granule staining

For visualization of the cortical granules, the Yoshida et al. (1993) method was used. After a 24 h period of maturation, the oocytes were mechanically denuded in a solution of PBS and 10% FCS, and washed three times in TCM 199 medium at room temperature. Next, the pellucida zone was removed with 0.5% pronase in PBS for 5 min. Thereafter, the oocytes were washed three times again, for 5 min each time, in a medium containing TCM 199 for removal of the pellucida zone remnants, and washed in blocking solution (BS – PBS supplemented with 0.1 mg/ml BSA, fraction V and 100 mM glycine). Next, the oocytes were fixed in 3% paraformaldehyde dissolved in PBS for 30 min at room temperature and washed in SB (three times, for 5 min each time), permeabilized with 0.1% Triton X-100 diluted in PBS for 5 min, washed again in SB (three times, for 5 min each time) and incubated in 10 μg/mL fluorescein isothiocyanate conjugated *Lens culinaris* agglutinin (FITC-LCA) in BS for 30 min at 38.5 °C under protection from light. After coloring, the oocytes were washed again, twice in BS (for 5 min each time) and once in distilled water. Slide and coverslip mounting was done by using a medium containing glycerol at 90% and TRIS supplemented with 0.5% of *n*-propyl at 10%, and was taken to the fluorescence microscope (400×, Eclipse TE300/TE200, Nikon) for visualization of the cortical granules. 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.

2.7. Measurement of NO concentration

Nitrate/nitrite (NO_3^-/NO_2^-) levels were determined by a method based on the colorimetric reaction of Griess (Ricart-Jané et al., 2002). Because very little or no NO_2^- is normally found in cells culture, no attempt was made to differentiate between the amounts of NO_3^- and NO_2^-, and therefore the results are reported as NO. Griess reagent is composed of a mixture of sulphanilamide 2% and *N*-(1-naphthyl) ethylene-diamine 0.2% in deionized water. The first reacts with the nitrite in the sample to form diazonium salt, which in turn reacts with the second reagent to give a purple-azo-dye product with a peak absorbance at 540 nm. To transform NO_3^- into NO_2^- samples (40 μl) were incubated in 96-well plates with the reduction solution (100 μl of 10 IU NO_3^- reductase

enzyme diluted in deionized water + 900 μl deionized water), 1000 μl cofactor NADPH (5 mg/ml) diluted in deionized water, 1000 μl phosphate potassium buffer (0.5 M) at 37 °C for 14–16 h. Afterwards, 80 μl Griess reagent were added to samples. The standard curve of $NaNO_3$ was diluted in TCM 199 ranging from 0.5 to 100 μM. The analysis was made in a spectrophotometer (Multiskan EX Primary EIA V 2.1-0). A dispersion graph was generated with the absorbance values. There was a linear ($R^2 = 0.98$, $P < 0.05$) relation between absorbance and NO concentration. All solutions were protected from light.

2.8. Fecundation and embryo development in vitro

After maturation, the COCs were submitted to *in vitro* fecundation (IVF). Frozen-thawed semen from the same bull was used, which was prepared by Percoll (Pharmacia, Uppsala, Sweden) gradient technique (Parrish et al., 1988). The semen was thawed at 36–37 °C for 1 min, and was then added to the upper portion of the Percoll gradient and centrifuged for 8 min at 600 × *g*. After removal of the supernatant, a new centrifugation process for 2 min at 150 × *g* with 5 ml of TALP-sp medium was performed to remove excess Percoll. Fecundation medium used was TALP-fec supplemented with 6 mg/ml of fatty-acid-free BSA, 2 μM of penicillamine, 1 μM hypotaurine, 250 μM of epinephrine and 20 μg/ml of heparine. The oocytes were washed (four times) in fecundation medium 30 min before the start of IVF. Separated motile spermatozoa were added to the fertilization droplet at a final concentration of 2.0×10^6 sperm cells/ml, and were co-incubated at 38.5 °C in an atmosphere of 5% CO_2 for 18 h. After this period, the presumed zygotes were washed four times in culture medium (TCM 199 without Hepes + 10% FCS and antibiotics) for removal of CC and spermatozoa, and then transferred to drops of 100 μl of culture medium under mineral oil, where they were cultured as above described. Assessment of embryonic development took place 40 h after insemination (cleavage) and 168–192 h after insemination (formation of blastocyst).

All media were prepared on the very day of the experiments, and all reagents used were of Sigma Chemical CO (St. Louis, MO, USA) brand, unless otherwise here mentioned.

2.9. Experimental design

2.9.1. Experiment 1: Effect of different AG concentrations

Different concentrations (0, 1, 10 and 100 mM) of AG were added to the maturation medium for assessing its action on CC and nuclear maturation of bovine oocytes. Only in the treatment with 100 mM AG, 10^{-5} M SNP was added with the intent to observe if the inhibitory effect of AG on NOS activity could be reversed by adding a NO donor. Such concentration has been used with basis on results by Viana et al. (2007) who observed that, in bovine, the addition of SNP at this concentration to the maturation medium significantly improved the percentage of blastocysts produced *in vitro*.

After the period of maturation (24 h), an assessment was made of the degree of CC expansion. Subsequently, the oocytes were denuded to enable observation of the stage of nuclear maturation and integrity of the plasma membrane of the oocyte and CC. The maturation medium of each treatment was collected and stocked at −20 °C until the day of NO_3^-/NO_2^- measuring.

2.9.2. Experiment 2: Effect of different AG concentrations

After maturation, an assessment was made of the location of cortical granules in the oocytes treated with the different AG concentrations. Subsequent experiments were performed solely with

the oocytes treated with inhibitor concentrations that had shown no interference in the nuclear maturation. After the IVF, the number of zygotes cleaved and subsequent embryonic development were observed and recorded.

Oocytes ($n = 30$–40) were used in each culture droplet for each concentration tested, and this procedure was repeated six to eight times, the same oocyte-to-medium ratio (1 oocyte/5 μl medium) being always maintained.

2.9.3. Statistical analysis

The results regarding the effect of addition of different AG concentrations on cumulus cells expansion, on the integrity of the oocyte plasma membrane, and on oocyte nuclear maturation were evaluated by χ^2-square test. Analysis of variance (ANOVA) was performed for the effect of different AG concentrations on membrane integrity, NO_3^-/NO_2^-concentration, cytoplasmic maturation and the means were compared by *t*-test at 5% level of significance. The results obtained from CC expansion grade 3 have been transformed for having a greater coefficient of variation.

3. Results

3.1. Experiment 1: Effect of different AG concentrations

The addition of different concentrations of AG to the maturation medium promoted a dose–response effect on the inhibition of CC expansion (Table 1) and on the viability (Table 2) of COC of cattle. After the addition of 10 mM AG, it has been verified that 100% of the COC cultivated presented expansion only of the CC outer-most layers (grade 2), with concurrent decrease of the viability of cumulus cells (26.8 ± 4.8%), therefore a different outcome from the results obtained in control (92.0 ± 7.9% grade 4 and 94.6 ± 3.1%, respectively) and with 1 mM AG (92.1 ± 6.3% grade 4 and 96.0 ± 2.8%, respectively) ($P < 0.05$). The addition of 100 mM AG increased the percentage of CC presenting grade 0 (92.8 ± 2.8%) of expansion, decreased membrane integrity of the CC (2.6 ± 1.1%) and oocyte (0%) (Tables 1 and 2). The addition of SNP (10^{-5} M) to 100 mM AG did not reverse the inhibitory effect of 100 mM AG on expansion nor on the viability of cumulus cells and oocyte, and it also blocked ($P < 0.05$) the meiosis progression (100% of the oocytes remained in pre-MI) (Tables 1–3).

Table 1
Effect of addition of different AG concentrations on cumulus cells expansion of bovine oocytes matured *in vitro* for 24 h

Treatment	*n*	Cumulus expansion (%)				
		0	1	2	3	4
Control	240	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0a	8.0 ± 7.9b	92.0 ± 7.9b
AG						
1 mM	240	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0a	7.9 ± 6.2b	92.1 ± 6.3b
10 mM	240	0.0 ± 0.0b	0.0 ± 0.0b	100 ± 0.0b	0.0 ± 0.0a	0.0 ± 0.0a
100 mM	240	92.8 ± 2.8a	7.2 ± 2.8a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
100 mM + 10^{-5} M SNP	240	93.5 ± 2.7a	6.5 ± 2.7a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a

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 with 40-oocyte drops.

Table 2
Effect of addition of different AG concentrations on viability of cumulus cells and oocyte of COC bovine matured *in vitro* for 24 h

Treatment/AG (mM)	Integrity of the membrane (%)			
	n	Cumulus cells	*n*	Oocyte
Control	230	94.6 ± 3.1a	235	100.0 ± 0.0b
1	276	96.0 ± 2.8a	250	100.0 ± 0.00b
10	216	26.8 ± 4.8b	231	100.0 ± 0.00b
100	260	2.6 ± 1.1c	216	00.0 ± 0.00a
AG + SNP (100 mM + 10^{-5}M)	240	2.1 ± 0.7c	235	00.0 ± 0.00a

Values with different alphabets (a,b,c) within columns are significantly different ($P < 0.05$). Data are presented as mean ± S.E.M. of six to seven replicates with 30–40-oocyte drops.

3.1.1. NO_3^-/NO_2^- concentration

Only the addition of 100 mM to the maturation medium has resulted in an increase (20.1 ± 3.1 mM) of the NO_3^-/NO_2 concentration as compared to control (13.0 ± 4.0 mM) and to groups treated with 1 (13.7 ± 1.7 mM) and 10 mM AG (17.0 ± 2.7 mM) ($P < 0.05$). The addition of 10^{-5} M of SNP to the group treated with 100 mM AG has resulted in an increase (29.7 ± 3.0 mM) of the NO_3^-/NO_2 concentration in relation to the group treated with 100 mM AG and in relation to the other AG concentrations ($P < 0.05$; Table 3).

3.2. Experiment 2: Effect of different AG concentrations

3.2.1. Cortical granule migration

A dose–response effect from the addition of AG to IVM medium was shown in cortical granule migration assessment (Table 4). An intermediary AG concentration (10 mM) promoted partial migration in 96.1 ± 6.4% of the oocytes treated, differing from control and from the oocytes treated with 1 and 100 mM AG ($P < 0.05$). The COCs treated with 100 mM AG did not present migration of cortical granules. The addition of SNP to 100 mM AG did not reverse the inhibitory effect of 100 mM AG on migration of cortical granules (Table 4).

Table 3
Effect of addition of different AG concentrations on meiosis progression in bovine oocytes matured *in vitro* for 24 h and in NO concentration in IVM medium

Treatment/AG (mM)	*n*	Stage of nuclear maturation		NO_3^-/NO_2^- (μM)
		%Pre-MI	%MII	
Control	210	0.0 ± 0.0b	100.0 ± 0.0b	13.0 ± 4.0c
1	204	0.0 ± 0.0b	100.0 ± 0.0b	13.7 ± 1.7c
10	220	0.0 ± 0.0b	100.0 ± 0.0b	17.0 ± 2.7bc
100	219	100.0 ± 0.0a	0.0 ± 0.0a	20.1 ± 3.1b
AG + SNP (100 mM + 10^{-5} M)	221	100.0 ± 0.0a	0.0 ± 0.0a	29.7 ± 3.0a

Values with different alphabets (a,b) within columns are significantly different ($P < 0.05$). Data are presented as mean ± S.E.M. of eight replicates with 30-oocyte drops.

Table 4
Effect of addition of different AG concentrations on cortical granule migration of bovine oocytes

Treatment/AG(mM)	*n*	Cortical granule migration (%)		
		None	Partial	Total
Control	118	0b	16.3 ± 8.3c	83.6 ± 8.2c
1	111	0b	16.0 ± 6.2c	83.9 ± 6.2c
10	97	0b	96.1 ± 6.4b	3.8 ± 6.44b
100	89	100a	0a	0a
AG + SNP (100 mM + 10^{-5}M)	90	100a	0a	0a

Values with different alphabets (a,b,c) within columns are significantly different ($P < 0.05$). Data are presented as mean ± S.E.M. of six replicates with 30-oocyte drops.

Table 5
Effect of addition of AG to the *in vitro* maturation medium of bovine oocytes on the cleavage and blastocyst rate (mean ± S.D.)

IVM treatment	*n*	CL%	BL/T%
Control	210	89.1 ± 3.4[b]	37.6 ± 7.3[b]
AG			
1 mM	204	80.9 ± 8.4 [ab]	41.5 ± 10.5[b]
10 mM	220	73.0 ± 8.1[a]	15.0 ± 8.9[a]

Values with different alphabets (a,b) within columns are significantly different ($P < 0.05$). Data are presented as mean ± S.E.M. of eight replicates with 30-oocyte drops.

3.2.2. Embryonic development

The addition of 10 mM AG decreased ($P < 0.05$) the cleavage and blastocyst percentages (73.0 ± 8.1%; 15.0 ± 8.9%, respectively) as compared to control group (89.1 ± 3.4%; 37.6 ± 7.3%, respectively) and to the group treated with 1 mM AG (80.9 ± 8.4%; 41.5 ± 10.5%, respectively; Table 5).

4. Discussion

In the present study, NO acts on *in vitro* maturation of oocyte-COC through inhibition of iNOS by aminoguanidine, a specific inhibitor for this enzyme. Through the use of AG, iNOS-derived NO has a role in the maturation of oocyte-COC of rats, mice, and pigs (Nakamura et al., 2002; Bu et al., 2004; Tao et al., 2004, 2005). This is the first report on the role of iNOS in maturation of the oocyte-COC of cattle.

Several members of the TGFβ superfamily secreted by the oocyte (OSF) such as GDF-9 and BMP15, mimic the paracrine actions of oocytes in the regulation of CC expansion *in vitro* (Dragovic et al., 2005; Elvin et al., 1999; Pangas et al., 2004; Shimizu et al., 2004). The addition of increasing amounts of AG has resulted in a dose–response effect on inhibition of CC expansion, where a concentration of 10 mM AG has inhibited the expansion of the inner-most layers of the CC (grade 2). Tao et al. (2005) verified that 10 mM AG has promoted minimal expansion (grade 1) of CC of pigs thus these CC are more sensitive to iNOS inhibition through AG than those of cattle.

The data from the present research suggest that addition of 10 mM AG may be decreasing hyaluronan synthesis and that of other factors that are synthetized by CC that are involved in expansion in response to one or more factors synthesized by the oocyte, because there was CC

expansion only in the most-outer layers (grade 2) and/or a decrease in the number of viable CC has been observed. Thus, production of those factors associated with expansion did not occur at a concentration that is adequate for full expansion to occur. This hypothesis is reinforced by data from Ralph et al. (1995) where CC from cows were stimulated to expand *in vitro* in the absence of oocytes, even though these also produce factors that stimulate CC expansion.

Many lipids contain "actived" allylic C–H bonds, therefore these are susceptible to oxidative damage that can lead to the destruction/modification of numerous lipid molecules, which can result in a loss of membrane integrity. Nitric oxide limits lipid peroxidation by acting as a terminating species (e.g., reaction) by altering the reactivity of metals known to serve as catalysts for their generation (Rubbo et al., 1996). AG addition promoted lesions to the CC membrane only when 10 mM AG was added to the medium, and to the CC and oocyte membrane when 100 mM AG was added. According to Tao et al. (2005), the addition of 10 mM AG increased the degeneration of pig oocytes and decreased percentage of COC at the MII stage, but did not alter CC viability as assessed through DNA fragmentation detected by flow cytometry analysis. In the present research, addition of AG at this concentration did not influence progression of COC until the MII stage, and also there was no lesion to oocyte membranes, which demonstrates existence of differences between species regarding sensitivity of CC and oocyte membranes to inhibition of iNOS and of the signaling pathways involved in COC maturation. As the viability assessment was performed with distinct methods, it is not possible to determine whether the results differ. Therefore, results from the present research suggest that the iNOS-derived NO may be involved in pathways associated with protection against lipid peroxidation, because inhibition of this enzyme activity led to a loss of membrane integrity for CC and oocyte, in spite of there not being any decrease in synthesis of NO by COC.

The addition of 100 mM AG has caused a decrease in percentage ($2.6 \pm 1.1\%$) of CC and oocytes (0%) with unaltered plasma membranes, and this may have been the main reason for expansion of the CC not occurring. Yet, resumption of meiosis was not inhibited, but there was indeed progression from pre-MI to MII. Thus, results of the present research suggest that, in cattle, meiosis resumption is not dependent on CC viability, because meiosis resumption did occur even when only $2.6 \pm 1.1\%$ of the CC were viable after addition of 100 mM AG. Nevertheless, for progression from MI to MII to take place, the existence of at least $26.8 \pm 4.8\%$ of viable cells was required, as occurred in the oocyte group treated with 10 mM AG.

Nuclear maturation of mammalian oocytes is associated with expansion of CC under both *in vivo* and *in vitro* conditions. In the present study, there was dissociation between the two events when adding 10 mM to the IVM medium, i.e., the oocyte reached MII, but there was partial inhibition both of cytoplasmic maturation and CC expansion, reflecting a decrease in cleavage percentage and in production of blastocytes. GDF-9 and BMP15, besides affecting CC expansion, impact the micro-environmental regulation of COC as an important determinant of oocyte developmental programming (Hussein et al., 2006). The results of the present experiment suggest that addition of 10 mM AG altered synthesis of one or more OSF, causing a decrease of CC expansion and viability and migration of CG, leading to alteration of the oocyte developmental competence, as observed from the decreased cleavage percentage and of blastocyte production. Therefore, there would be another pathway linked to the iNOS/NO, in parallel to the ones already described in the control of cumulus–oocyte complex maturation (Sirard et al., 1998; van den Hurk and Zhao, 2005).

While evaluating the data associated with nuclear maturation in the group treated with 100 Mm AG in the present study, 100% of oocytes were in pre-MI stage when 100 mM AG was added to the medium, and this effect was not reversed with the addition of SNP. Nakamura et al. (2002), while

evaluating the effect of iNOS inhibition by AG in pieces of rat ovaries containing four to seven preovulatory follicles, observed results that were inconsistent to those in the present study, where the addition of 10 and 100 mM AG stimulated resumption of meiosis, and this effect was reversed by addition of 500 μM SNAP (*S*-nitroso-L-acetil penicilamine), an NO donor. These differences may be due to the use of different systems of culture, as in the culture of ovarian pieces there are several other types of cells (intrafollicular and ovary stroma) interacting among each other, the direct effect of AG on COC not being observed. Nevertheless, results of the present research are similar to those observed by Huo et al. (2005), where effects of adding AG to the maturation medium for mouse oocytes was evaluated, and VGBD and liberation of the first polar body were significantly inhibited in a dose-dependent fashion.

During the cell cycle, the M-phase is controlled through activation and inactivation of the MPF (Masui and Markert, 1971). The modulation of its regulatory sub-unit, cyclin B concentration by synthesis and degradation is of central importance for the control of MPF activity (Murray and Kirschner, 1989). MPF is actived at VGBD and increases until it reaches a plateau at the end of the first meiotic M-phase (Choi et al., 1991; Verlhac et al., 1994). The immature oocyte contains only a small amount of cyclin B, just enough to induce entry into the first meiotic M-phase (Hampl and Eppig, 1995; Winston, 1997). It enters the germinal vesicle just prior to GVBD (Marangos and Carroll, 2004). After GVBD, the amount of synthesis of cyclin B increases progressively, reaching maximum at the end of MI, and the newly synthesized protein becomes associated immediately with the $p34^{cdk1}$ kinase to form an active complex (Hampl and Eppig, 1995; Ledan et al., 2001). In the present experiment, aminoguanidine added to medium at a concentration of 100 mM did not inhibit resumption of meiosis in contrast to the effects observed by Huo et al. (2005) in mouse COC and Tao et al. (2005) in pig COC using AG at concentrations 2–10 times less, respectively, but it did inhibit meiosis progression to the MII stage. The data from the present study suggest that addition of 100 mM AG did not inhibit cyclin B entry into the germinal vesicle for MPF activation at meiosis resumption, but it did allow more cyclin B synthesis, sufficient for entry into MII. Therefore, the action of AG on meiosis progression varies depending on the species and culture system (ovary and COC or oocyte), and oocytes of cattle are less sensitive to AG treatment than those of mice and pigs.

In the present experiment, no significant difference in NO_3^-/NO_2^- concentration was determined 24 h after the addition of 1 and 10 mM AG, but the addition of 100 mM AG did increase the concentration of NO_3^-/NO_2^- significantly, as compared to control and other concentrations. A negative feedback between iNOS and further isoforms is likely to have occurred. Had iNOS been inhibited, there would have been an increase in activity of the other three isoforms or of one or two isoforms. Thus, the addition of NO to the maturation medium did not reverse inhibition of progression from MI to MII, even if the COC remained viable, for there was no decrease of NO synthesis, but blocking of the pathway connected to NO synthesis by iNOS indicates it is involved in various events of COC maturation in cattle.

In immature oocytes in the VG stage, the cortical granules are distributed throughout the cytoplasm, forming clusters. Shortly after GVBD, MPF activation stimulates vesicle association with microfilaments, and is a key regulatory step is the coordinated translocation of CG to the oocyte cortex (Connors et al., 1998; Wessel et al., 2002). In the present study, there was a dose–response effect in migration of cortical granules due to inhibition of iNOS by AG. Although the 10 mM AG concentration caused a partial migration of the cortical granules, it did not alter nuclear maturation, suggesting that the use of AG at 10 mM concentration may be altering the pathway stimulated by the MPF that promotes the association of CG with the microfilaments, but not the resumption of meiosis and progression into MII. This may be one of the causes for the observed decrease

of cleavage (73.0 ± 8.1%) and blastocyst (15.0 ± 8.9%) percentages when compared to the control (89.1 ± 3.4% and 37.6 ± 7.3%, respectively), because the decrease in total rate of cortical granule migration may have influenced the amount of protein released by the cortical granules, necessary for pre-implantation embryo development (Hoodbhoy et al., 2001). Viana et al. (2007) have demonstrated that the addition of 10^{-5} M SNP to maturation medium increased the rate of cortical granule migration and of blastocyte production. Nevertheless, in the present experiment, the addition of 10 mM AG did not cause NO synthesis to decrease in relation to the control as expected. These data lead us to suggest that the iNOS-derived NO is part of one, or more than one, signaling pathway during *in vitro* cytoplasmic maturation of cattle oocytes, indispensable for the occurrence of cortical granule migration as well as for the initial phase of embryo development.

The results of the present experiment allow us to conclude that inhibition of iNOS activity by AG affects the COC maturation, modulating CC and oocyte viability, meiosis progression after GVBD, migration of cortical granules, and cleavage and blastocyst development. Further studies should also be aimed at verifying whether some dysfunctions of the COC maturation are associated with an altered synthesis of iNOS-derived NO to clarify whether these defects can be corrected through use of biological and pharmacological agents that down-regulate NO synthesis by iNOS, mainly at iNOS mRNA transcription event (Ganster and Geller, 2000), thereby improving the outcome when *in vitro* technologies are used for reproduction in cattle.

Acknowledgments

The present research was supported by FAPERJ and CAPES. S.G.C. Matta – recipient of a fellowship from UENF.

References

Blashkiv, T.V., Korniichuk, A.N., Voznesenskaya, T.Y., Pornichenko, A.G., 2001. Role of nitric oxide in ovulation, meiotic maturation of oocytes, and implantation in mice. Bull. Exp. Biol. Med. 132, 1034–1036.

Brunet, S., Maro, B., 2005. Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. Reproduction 130, 801–811.

Bu, S., Xie, H., Tao, Y., Wang, J., Xia, G., 2004. Nitric oxide influences the maturation of cumulus cell-enclosed mouse oocytes cultured in spontaneous maturation medium and hypoxanthine-supplemented medium through different signaling pathways. Mol. Cell. Endocr. 223, 85–93.

Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y., Kohmoto, K., 1991. Activation of p34cdc2 protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. Development 113, 789–795.

Connors, S.A., Kanatsu-Shinohara, M., Schultz, R.M., Kopf, G.S., 1998. Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs. Dev. Biol. 200, 103–115.

Corbett, J.A., Tilton, R.G., Chang, K., Hasan, K.S., Ido, Y., Wang, J.L., Sweetland, M.A., Lancaster Jr., J.R., Williamson, J.R., McDaniel, M., 1992. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. Diabetes 41, 552–556.

de Loos, F., Van Vilet, C., Van Maurik, P., Kruip, T.A., 1989. Morphology of immature bovine oocytes. Gamete Res. 24, 197–204.

Dragovic, R.A., Ritter, L.J., Schulz, S.J., Amato, F., Armstrong, D.T., Gilchrist, R.B., 2005. Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. Endocrinology 146, 2798–2806.

Elfering, S.L., Sarkela, T.M., Giulivi, C., 2002. Biochemistry of mitochondrial nitric-oxide synthase. J. Biol. Chem. 277, 38079–38086.

Elvin, J.A., Clarck, A.T., Wang, P., Wolfman, N.M., Matzuk, M.M., 1999. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol. Endocr. 13, 1035–1048.

Eppig, J.J., Downs, S.M., 1984. Chemical signals that regulate mammalian oocyte maturation. Biol. Reprod. 30, 1–11.

Fissore, R.A., Kurokawa, M., Knott, J., Zhang, M., Smyth, J., 2002. Mechanisms underlying oocyte activation and postovulatory ageing. Reproduction 124, 745–754.

Ganster, R.W., Geller, D.A., 2000. Molecular regulation of inducible nitric oxide synthase. In: Ignarro, L.J. (Ed.), Nitric Oxide. Biology and pathology. Academic Press, San Diego, pp. 129–156.

Gath, I., Closs, E.I., Gödtel-Armbrust, U., Schmitt, S., Nakane, M., Wesler, I., Förstermann, U., 1996. Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: implications for contractile function. FASEB J. 10, 1614–1620.

Hampl, A., Eppig, J.J., 1995. Translational regulation of the gradual increase in histone H1 kinase activity in maturating mouse oocytes. Mol. Reprod. Dev. 40, 9–15.

Hoodbhoy, T., Dandekar, P., Calarco, P., Talbot, P., 2001. p62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development. Mol. Reprod. Dev. 59, 78–89.

Huo, J.-L., Liang, C.-G., Yu, L.-Z., Zhong, Z.-S., Yang, Z.-M., Fan, H.-Y., Chen, D.-Y., Sun, Q.-Y., 2005. Inducible nitric oxide synthase-derived nitric oxide regulates germinal vesicle breakdown and first polar body emission in the mouse oocyte. Reproduction 129, 403–409.

Hussein, T.S., Thompson, J.G., Gilchrist, R.B., 2006. Oocyte-secreted factors enhance developmental competence. Dev. Biol. 296, 514–521.

Jablonka-Shariff, A., Olson, L.M., 1998. The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock-out mouse oocytes. Endocrinology 139, 2944–2954.

Jablonka-Shariff, A., Basuray, R., Olson, L.M., 1999. Inhibitors of nitric oxide synthase influence oocyte maturation in rats. J. Soc. Gynecol. Invest. 6, 95–101.

Ledan, E., Polanski, Z., Terret, M.-E., Maro, B., 2001. Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation. Dev. Biol. 232, 400–413.

Matta, S.G.C., Bussiere, M.C.C., Viana, K.S., Quirino, C.R., 2002. Efeito de diferentes concentrações do inibidor da síntese de óxido nítrico na maturação nuclear *in vitro* de oócitos bovinos. Rev. Bras. Reprod. Anim. 26, 149–151.

Marangos, P., Carroll, J., 2004. The dynamics of cyclin B1 distribution during meiosis I in mouse oocytes. Reproduction 128, 153–162.

Masui, Y., Markert, C.L., 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. 117, 129–146.

Michell, L.M., Kennedy, C.R., Hartshorne, G.M., 2004. Expression of nitric oxide synthase and effect of substrate manipulation of the nitric oxide pathway in mouse ovarian follicles. Hum. Reprod. 19, 30–40.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Murray, A.W., Kirschner, M.W., 1989. Cyclin synthesis drives the early embryonic cell cycle. Nature 339, 275–280.

Nakamura, Y., Yamagata, Sugino, N., Takayama, H., Kato, H., 2002. Nitric oxide inhibits oocyte meiotic maturation. Biol. Reprod. 67, 1588–1592.

Pangas, S.A., Jorgez, C.J., Mattttzuk, M.M., 2004. Growth differentiation factor 9 regulates expression of the bone morphogenetic protein antagonist gremlin. J. Biol. Chem. 279, 32281–32286.

Park, C.S., Park, R., Krishna, G., 1996. Constitutive expression and structural diversity of inducible isoform of nitric oxide synthase in human tissues. Life Sci. 59, 219–225.

Parrish, J.J., Susko-Parrish, J.L., Winer, M.A., First, N.L., 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38, 1171–1180.

Ralph, J.H., Telfer, E.E., Wilmut, I., 1995. Bovine cumulus cells expansion does not depend on the presence of an oocyte secreted factor. Mol. Reprod. Dev. 42, 248–253.

Ricart-Jané, D., Llobera, M., López-Tejero, M.D., 2002. Anticoagulants and other preanalytical factors interfere in plasma nitrate/nitrite quantification by Griess method. Nitric Oxide 6, 178–185.

Rubbo, H., Darley-Usmar, V., Freeman, B.A., 1996. Nitric oxide regulation of tissue free radical injury. Chem. Res. Toxicol. 9, 809–820.

Schwarz, K.R.L., Pires, P.R.L., Adona, P.R., Leal, C.L.V., 2006. Efeito da inibição do NO no desenvolvimento *in vitro* de embriões bovinos: resultados parciais. Act. Sci. Vet. 34, 446.

Sengoku, K., Takuma, N., Horikawa, M., Tsuchiya, K., Komori, H., Sharifa, D., Tamate, K., Ishikawa, M., 2001. Requirement of nitric oxide for murine oocyte maturation, embryo development, and trophoblast outgrowth *in vitro*. Mol. Reprod. Dev. 58, 262–268.

Sirard, M.-A., Richard, F., Mayes, M., 1998. Controlling meiotic resumption in bovine oocytes: a review. Theriogenology 49, 483–497.

Shimizu, T., Miyaahayashi, Y., Yokoo, M., Hoshino, Y., Sasada, H., Sato, E., 2004. Molecular cloning of porcine growth differentiation factor 9 (GDF-9) cDNA and its role in early folliculogenesis: direct ovarian injection of GDF-9 gene fragments promotes early folliculogenesis. Reproduction 128, 537–543.

Tao, Y., Fu, Z., Zhang, M., Xia, G., Yang, J., Xie, H., 2004. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Mol. Cell. Endocr. 222, 93–103.

Tao, Y., Xie, H., Hong, H., Chen, X., Xia, J.J.G., 2005. Effects of nitric oxide synthase inhibitors on porcine oocyte meiotic maturation. Zygote 13, 1–9.

Tesfaye, D., Kadanga, A., Rings, F., Bauch, K., Jennen, D., Nganvongpanit, K., Hölker, M., Tholen, E., Ponsuksili, S., Wimmers, K., Montag, M., Gilles, M., Kirfel, G., Herzog, V., Schellander, K., 2006. The effect of nitric oxide inhibition and temporal expression patterns of the mRNA and protein products of nitric oxide synthase genes during *in vitro* development of bovine pre-implantation embryos. Reprod. Dom. Anim. 41, 501–509.

van den Hurk, R., Zhao, J., 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology 63, 1717–1751.

van Voorhis, B.J., Moore, K., Strijbos, P.J., Nelson, S., Bayles, S.A., Grzybicki, D., Weiner, C.P., 1995. Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary, effects of gonadotropin stimulation *in vivo*. J. Clin. Invest. 96, 2719–2726.

Vanderhyden, B.C., 1993. Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). J. Reprod. Fertil. 98, 219–227.

Verlhac, M.H., Kubiak, J.Z., Clarke, H.J., Maro, B., 1994. Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development 120, 1017–1025.

Viana, K.S., Caldas-Bussiere, M.C., Matta, S.G.C., Faes, M.R., Carvalho, C.S.P., Quirino, C.R., 2007. Effect of sodium nitroprusside, a nitric oxide donor, on the *in vitro* maturation of bovine oocytes. Anim. Reprod. Sci. 102, 217–227.

Wessel, G.M., Conner, S.D., Berg, L., 2002. Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte. Development 129, 4315–4325.

Winston, N.J., 1997. Stability of cyclin B protein during meiotic maturation and the first meiotic cell cycle division in mouse oocyte. Biol. Cell 89, 211–219.

Yoshida, M., Cran, D.G., Pursel, V.G., 1993. Confocal and fluorescence microscopic study using lectins of the distribution of cortical granules during the maturation and fertilization of pig oocytes. Mol. Reprod. Dev. 36, 462–489.