ELSEVIER

Animal Reproduction Science 110 (2009) 222–236

ANIMAL REPRODUCTION SCIENCE

www.elsevier.com/locate/anireprosci

Nitric oxide regulates steroid synthesis by bovine antral granulosa cells in a chemically defined medium

M.R. Faes [a], M.C. Caldas-Bussiere [a,*], K.S. Viana [a], B.L. Dias [a], F.R. Costa [b], R.M. Escocard [c]

[a] *Laboratório de Reprodução e Melhoramento Genético Animal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, 2000 Alberto Lamego Avenue, Parque Califórnia, Campos dos Goytacazes, RJ 28013-602, Brazil*

[b] *Laboratório de Citogenética, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, 2000 Alberto Lamego Avenue, Parque Califórnia, Campos dos Goytacazes, RJ 28013-602, Brazil*

[c] *Laboratório de Biologia do Reconhecer, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Centro de Ciências e Tecnologias Agropecuárias, 2000 Alberto Lamego Avenue, Parque Califórnia, Campos dos Goytacazes, RJ 28013-602, Brazil*

Received 17 July 2007; accepted 28 January 2008
Available online 7 February 2008

Abstract

Nitric oxide (NO) in bovine ovary has been characterized as one of the controllers of granulosa cells' (GC) steroidogenesis and apoptosis. One of the pathways used by NO to have these effects is cGMP. The objectives of the present study were to verify the effect of sodium nitroprusside (SNP), a NO donor, on steroidogenesis, cell viability (mitochondrial activity) and GC cell cycle distribution and if this effect occurs by the NO-cGMP signaling pathway with the addition of SNP with or without 1H-[1,2,3] oxadiaziolo[4,3a]quinoxaline-1-one (ODQ), a selective soluble guanylate cyclase inhibitor. The antral GC from 3 to 5 mm diameter cattle follicles was cultured without treatment (control), with ODQ (10^{-4} M) and 10^{-5}, 10^{-3} and 10^{-1} M SNP with or without ODQ for 24 h. Nitrate/nitrite ($NO_3^-/N0_2^-$) concentrations were evaluated by Griess method, progesterone (P_4) and 17β-estradiol (E_2) concentrations by chemiluminescence, viability and cell cycle stage by MTT method (3-[4,5-dimethylthiazol-2yl]-2,3 diphenyltetrazolium bromide) and flow cytometry, respectively. Nitrate/nitrite concentration in culture medium increased ($P < 0.05$) in a dose-dependent manner according to SNP concentration added to the culture medium. The GC cultured without treatment, with ODQ and with 10^{-5} M SNP in the presence or absence of ODQ developed into cell aggregates and did not vary in cell viability ($P > 0.05$), while GC cultured with 10^{-3} and 10^{-1} M SNP with or without ODQ presented disorganized GC aggregates or did not develop into cell aggregates and also had substantially decreased cell

* Corresponding author. Tel.: +55 22 2726 1665; fax: +55 22 2726 3156.
E-mail address: claracal.@uenf.br (M.C. Caldas-Bussiere).

0378-4320/$ – see front matter
doi:10.1016/j.anireprosci.2008.01.018

viability (mitochondrial activity inhibition) and steroids synthesis ($P < 0.05$), and effects were not reversed with us of ODQ. Most GC cultured without treatment (control) or with ODQ, 10^{-5} and 10^{-3} M SNP with or without ODQ were in the G0/G1 (80–75%) stage and in a lesser proportion (20–25%) in the S + G2/M stage of the cell cycle, while the 10^{-1} M SNP treatment resulted in GC in G1 phase arrest. The treatment with 10^{-5} M SNP increased ($P < 0.05$) E_2 synthesis and inhibited ($P < 0.05$) progesterone synthesis. The addition of ODQ reversed ($P < 0.05$) the stimulatory effect of 10^{-5} M SNP treatment on E_2, but not on P_4 synthesis ($P > 0.05$). These results demonstrated that E_2 synthesis by antral GC from small follicles is modulated by lesser NO concentrations via the cGMP pathway, but not P_4 while steroids inhibition cGMP pathway independent, mitochondrial damage and the interference on cell cycle progression caused by greater NO concentration can lead to cell death.

Keywords: Granulosa cells; Nitric oxide; Progesterone; 17β-Estradiol; cGMP; Cattle

1. Introduction

17β-Estradiol (E_2) has an essential role to establish and maintain the reproductive system. Estradiol synthesis by granulosa cells (GC) depends on enzyme P450 aromatase (P450arom), which metabolizes androgens into E_2. Its increase is induced by FSH, and when bound to the receptor attaches to G protein, activates adenylyl cyclase and stimulates the production of the second messenger cAMP (Richards et al., 1995). Besides FSH, there are other hormones, growth factors, proteins, peptides, intercellular and intracellular factors that modulate GC functions (Chun et al., 1995; Lapolt and Hong, 1995).

Nitric oxide (NO) is a colorless gas at room temperature and pressure. The maximum solubility of NO in water at room temperature and pressure is slightly greater than the solubility of dioxygen (O_2). Similar to O_2, NO is somewhat lipophilic and is 6–8 times more soluble in nonpolar solvents and lipid membranes than in water. Thus, NO reaction rates can be dramatically greater in a hydrophobic environment than in water due to its concentration increase (Liu et al., 1998), because it has a greater solubility in biological membranes.

NO is produced from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS) that exists in three isoforms: endothelial (eNOS) and neuronal (nNOS), which are Ca^{2+}/CaM-dependent and inducible (iNOS), regulated by cytokines (Snyder et al., 1996). The presence of eNOS and iNOS as a source of NO in ovarian compartments and its function as a steroidogenesis regulator in GC has previously been demonstrated in mice (Janblonka-Shariff and Olson, 1997; Matsumi et al., 1998; Hurwitz et al., 2002) and cattle (Basini et al., 1998). However, there are still inconsistencies with regard to the mechanism by which NO effects GC.

NO is a negative regulator of steroids synthesis. NO exerts its effects by binding to the prosthetic heme group of enzymes. Thus, NO may directly bind to the enzyme, P450 aromatase, and inhibit gonadotropin-stimulated steroidogenesis (Van Voorhis et al., 1994; Hanke et al., 1998).

Another mechanism of action mediated by NO is the activation of soluble guanylate cyclase that converts GTP (guanosine triphosphate) to cGMP and inorganic pyrophosphate (Murard, 1999; Hanafy et al., 2001) in a heme-dependent manner (Craven and DeRubertis, 1978). It has been suggested that cGMP increases phosphodiesterase 2 (PDE2) activity which in turn increases cAMP hydrolysis, the FSH intracellular second messenger.

In cultured GC from mice (Ishimaru et al., 2001) and pigs (Grasselli et al., 2001) the NO/cGMP pathway has been suggested as one of the mechanisms used by NO to inhibit steroidogenesis.

The use of cGMP analogs in cows, however, did not efficiently inhibit steroidogenesis (Basini et al., 2000).

These results are conflicting, probably due to variations among culture systems, presence of blood cells, differences among species and variation in GC differentiation stages of cells that are used. BSA is normally used in most culture systems, including those previously reported, and is contaminated with steroids, cholesterol, peptides and other yet to be recognized molecules (Wang et al., 1997; Mingoti et al., 2002). For this reason, culture medium becomes semi-defined possibly causing interference in the results. Moreover, the presence of blood cells can also alter GC steroidogenesis and NO synthesis/concentration (Beckmann et al., 1991; Shakil and Whitehead, 1994) in culture medium. Therefore, in the present experiment, BSA was replaced by polyvinyl alcohol (PVA) in a culture system which maintains GC P450arom activity without undergoing luteinization (Piccinato et al., 2000). Methods to extract red blood cells and macrophages that are accidentally collected during ovarian follicle aspiration were also performed. Using this culture system, the objectives were: (1) to verify NO effect using sodium nitroprusside (SNP), a NO donor, on antral GC steroid synthesis, viability and cell cycle distribution obtained from small follicles (3–5 mm) of cattle, because these have greater steroidogenic activity (Rouillier et al., 1996, 1998) and are not yet differentiated having greater proliferative activity (Gutierrez et al., 1997); (2) to verify if these effects are modulated by cGMP pathway using a treatment with 1H-[1,2,3] oxadiaziolo[4,3a]quinoxaline-1-one (ODQ), a selective soluble guanylate cyclase inhibitor.

2. Materials and methods

2.1. Reagents

Most reagents were obtained from Sigma (St. Louis, MO, USA). Reagents from other laboratories are specified.

2.2. Experimental design

GC were cultured with 0, 10^{-5}, 10^{-3}, 10^{-1} M sodium nitroprusside without or with 10^{-4} M ODQ (Basini et al., 2000). The synthesis of NO_3^-/NO_2^-, progesterone (P_4) and E_2 by GC and GC viability and cell cycle status were evaluated at the end of 24 h of culture.

2.3. GC collection

Ovaries were collected postmortem at a local abattoir from mixed-age cows in the luteal and follicular phase of the estrous cycle and the absence of palpable products of pregnancy (Ireland et al., 1980) and were immediately transported (30 °C) to the laboratory in sterile saline solution (NaCl 0.9%) supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin sulphate). Excess tissue was trimmed. Ovaries were washed with saline solution containing antibiotics and were briefly rinsed with 70% ethanol and then washed (three times) with saline solution supplemented with antibiotics.

Small well-vascularized follicles (3–5 mm) having more than 70% of clear exposed surface (Grimes et al., 1987) were punctured. Pool of antral GC were then aspirated (Rouillier et al., 1998) and placed in tubes of 15 ml containing 2 ml base medium [Dulbecco's Modification of Eagle's Medium/Ham's F-12 containing Hepes 15 mM (Gibco-BRL Life Technologies), supplemented

with 10 mM sodium bicarbonate; 100 UI/ml penicillin G and 100 μg/ml streptomycin sulphate (Merck Indústrias SA, Rio de Janeiro, Brazil)] and 50 UI heparin to prevent cell aggregates.

GC suspension was filtered through nylon mesh to extract oocytes and cellular debris and then centrifuged (300 × *g*/10 min) at room temperature. Supernatant was decanted and GC were washed (twice) using 1 ml base medium. Regarding the fact that red blood cells can bind to NO (McMahon, 2002), that macrophages can synthesize NO (Whitehead and Lacey, 1996) and that during follicle puncture blood may also be aspirated, two procedures were accomplished. GC pellet was treated with 300 μl 0.9% prewarmed ammonium chloride solution at 37 °C and incubated for 1 min to extract red blood cells. Following, GC isotonicity was recovered by adding base medium up to 12 ml into the tubes containing GC. GC were centrifuged (300 × *g*/10 min) and the pellet was washed with base medium. The GC pellet was re-suspended in 1 ml base medium and GC were seeded in a 32 mm tissue culture dish (Corning Incorporated, Corning, NY, USA) containing 2 ml base medium. GC were incubated for 2 h at 37.5 °C in a 5% CO_2 atmosphere to remove macrophages, because these cells can easily adhere to culture plate (Beckmann et al., 1991).

Remaining GC (not adhered) were collected from culture plate, centrifuged (300 × *g*/10 min) and re-suspended in 2 ml culture medium [Dulbecco's Modification of Eagle's Medium/Ham's F-12 containing Hepes 15 mM, 10 mM sodium bicarbonate, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulphate (Merck Indústrias SA); 10^{-7} M androstenedione; 0.1% PVA; 1% MEM nonessential amino acid mixture 100% and 1% solution ITS 100% (1.4 ng/ml sodium selenite, 5.0 μg/ml transferrin, 10 ng/ml insulin)].

Viability and cell number were estimated under an inverted microscope (Telaval 31, Zeiss) at 400× magnification using a hemocitometer and Trypan blue (0.4%) exclusion method. Cellular viability average was 70%.

Smears of the cellular suspension were realized before and after red blood cells and macrophages removal. After staining with hematoxyline, 100 cells were counted in 10 replicates (1000 total cells). For each 100 cells counted before the treatment an average of 39.8 ± 7.7% red blood cells and 8.5 ± 3% macrophages were found. After treatment 9 ± 4% red blood cells and 0.4 ± 0.5% macrophages were found.

2.4. GC culture

5.0×10^5 antral GC of follicles of 3–5 mm diameter were seeded in 24-well plates with 1000 μl culture medium containing the treatments. GC were then incubated at 37.5 °C in a 5% CO_2 atmosphere.

2.5. Cell viability determination

The NO effect on cell viability was evaluated by MTT method. This assay indirectly determines cell viability by measuring the activity of mitochondrial enzymes. Yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,3 diphenyltetrazolium bromide (MTT) is metabolized by mitochondrial dehydrogenases to form the final product blue formazan (metyltriazole) (Mosmann, 1983). At the end of 24 h of culture period, after the removal of 700 μl culture medium for hormone and NO analysis, 30 μl MTT (5 mg/ml) were added to each well and GC were held in incubation for 3 h at 37 °C in CO_2:95% air. After incubation 300 μl isopropanol acidified with HCl (0.04 N) containing 10% Triton X 100 were added to dissolve formed crystals and remained 14–16 h at room temperature. After this period, 200 μl blue formazan/mixture were transferred to 96-

well plates and analyses were determined by a spectrophotometer (Multiskan EX Primary EIA V 2.1–0) at wavelength of 570 nm, with background subtraction (620 nm). The relationship between absorbance and cell number was determined by the previous incubation of a known cell number with MTT to establish a standard curve. A linear relationship between cell number and absorbance values ($R^2 = 0.99$; $P < 0.05$) was found.

2.6. Cell cycle

To determine the effect of different treatments on GCs cell cycle distribution the flow cytometry determination was used. For that reason, the method described by Blondin et al. (1996) was used with some modifications. GC were collected after incubation with 1 ml PBS Ca^{2+}/Mg^{2+}-free and EDTA (125 mg/l) for 10 min and then centrifuged at 400 × *g* at 4 °C for 5 min. The supernatant was decanted and the pellet was re-suspended in 500 μl PBS Ca^{2+}/Mg^{2+}-free. GC were fixed with 1 ml absolute ethanol during agitation in vortex for at least 1 h at 4 °C. Afterwards, GC were centrifuged and the pellet re-suspended with 500 μl ethanol 70% and stored at −20 °C until flow cytometry analysis. Prior to staining GC were centrifuged, the supernatant was decanted and cells were washed twice with PBS (400 × *g* at 4 °C for 5 min). Fixed GC were re-suspended with 1 ml PBS containing RNAse at 50 μg/ml (Merck Indústrias SA), propidium iodide (50 μg/ml) and incubated for 4 h at 4 °C. Samples were filtered through 40 μm nylon mesh. The percentage of cells with degraded DNA and cell cycle distributions for single cells was determined using a ploidy analyzer (PARTEC PA) with UV filter. Data derived from DNA histogram were analyzed by flow cytometer consisting of GC percentage in stage G0/G1 (diploid cells), S + G2/M (DNA synthesis and mitosis).

2.7. Nitrate/nitrite and steroids dosages

After a 24 h period, culture medium was collected and stored at −20 °C to dose nitrate/nitrite (NO_3^-/NO_2), P_4 and E_2.

2.7.1. NO_3^-/NO_2^-

Nitrate/nitrite concentrations 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 GC cultures, 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 UI 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 DMEM/Ham's-F12 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.

2.7.2. Steroids

Progesterone and E_2 concentrations were determined by an automated chemiluminescence system (ACS:180®, Bayer Corporation, Tarrytown, NY, USA) which is a competitive immunoassay that uses direct chemiluminescent technology (progesterone and estradiol derivative are labeled with acridinium ester). The monoclonal mouse anti-rabbit antibodies are coupled to paramagnetic particles and are highly specific, possesses a cross-reactivity of less than 0.95% with other compounds. Progesterone and E_2 concentrations ranged from 0.86 to 29.80 ng/ml and 88 to 785 pg/ml, respectively. Samples containing 0 and 10^{-5} M SNP with or without ODQ were diluted (1/400), however, culture medium samples with 10^{-3} and 10^{-1} M SNP with or without ODQ were not. The average intra-assay coefficient of variation was 1.6% and 3.0% for P_4 and E_2, respectively.

2.8. Statistical analysis

Each experiment assessing cell viability, cell cycle, steroidogenesis and NO concentration was repeated two times independently and in each experiment treatment two replicates culture wells. Data are expressed as mean ± S.E.M. Statistical differences between treatments and interactions were calculated with multifactorial ANOVA. When significant differences were found, means were compared by Tukey test considered significant at $P < 0.05$. SAS (1996) (Statistical Analysis System) software was used.

3. Results

3.1. Morphological characteristics of GC

At the end of 24 h, GC cultured with 0 (control), 10^{-5} M SNP with or without ODQ (Fig. 1, panels a–d) maintained the typical round shape of steroidogenically active GC and formed cell groups, while GC cultured with 10^{-3} M SNP with or without ODQ (Fig. 1, panels e and f) formed disorganized groups, had dark color with little adherence to the culture plate and the cultured with 10^{-1} M SNP with or without ODQ (Fig. 1, panels g and h) did not form groups, had dark color and were flattened in the culture plate.

3.2. NO_3^-/NO_2^- concentration

NO concentration in culture medium increased ($P < 0.05$) in a dose-dependent manner according to the SNP concentration added in culture medium: SNP 10^{-5} M with or without ODQ (6.7 ± 2.27, 6.6 ± 2.3 μM, respectively), SNP 10^{-3} M with or without ODQ (48.6 ± 11, 50.9 ± 2.6 μM, respectively) and SNP 10^{-1} M with or without ODQ (65.6 ± 2.9, 68.3 ± 11.7 μM, respectively) (Fig. 2a).

3.3. Cell viability

Cell viability was not altered ($P > 0.05$) among control group ($6.8 \times 10^5 \pm 0.27$ viable cells) and groups cultured with ODQ ($6.6 \times 10^5 \pm 0.75$ viable cells) and 10^{-5} M SNP with ODQ ($7.0 \times 10^5 \pm 0.5$ viable cells) or without ODQ ($7.6 \times 10^5 \pm 0.8$ viable cell). However, after the addition of 10^{-3} and 10^{-1} M SNP with or without ODQ, 100% GC ($P < 0.05$) became non-viable (Fig. 2b).

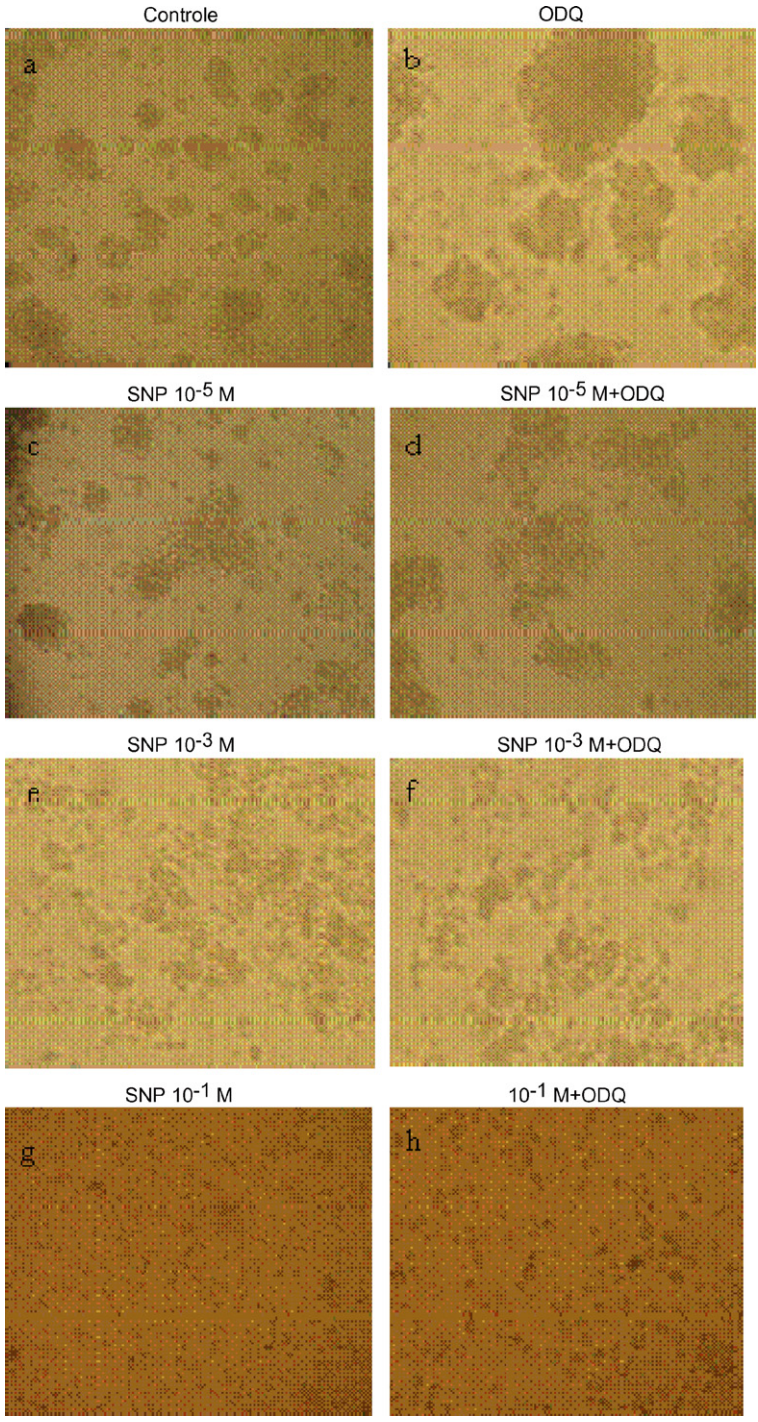

Fig. 1. Granulosa cells cultured without SNP and ODQ (control) (a) or with 10^{-4} M ODQ (b); 10^{-5} M SNP (c); 10^{-5} M SNP + 10^{-4} M ODQ (d); 10^{-3} M SNP (e); 10^{-3} M SNP + 10^{-4} M ODQ (f); 10^{-1} M SNP (g); 10^{-1} M SNP + 10^{-4} M ODQ (h) (at 400× magnification).

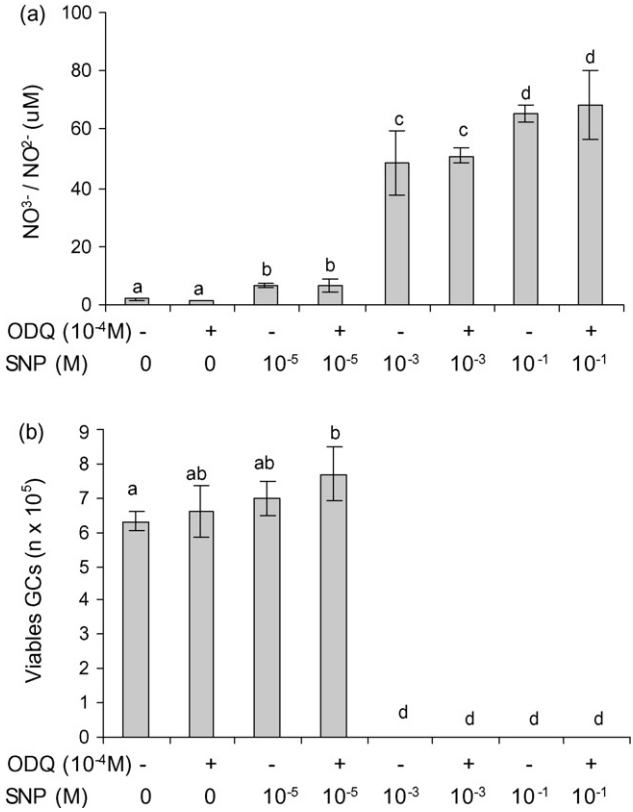

Fig. 2. Effect of the addition of SNP (0, 10^{-5}, 10^{-3} and 10^{-1} M) with or without ODQ (10^{-4} M) in NO_3^-/NO_2^- concentration in culture medium and in cell viability (number of viable GCs $\times 10^5$) after 24 h culture. Values with different superscripts (a–d) are different ($P < 0.05$) by Tukey test. Each value represents mean ± S.E.M. of a total of 4 determinations from two independents experiments.

3.4. Cell cycle

Most GC cultured with 0 (control), 10^{-5} and 10^{-3} M SNP with or without ODQ were in G0/G1 (80–70%) stage after 24 h culture, while 20–30% were in the stage S + G2/M. The GC cultured with 10^{-1} M SNP, however, had a decreased cell count and a G1 phase arrest (Fig. 3).

3.5. Steroidogenesis

SNP at 10^{-3} and 10^{-5} M inhibited ($P < 0.05$) P_4 synthesis (14.39 ± 0.003; 129.1 ± 0.006 ng/ml, respectively) compared to control (174.7 ± 0.02 ng/ml): 10^{-3} SNP had a greater effect ($P < 0.05$) than SNP 10^{-5} M. The addition of 10^{-4} M ODQ did not influence the negative effect of 10^{-3} and 10^{-5} M SNP on P_4 synthesis (16.35 ± 0.0007; 126.68 ± 0.044 ng/ml, respectively) and ODQ also inhibited P_4 synthesis (91.3 ± 0.02 ng/ml)(Fig. 4, panel a).

Although the addition of both 10^{-4} M ODQ and 10^{-5} M SNP stimulated ($P < 0.05$) E_2 synthesis (3.4; 6.8 ± 0.5 ng/ml, respectively) when compared to the control (2.0 ng/ml, $P < 0.05$), E_2 synthesis was greater ($P < 0.05$) for the 10^{-5} M SNP than for ODQ treatments. Even though the

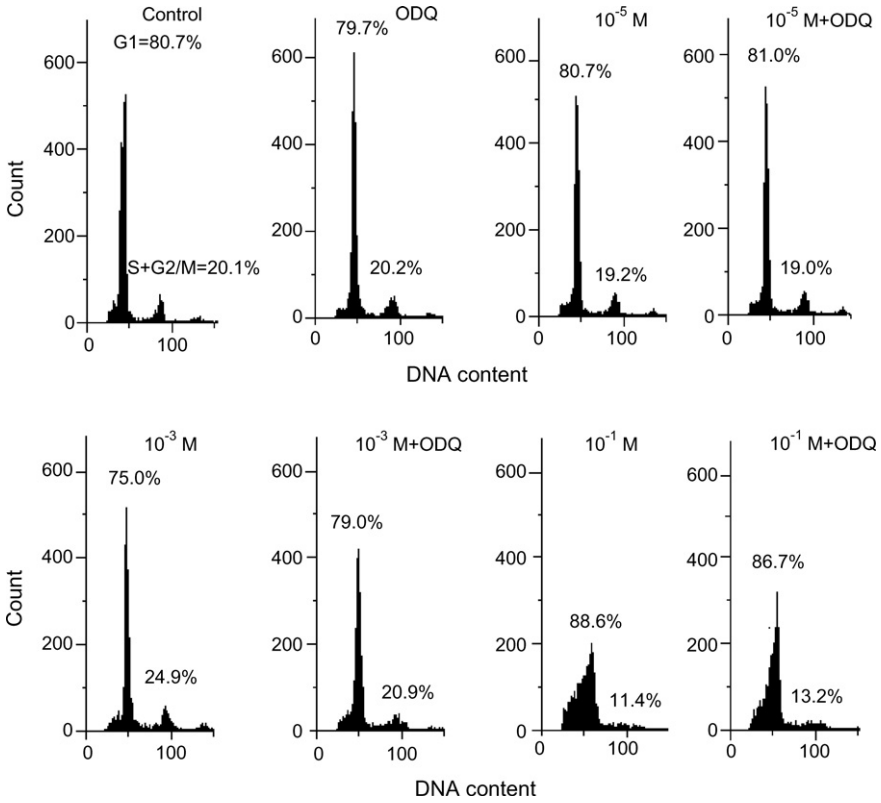

Fig. 3. Effect of SNP addition on cell cycle of GC cultured without SNP and ODQ (control); 10^{-4} M ODQ; 10^{-5} M SNP, 10^{-5} M SNP + 10^{-4} M ODQ; 10^{-3} M SNP; 10^{-3} M SNP + 10^{-4} M ODQ; 10^{-1} M SNP; 10^{-1} M SNP + 10^{-4} M ODQ. G0: subdiploid DNA levels; G0/G1: diploid cells; S and G2/M: DNA synthesis/cell mitosis.

addition of ODQ inhibited ($P < 0.05$) the increase of E_2 synthesis (3.3 ± 0.3 ng/ml) which was stimulated by the addition of 10^{-5} M SNP, no difference ($P > 0.05$) in E_2 synthesis between cultures containing ODQ and ODQ with 10^{-5} M SNP was observed. The addition of 10^{-3} M SNP inhibited ($P < 0.05$) E_2 synthesis (0.02 ± 0.001 ng/ml) which was not reversed by the addition of ODQ (0.02 ± 0.1 ng/ml) (Fig. 4, panel b).

Progesterone and E_2 synthesis in cultures containing 10^{-1} M SNP was less than the minimum concentration (>0.86 ng/ml and >88.0 pg/ml, respectively) detected by the method utilized and, therefore, is not included in the figures.

4. Discussion

The current research demonstrated that the culture system containing PVA maintained the viability of GC, allowed cells to progress though the cellular cycle, and maintained the morphological characteristics of steroidogenicaly actives GC described by Gutierrez et al. (1997), such as a round shape and cell groups adhered to the plate by fibroblast-like cells. In addition, the treatment to remove red blood cells and macrophages was efficient. Blood cells may interfere in steroidogenesis and macrophages may synthesize NO (Beckmann et al., 1991; Shakil and

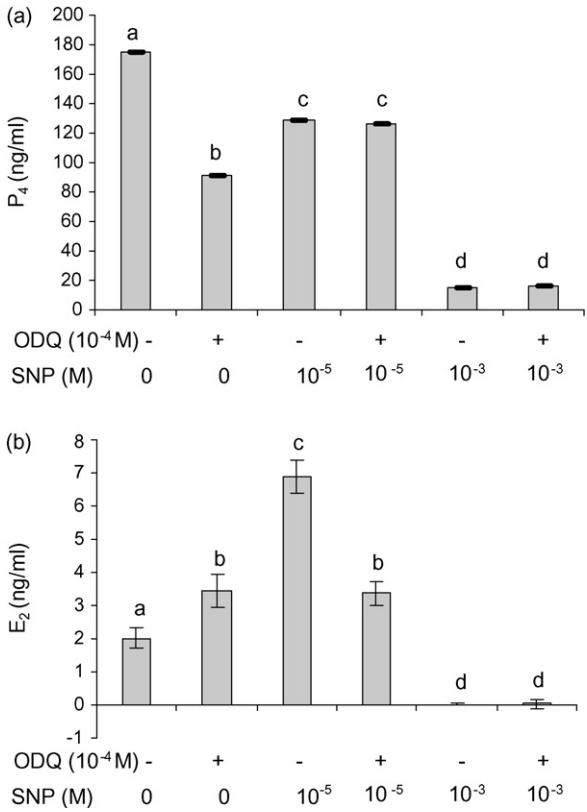

Fig. 4. Effect of SNP addition (0, 10^{-5}, 10^{-3} M) with or without ODQ (10^{-4} M) on P_4 and E_2 synthesis by GC after 24 h culture. Values with different superscripts (a–d) are different ($P < 0.05$) by Tukey test. Each value represents mean ± S.E.M. of a total of four determinations from two independents experiments.

Whitehead, 1994). Consequently, this treatment becomes important to verify NO effects in GC steroidogenesis.

An inhibitory effect on E_2 synthesis without the cGMP pathway activation (Basini et al., 2000) in cattle GC cultured after the addition of increasing concentrations of NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), has been verified. Some studies, however, suggest that the NO/cGMP pathway is a GC steroidogenesis inhibition mechanism in rats (Ellman et al., 1993) and pigs (Grasselli et al., 2001). Although the E_2 synthesis inhibition mechanism that has been proposed is different, they have in common the fact that NO inhibits steroid synthesis. On the contrary, the results of the current experiment indicates that at least during the first 24 h culture NO stimulates E_2 synthesis by antral GC through the activation of soluble nitric oxide-sensitive guanylyl cyclase, because 10^{-5} M SNP stimulated E_2 synthesis, which was inhibited by the addition of ODQ. In spite of these inconsistences, there are reports the increase of nitrite concentration (NO metabolite) is correlated with follicular volume and E_2 concentration, but not with progesterone concentration in human follicular fluid during follicular phase of the reproductive cycle (Anteby et al., 1996). Additionally, Bonello et al. (1996) found a decrease in E_2 synthesis in rats through ovarian perfusion using the inhibitor of an NOS enzyme (Nω-nitro-L-arginine methyl ester, L-NAME), suggesting that NO may positively regulate E_2 synthesis. The present results

agree with these latter results suggesting that NO may take part in the positive regulation of E_2 synthesis by GC of cattle.

Most NO biological effects occur by the soluble guanylyl cyclase activation that catalyzes the conversion of GTP into cGMP (Denninger and Marletta, 1999). cGMP causes cAMP concentrations to decrease by the phosphodiesterase-cAMP (PDE-cAMP) activation that cleaves cAMP (MacFarland et al., 1991). However, there are two PDE categories: PDE 2 activated by NO which cause cAMP hydrolysis (MacFarland et al., 1991), and PDE 3 (Conti et al., 1995) inhibited by NO and cGMP, resulting in cAMP increase and in the activation of the camp-dependent pathway (Draijer et al., 1995; Kurtz et al., 1998). Oocyte PDE 3 gene expression and GC PDE 4 gene expression has been reported (Tsafriri et al., 1996). Thus, it becomes clear that other components of the cGMP signaling pathway must be elucidated to comprehend NO actions on cGMP and cAMP pathway.

Besides the previously mentioned considerations, the culture system used in the present study was different from that in previous studies because of GC type and differentiation stage, and also because of the different supplementations added to the culture (Basini et al., 2000; Grasselli et al., 2001). Cultures supplemented with BSA become undefined because BSA contains cholesterol, testosterone, progesterone, various growth factors and other yet to be determined components. Moreover, there are variations in the component concentrations, because these are biological materials (Wang et al., 1997; Maurer, 1992; Keskintepe and Brackett, 1996; Mingoti et al., 2002). Mingoti et al. (2002) also demonstrated a decrease on estradiol synthesis by *cumulus* cells when bovine *cumulus*–oocyte complexes (COC) were matured in culture medium supplemented with BSA and testosterone, suggesting that BSA increases androgen concentrations in the medium, causing a negative effect on estradiol synthesis, and hence influencing the results obtained in such studies. Additionally, the culture supplementation with insulin, not verified in the culture system utilized by Basini et al. (2000), might have resulted in the differences observed in results regarding the NO role on steroid synthesis by GC of cattle, because insulin maintains GC steroidogenic activity (Gutierrez et al., 1997).

Although 10^{-5} M SNP stimulated E_2 synthesis, it inhibited progesterone without soluble nitric oxide-sensitive guanylyl cyclase activation, in accordance with Basini et al. (2000). Besides the cGMP pathway, NO can activate enzymes containing heme groups such as cyclooxygenases (Rettori et al., 1992) and cytochrome P450 (Wink et al., 1993). Snyder et al. (1996) suggested that NO regulates the stedoidogenesis by the direct inhibition of cytochrome P450 enzymes. Accordingly, the results for the present experiment showed that NO inhibited progesterone synthesis by GC through cGMP independent mechanisms, but it can also positively regulate E_2 synthesis by cGMP in the first 24 h culture.

It was also shown that the NO independent cGMP pathway is involved with steroids synthesis, because of the cGMP pathway inhibition by the addition of ODQ without SNP stimulated estradiol synthesis in comparison with the control. Therefore, the 50% estradiol increase was due to the 10^{-5} M SNP addition that stimulated estradiol synthesis by cGMP pathway, while the 20% estradiol increase was due to cGMP pathway inhibition which is stimulated by factors not elucidated in this experiment. It was also observed that there are other factors regulating progesterone synthesis through the cGMP pathway, because its inhibition decreased progesterone synthesis, however, no negative effect was detected on progesterone synthesis when ODQ was added to 10^{-5} M SNP treatment. Even though NO is the major guanylyl cyclase physiological activator (Ignarro, 1991) there are other physiological regulators involved in its activation such as calcium (Sitaramayya, 2002) and protoporfirin IX (Mingone et al., 2006). Little is known about guanylyl cyclase regulators in ovaries (Lapolt et al., 2002), and thus research in this field must be done

to verify other cGMP pathway regulators which will provide a greater understanding about the events that control ovarian function.

Results from the present study also showed that greater SNP concentrations (10^{-3} and 10^{-1} M) inhibit both progesterone and estradiol synthesis by independent cGMP pathway mechanisms as previously detected by Basini et al. (2000). Basini et al. (1998) showed that NO in greater concentrations (10^{-3} M SNAP) protects GC from apoptosis. Results of the present study also did not demonstrate that GC apoptosis was induced by greater concentrations of SNP (10^{-3} and 10^{-1} M), but it did indicate that greater NO concentrations did not protect GC from cellular damage, on the contrary, these cells had a lesser viability as verified by substantial mitochondrial damage and altered the morphological characteristics of steroidogenicaly active GC which were previously described by Gutierrez et al. (1997), and had disorganized GC aggregates (10^{-3} M) or did not develop into cell aggregates (10^{-1} M).

NO in greater concentrations induces apoptosis and/or necrosis in rat ovaries (Ellman et al., 1993), human epithelial-endometrial cells (Li et al., 2001) and cattle embryos (Orsi, 2006). NO in greater concentrations compete with oxygen and binds to enzyme heme groups from the respiratory chain, as well as cytochrome oxidase (Clementi et al., 1999; Sarti et al., 1999). After several hours of NO treatment, cytochrome oxidase enzyme can be irreversibly inhibited due to NO conversion into nitrogen reactive species (peroxynitrite), which inhibits various cellular respiratory sites (Clementi et al., 1999; Sarti et al., 1999). This mechanism triggers calcium release (Richter, 1993). Disturbances in mitochondrial calcium concentrations can kill cells through excessive calcium cycling across the inner mitochondrial membrane which can have serious consequences such as disruption of mitochondrial membrane potential, ATP depletion and cell death (Richter, 1993). Therefore, the stable maintenance of mitochondrial membrane potential can inhibit the onset of apoptosis. Thus, the present data suggest that mitochondrial damage caused by toxic effect of greater SNP concentration perturbs the Krebs cycle which in normal physiologic conditions generated nicotinamide adenine dinucleotide phosphate (NADPH) utilized by P450scc and P450arom to P_4 and E_2 synthesis, respectively (Strauss et al., 1981; Lieberman et al., 1984). The possibility cannot be ignored that NO may also bind with the heme group of P450scc and P450arom as an inhibition mechanism of steroid synthesis (Van Voorhis et al., 1994; Snyder et al., 1996; Hanke et al., 1998).

Although 10^{-3} M SNP concentrations kept most GC in the G0/G1 stage, these cells progressed though the cell cycle similarly to control and treatments containing 10^{-5} M SNP with or without ODQ while 10^{-1} M SNP induced G1 phase arrest as demonstrated by flow cytometry. NO inhibitory effects on cell proliferation were demonstrated in vascular smooth muscle cells (VSMCS) (Tanner et al., 2000), and T cells (Sato et al., 2007). NO is involved on over-expression of p21 (cyclin-dependent kinase inhibitors) which induces G1 arrest in VSMCS (Tanner et al., 2000), and this elevated concentration is consistent with the anti-proliferative effect of NO (Yang et al., 1996). A disruption of DNA synthesis can interfere with cell replication and lead to cell death (Burney et al., 1997). The present results demonstrated that greater NO concentrations inhibited GC progression though the cell cycle suggesting a cytostatic effect.

The results from the current experiment, that utilized the culture system previously described, demonstrated that lesser NO concentrations can negatively regulate P_4 synthesis by a cGMP independent pathway; however, this pathway is involved in the negative regulation of progesterone synthesis by factors not elucidated. The NO/cGMP pathway is, however, involved with synthesis, but this pathway also seems to be involved in inhibition of estradiol synthesis by independent NO mechanisms. NO may, therefore, be involved in steroidogenesis regulation, GC differentiation by independent or dependent cGMP mechanisms. In addition, cytotoxic effects verified by greater

NO concentration treatments in the present experiment likely initiated with mitochondrial function inactivation and subsequently with interference on cell cycle progression and P_4 and E_2 synthesis inhibition. Probably this NO toxicity that was induced could lead a cell death by apoptosis or necrosis. Therefore, these findings have important implications regarding NO concentrations in GC function.

Acknowledgments

Authors thank Carla Sobrinho Paes de Carvalho and Sílvia Gonsalves de Carvalho Matta for their technical support, Professora Telma Nair Pereira (flow cytrometry) for her collaboration. This work was supported by FAPERJ. M.R. Faes—recipient of a fellowship from CNPq.

References

Anteby, E.Y., Hurwitz, A., Korach, O., Revel, A., Simon, A., Finci-Yeheskel, Z., Mayer, M., Laufer, N., 1996. Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. Hum. Reprod. 11, 1947–1951.

Basini, G., Baratta, M., Ponderato, N., Bussolati, S., Tamanini, C., 1998. Is nitric oxide an autocrine modulator of bovine granulosa cell function? Reprod. Fertil. Dev. 10, 471–478.

Basini, G., Grasselli, F., Ponderato, N., Bussolati, S., Tamanini, C., 2000. Lipid hydroperoxide and cGMP are not involved in nitric oxide inhibition of steroidogenesis in bovine granulosa cells. Reprod. Fertil. Dev. 12, 289–295.

Beckmann, M.W., Polacek, D., Seung, L., Schreiber, J.R., 1991. Human ovarian granulosa cell culture: determination of blood cell contamination and evaluation of possible culture purification steps. Fertil. Steril. 56, 881–887.

Blondin, P., Dufour, M., Sirard, M.A., 1996. Analysis of atresia in bovine follicles using different methods: flow cytometry, enzyme-linked immunosorbent assay, and classic histology. Biol. Reprod. 54, 631–637.

Bonello, N., McKie, K., Jasper, M., Andrew, L., Ross, N., Braybon, E., Brännstrom, M., Norman, R.J., 1996. Inhibition of nitric oxide: effects on interleukin-1β-enhanced ovulation rate, steroid hormones, and ovarian leukocyte distribution at ovulation in rat. Biol. Reprod. 54, 436–445.

Burney, S., Tamir, S., Gal, A., Tannenbaum, S.R., 1997. A mechanistic analysis of nitric oxide-induced celluar toxicity. Nitric Oxide 1, 130–144.

Chun, S.Y., Eisenhauer, K.M., Kubo, M., Hsueh, A.J.W., 1995. Interleukin-1β suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. Endocrinology 136, 3120–3127.

Clementi, E., Brown, G.C., Foxwell, N., Moncada, S., 1999. On mechanism by which vascular endothelial cells regulate their oxygen consumption. Proc. Natl. Acad. Sci. U.S.A. 96, 1559–1562.

Conti, M., Nemoz, G., Sette, C., Vicini, E., 1995. Recent progress in understanding the hormonal regulation of phosphodiesterases. Endocr. Rev. 16, 370–389.

Craven, P.A., DeRubertis, F.R., 1978. Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and hemeproteins. Evidence for involvement of the paramagnetic nitrosyl–heme complex in enzyme activation. J. Biol. Chem. 253, 8433–8443.

Denninger, J.W., Marletta, M.A., 1999. Guanylate cyclase and the NO/cGMP signaling pathway. Biochem. Biophys. Acta 5, 339–350.

Draijer, R.J., Atsma, D.E., van der Laarse, A., van Hinsberg, V.W., 1995. cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. Circulation Res. 76, 199–208.

Ellman, C., Corbett, J.A., Misko, T.P., McDaniel, M., Beckerman, K.P., 1993. Nitric oxide mediates interleukin-1-induced cellular cytotoxicity in rat ovary: potential role for nitric oxide in the ovulatory process. J. Clin. Invest. 92, 3053–3056.

Grasselli, F., Ponderato, N., Basini, G., Tamanini, C., 2001. Nitric oxide synthase expression and nitric oxide/cyclic GMP pathway in swine granulosa cells. Dom. Anim. Endocr. 20, 241–252.

Grimes, R.W., Matton, P., Ireland, J.J., 1987. A comparison of histological and non-histological and non-histological indices of atresia and follicular function. Biol. Reprod. 37, 82–88.

Gutierrez, C.G., Campbell, B.K., Webb, R., 1997. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characterisics. Biol. Reprod. 56, 608–616.

Hanafy, K.A., Krumenacker, J.S., Murad, F., 2001. NO, nitrotyrosine, and cyclic GMP in signal transduction. Med. Sci. Monitor 7, 801–819.

Hanke, C.J., Drewtt, J.G., Myers, C.R., Campbell, W.B., 1998. Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. Endocrinology 139, 4053–4060.

Hurwitz, A., Finci-Yeheskel, Z., Milwidsky, A., Mayer, M., 2002. Regulation of cycooxygenase activity and progesterone production in the rat corpus luteum by inducible nitric oxide synthase. Reproduction 123, 663–669.

Ignarro, L.J., 1991. Signal transduction mechanism involving nitric oxide. Biochem. Pharm. 41, 485–490.

Ireland, J.J., Murphee, R.L., Coulson, P.B., 1980. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J. Dairy Sci. 63, 155–160.

Ishimaru, R.S., Leung, K., Hong, L.-S., LaPolt, P.S., 2001. Inhibitory effects of nitric oxide on estrogen production and cAMP levels in rat granulosa cells cultures. J. Endocrinol. 168, 249–255.

Janblonka-Shariff, A., Olson, L.M., 1997. Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. Endocrinology 138, 460–468.

Keskintepe, L., Brackett, B.G., 1996. In vitro developmental competence of in vitro-matured bovine oocytes fertilized and cultured in completely defined media. Biol. Reprod. 55, 333–339.

Kurtz, A., Gotz, K.H., Hamann, M., Wagner, C., 1998. A stimulation of rennin secretion by nitric oxide is mediated by phosphodiesterase3. Proc. Natl. Acad. Sci. U.S.A. 95, 4743–4747.

Lapolt, P.S., Hong, L.S., 1995. Inibitory effects of superoxide dismutase and cyclic guanosina 3′, 5′-monophosphate on estrogen production in cultured rat granulosa cells. Endocrinology 136, 5533–5539.

Lapolt, S.P., Leung, K., Ishimaru, R., Tafoya, M.A., Chen, Y.J., 2002. Roles of cyclic GMP in modulating ovarian functions. In: Symposium: Genetic Aspects of Follicular and Oocyte Growth. Reprod. Biomed., pp. 15–23 (Online 6).

Li, H.Y., Chang, S.P., Yuan, C.C., Chao, H.T., 2001. Nitric oxide induces apoptosis in endometrial epithelial cells in the presence of progesterone: involvement of mitogen-activated protein kinase pathway. Mol. Hum. Reprod. 7, 755–763.

Lieberman, S., Greenfield, N.J., Wolfson, A., 1984. A heuristic proposal for understanding steroidogenic process. Endocr. Rev. 5, 128–148.

Liu, X., Miller, M.J., Thomas, D.D., Lancaster, J.R., 1998. Accelerated reaction of nitric oxide with oxygen within the hydrophobic interior of biological membranes. Proc. Natl. Acad. Sci. U.S.A. 95, 2175–2179.

MacFarland, R.T., Zelus, B.D., Beavo, J.A., 1991. High concentrations of a cGMP-stimulated phosphodisterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. J. Biol. Chem. 266, 136–142.

Matsumi, H., Yano, T., Koji, T., Ogura, T., Tsutsumi, O., Taketani, Y., Esumi, H., 1998. Expression and localization of inducible nitric oxide synthase in the rat ovary: a possible involvement of nitric oxide in the follicular development. Biochem. Biophys. Res. Commun. 243, 67–72.

Maurer, H.R., 1992. Towards serum-free, chemical defined media for mammalian cell culture. In: Freshney, R.I. (Ed.), Animal Cell Culture: A Practical Approach, second ed. Oxford University Press, pp. 15–46.

McMahon, T.J., 2002. Nitric oxide in the human respiratory cycle. Nat. Med. 8, 711–717.

Mingone, C.J., Gupte, S.A., Chow, J.L., Ahmad, M., Abraham, N.G., Wolin, M.S., 2006. Am. J. Physiol. Lung Cell. Mol. Physiol. 29, 334–336.

Mingoti, G.Z., Garcia, J.M., Rosa e Silva, A.A.M., 2002. Steroidogenesis in cumulus cells of bovine cumulus-oocyte-complexus matured in vitro with BSA and different concentrations of steroids. Anim. Reprod. Sci. 69, 175–186.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. 65, 55–63.

Murard, F., 1999. Cellular signaling with nitric oxide and cyclic GMP. Braz. J. Med. Biol. Res. 32, 1317–1327.

Orsi, N.M., 2006. Embryotoxicity of the nitric oxide donor sodium nitroprusside in preimplantation bovine embryons in vitro. Anim. Reprod. Sci. 91, 225–236.

Piccinato, C.A., Montrezor, L.H., Rosa e Silva, A.A.M., 2000. Produção de óxido nítrico: padronização do modelo de cultura das células da granulosa de folículos ovarianos bovinos. In: Abstracts of the XV Reunião Anual da Sociedade de Transferência de Embriões, Caldas Novas, Brazil. Porto Alegre: Arq. Fac. Vet. UFRGS, 308 pp.

Rettori, V., Gimero, M., Lyson, K., McCann, S.M., 1992. Nitric oxide mediates norepinephrine-induced prostaglandin estradiol release from the hypothalamus. Proc. Natl. Acad. Sci. U.S.A. 89, 543–546.

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

Richards, J.S., Fitzpatrick, S.L., Clemens, J.W., 1995. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulates genes. Rec. Prog. Horm. Res. 50, 23–254.

Richter, C., 1993. Pro-oxidants and mitochondrial Ca^{+2}: their relationship to apoptosis and oncogenesis. FEBS Lett. 325, 104–107.

Rouillier, P., Matton, P., Sirard, M.-A., Guilbault, L.A., 1996. Follicle-stimulating hormone-induced estradiol and progesterone production by bovine antral and mural granulosa cells cultured in vitro in a completely defined medium. J. Anim. Sci. 74, 3012–3019.

Rouillier, P., Matton, P., Dufour, M., Sirard, M.-A., Guilbault, L.A., 1998. Steroid production, cell proliferation, and apoptosis in cultured bovine antral and mural granulose cells: developmental of an in vitro model to study estradiol production. Mol. Reprod. Dev. 50, 170–177.

Sarti, P., Lendaro, E., Ippoliti, R., Bellelli, A., Benedetti, P.A., Brunori, M., 1999. Modulation of mitochondrial respiration by NO: investigation by single cell fluorescence microscopy. FASEB J. 13, 191–197.

SAS, 1996. Statistical Analysis System.

Sato, K., Ozaki, K., Oh, L., Meguro, A., Hatanaka, K., Nagai, T., Muroi, K., Ozawa, K., 2007. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood 109, 228–234.

Shakil, T., Whitehead, S.A., 1994. Inhibitory action of peritoneal macrophages on progesterone secretion from co-cultured rat granulose cells. Biol. Reprod. 50, 1183–1189.

Sitaramayya, A., 2002. Calcium-dependent activation of guanylato cyclase by S100b. Adv. Exp. Med. Biol. 514, 389–398.

Snyder, G.D., Holmes, R.W., Bates, J.N., Van Voorhis, B.J., 1996. Nitric oxide inhibits aromatase activity: mechanisms of action. J. Steroid Biochem. Mol. Biol. 58, 63–69.

Strauss III, Schuler, L.A., Rosenblum, M.F., Tanaka, T., 1981. Cholesterol metabolism by ovarian tissue. Adv. Lipid Res. 18, 99–157.

Tanner, F.C., Meier, P., Greutert, H., Champion, C., Nabel, E.G., Lüscher, T.F., 2000. Nitric oxide modulates expression of cell cycle regulatory proteins. Acytostatic strategy for inhibition of human vascular smooth muscle cell proliferation. Circulation 101, 1982–1989.

Tsafriri, A., Chun, S.Y., Zhang, R., Hsueh, A.J., Conti, M., 1996. Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors. Dev. Biol. 140, 307–317.

Van Voorhis, B.J., Dunn, M.S., Snyder, G.D., Weiner, C.P., 1994. Nitric oxide: an autocrine regulator of human granulosa-luteal cell steroidogenesis. Endocrinology 135, 1779–1806.

Wang, S., Liu, Y., Holyoak, G.R., Bunch, T.D., 1997. The effects of bovine serum albumin and fetal bovine serum on the development of pre- and post-cleavage-stage bovine embryos cultured in modified CR2 and M199 media. Anim. Reprod. Sci. 48, 37–45.

Whitehead, S., Lacey, M., 1996. Inhibitory effect of peritoneal macrophages on progesterone release from co-cultured rat granulosa cells is reversed by dexamethasone: evidence for an action independent of nitric oxide and distal to cyclic adenosine 3′, 5′-monophosphate generation. Biol. Reprod. 54, 1317–1325.

Wink, D.A., Osawa, Y., Darbysbire, J.F., Jones, C.R., Eshenaur, S.C., Nims, R.W., 1993. Inhibition of cytochomes P450 by nitric oxide and a nitric oxide—releasing agent. Arch. Biochem. Biophys. 300, 115–123.

Yang, Z., Simari, R.D., Perkins, N.D., San, H., Gordon, D., Nabel, G.J., Nabel, E.G., 1996. Role of p21 cyclin-dependent kinase inhibitory in limiting intimal cell proliferation in response to arterial injury. Proc. Natl. Acad. Sci. U.S.A. 93, 7905–7910.