Animal Reproduction Science 116 (2009) 38–49

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Role of nitric oxide on quality of freshly ejaculated bull spermatozoa during heparin-induced *in vitro* capacitation

A.C.M.S. Leal, M.C. Caldas-Bussiere*, C.S. Paes de Carvalho, K.S. Viana, C.R. Quirino

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

ARTICLE INFO

Article history:
Received 7 February 2008
Received in revised form 18 December 2008
Accepted 23 December 2008
Available online 31 December 2008

Keywords:
Bull
Nitric oxide
Quality fresh sperm
Capacitation *in vitro*

ABSTRACT

The objective of this study was to assess the effects of nitric oxide (NO) on heparin-induced capacitation *in vitro* of fresh bull sperm, through the addition of Nω-nitro-L-arginine methyl ester (L-NAME, a NO-synthesis inhibitor) and L-arginine (L-Arg, a NO-synthesis precursor) to the capacitation medium. In Experiment 1, different concentrations of L-NAME (0.1, 1, 10 mM) and of L-Arg (10 mM) were added to the capacitation medium. Sperm motility and vigor were subjectively appraised using direct light microscopy; sperm membrane integrity was examined using a 2% Trypan blue solution while the concentration of nitrate/nitrite (NO_3^-/NO_2^-) was determined by using the Griess method over a 5 h capacitation period. The addition of 10 mM L-NAME has inhibited NO synthesis, sperm progressive motility, sperm vigor and sperm membrane integrity ($P < 0.05$) as compared to control. The addition of 10 mM L-Arg to the capacitation medium increased all variables evaluated in comparison to the control ($P < 0.05$). In Experiment 2, mitochondrial activity was assessed through the MTT test (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and sperm capacitation was assessed through the test of penetration in homologous oocytes after addition of the 10 mM L-NAME, and of the 10 mM L-Arg. The addition of 10 mM L-NAME caused mitochondrial activity (40%) and the percentage of oocytes penetrated (77%) to decrease in relation to the control ($P < 0.05$). After addition of 0.6 mM L-Arg + 10 mM L-NAME, partial reversal of mitochondrial activity did occur (only 20%). The addition of 10 mM L-Arg increased the percentage of oocytes penetrated as compared to control (21%) ($P < 0.05$). These

* Corresponding author. Tel.: +55 22 27261665; fax: +55 22 27262003.
E-mail address: claracal@uenf.br (M.C. Caldas-Bussiere).

0378-4320/$ – see front matter
doi:10.1016/j.anireprosci.2008.12.020

results indicate that: (1) NO is involved in control of progressive sperm motility, vigor, membrane integrity, and mitochondrial activity along the period of heparin-induced capacitation of fresh bovine sperm via NOS/NO; (2) adequate L-Arg/NO concentrations into the capacitation medium can potentiate heparin action or act independently for increasing the number or the quality of capacitated sperm.

1. Introduction

Nitric oxide (NO) is a reactive nitrogen species that may act as an antioxidant or free radical in inter- and intra-cellular signaling (Dixit and Parvizi, 2001). It is a gas that is colorless at room temperature and pressure; it is biologically active in almost all cells of the organism and travels freely through the membranes because of its low molecular weight (Lowenstein et al., 1994).

NO is synthesized during the conversion of L-arginine (L-Arg) into L-citrulline, through oxidative reactions catalyzed by the enzyme nitric oxide synthase (NOS), by using oxygen, nicotinamide adenine dinucleotide phosphate (NADPH) (Moncada and Higgs, 1993), flavine adenine dinucleotide (FAD), flavine mononucleotide (FMN) (Donnelly et al., 1997) and calcium/calmodulin (CaM) as cofactors. There are four isoforms of NOS: three that are constitutive and calcium-dependent – neuronal (nNOS), endothelial (eNOS), mitochondrial (mtNOS) – and one that is inducible (iNOS) and calcium-independent (Moncada et al., 1991). However, some research indicates that constitutive expression for the iNOS gene in some cells (Gath et al., 1996).

Localization and function of some NOS isoforms have been described for sperm of several animal species, such as cattle (Meiser and Schulz, 2003; O'Flaherty et al., 2004), rodent (Herrero et al., 1994, 1996; Bredt et al., 1991), human (O'Bryan et al., 1998; Francavilla et al., 2000), though most of those experiments have been performed on humans and laboratory animals (rodents).

Sperm capacitation and acrosome reaction (AR) are indispensable events for the occurrence of successful fertilization, both *in vitro* and *in vivo*, depending on the NO action, in cattle (Rodriguez et al., 2005a; O'Flaherty et al., 2004), swine (Funahashi, 2002) and buffalo (Roy and Atreja, 2008). Increased NO in the capacitation medium induces AR in cattle (Rodriguez et al., 2005a), human (Herrero et al., 2000) and rodent (Guzman-Grenfell et al., 1999) sperm.

Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO production, has been used in the studies evaluating the role of NO in sperm capacitation and AR, because it competes with the NOS for the same binding site of the L-Arg, a precursor of the NO synthesis (Moncada et al., 1991). The literature contains little about the addition of L-NAME and L-Arg in bull sperm capacitation medium. However, recent works indicate addition of L-NAME to cryopreserved bull sperm culture medium has inhibited capacitation after 45 min, although sperm motility has not been assessed after treatment with L-NAME (0.001, 0.01 and 0.1 mM; Rodriguez et al., 2005b). O'Flaherty et al. (2004) have added L-Arg (10, 20, 30, 40, 50 mM) to culture medium and have observed that a 10 mM addition improved sperm motility, capacitation, and acrosome reaction of bull sperm after 45 min. Greater concentrations have inhibited sperm motility and have not shown significant difference from the control with respect to integrity and AR, but have maintained the stimulatory effect on sperm capacitation. Roy and Atreja (2008) verified that the addition of L-NAME (0.5 mM) to the culture medium for buffalo has inhibited sperm capacitation, and that the addition of L-Arg (15 and 20 mM) has decreased sperm motility and integrity, while lesser concentrations (5 and 10 mM) caused capacitation to increase.

In a present study, assessment of sperm motility, vigor, membrane integrity, sperm capacitation, mitochondrial activity and dosage of nitrate/nitrite over a 5 h period were combined (Chamberland et al., 2001) in assessing bull sperm capacitation, using fresh semen, after addition of 0.1, 1, and 10 mM L-NAME and 10 mM L-Arg. With this approach, the role of NO on sperm quality during capacitation was assessed to optimize *in vitro* embryo production in cattle.

2. Materials and methods

2.1. Culture media and reagents

The basic medium used in sperm capacitation was the Modified Tyrode's (sp-TALP) (Parrish et al., 1988), supplemented with 6 mg/mL free of fatty acids BSA, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The washing medium employed in the sperm capacitation was that of Tyrode's, modified (Chamberland et al., 2001).

All reagents used were purchased from Sigma–Aldrich (St. Louis, MO, USA). Media were sterilized by filtration before use, with 0.22 μm sterile filtering units (Millipore Indústrias e Comércio Ltda, São Paulo, Brazil) having gone through a 30–60 min pre-incubation period in a chamber at 38.5 °C with a humidified atmosphere of 95% of air and 5% CO_2 for stabilization. After this process, they have been stored at 4 °C. L-NAME ($C_7H_{15}N_5O_4$·HCl and FW 269.7) and L-arginine ($C_6H_{14}N_4O_2$·HCl and FW 174.2) were not diluted and added to capacitation medium until the start of the experiments.

2.2. Semen collection

Repeated measurement ($n = 18$) of fresh semen was used. The single Nelore (*Bos indicus*) bull used in this study was maintained on pasture away from females. A serial collection of semen was performed weekly, even when the semen would not be used, with the purpose of maintaining the semen quality.

Right after manual massage of vesicle glands, the semen was collected with an electro-ejaculator (HAYONIK, FTM 1201, PR-Brazil) and was immediately transported in an insolated container (30–35 °C) to the laboratory.

2.3. Preparation of sperm and sperm capacitation

The semen was submitted to centrifugation at 700 × *g* for 10 min, on a 45 and 90% Percoll gradient (Parrish and Eid, 1994), to separate those sperm with greater integrity for the different treatments. After centrifugation, the supernatant was stored at −20 °C for later dosing of nitrate/nitrite (NO_3^-/NO_2) and the pellet formed was washed in a 3 mL washing medium (Chamberland et al., 2001) and then submitted to one more centrifugation at 200 × *g* for 3 min. From the resultant sediment, sperm motility and vigor were assessed, and then sperm concentration was calculated.

Sperm capacitation *in vitro* was induced in a 200 μL sp-TALP capacitation medium containing 20 μg/mL heparin, within a 38.5 °C chamber with humidified atmosphere, 95% air and 5% CO_2, for a 5 h period (Parrish et al., 1988). The sperm concentration used for each experiment was adjusted to 50×10^6 sperm/mL.

2.4. In vitro assessment of sperm quality during capacitation

2.4.1. Sperm motility and vigor

Assessment of sperm motility (percentage of sperm with progressive motility) and of sperm vigor (the strength of motion, which influences motion speed) were evaluated by direct light microscopy at 400× magnification (ZEISS, Jenamed, Germany). Vigor has been rated from zero to five, zero representing the absence of progressive motility with little or negligible lateral tail displacement, and five representing energetic, fast and, most often, progressive sperm motility (Manual for Andrologic Testing and Assessment of Animal Semen, of CBRA—Colégio Brasileiro de Reprodução Animal, 1998).

Because after 1 h in the capacitation medium, sperm aggregation occurs in the lateral region of the head, and agglutination occurs after 4 h, it has not been possible to evaluate sperm motility by using a computerized method (Hamilton Thorne Research, version 10.8q, Beverly, MA, USA), because the program recognizes the sperm by its pre-defined size. Still, in spite of the sperm being aggregated head-to-head, we were able to evaluate the percentage of sperm that had progressive motility, by using the subjective method.

2.4.2. Sperm plasma membrane integrity

The sperm were assessed for membrane integrity, by using vital staining with Trypan blue at 2% (Didion et al., 1989). Sperm ($n = 200$) were assessed, being classified either as intact (not stained) or damaged (blue-stained).

2.4.3. Dosage of nitrate/nitrite (NO_3^-/NO_2^-)

The NO_3^-/NO_2^- concentration in capacitation medium was measured by using the Griess colorimetric method (Ricart-Jane et al., 2002), for 5 h (0.25, 1, 2, 3, 4 and 5). The Griess reagent is composed of a mixture of 2% sulfanilamide and 0.2% NNED in deionized water. The first reaction in the sample occurs with the nitrite to form the diazonium salt, which reacts together with the second reagent to produce the purple color with an absorbance peak of 540 nm. To reduce the nitrate to nitrite, the samples (40 μL) were incubated with a 40 μL mixture containing 1000 μL of the nitrate reductase enzyme originated from bacteria (100 μL of the enzyme (10 IU) diluted in deionized water + 900 μL of deionized water), 1000 μL of the cofactor NADPH (5 mg/mL diluted in deionized water) and 1000 μL of potassium phosphate buffer (0.5 M). The samples were incubated at 37 °C for 14–16 h in 96-well plate. Thereafter, 80 μL of the Griess reagent were added to the samples. All solutions were protected from light during stocking and assay period.

A standard curve with sodium nitrite diluted in sp-TALP containing known values of nitrite was performed, where the minimum value of nitrite was 0.5 μM and the maximum, 100 μM. With the absorbance values a dispersion graph was drawn. The relationship between absorbance and concentration of NO_3^-/NO_2^- was found to be linear ($R^2 = 1$; $P < 0.05$).

On inhibiting capacitation with L-NAME, one expects a reduction in NO production to occur, because one is inhibiting the constitutive NOS, an enzyme that is responsible for the conversion of L-Arg into L-citrulline and the liberation of NO.

2.4.4. Determination of mitochondrial activity

Mitochondrial activity was verified right after the 5 h capacitation period, by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) method. Kinetics analysis has not been performed, as the large number of samples made it impossible to proceed with mitochondrial activity assessment for all treatments simultaneously and along the 5 h period of capacitation. This assay is based on the ability of live cells that have active mitochondria to reduce yellow tetrazolium salt (MTT) to the formazan blue final product (dietylthiazol) (Mosmann, 1983). At the end of capacitation, 100 μL of culture medium were removed in order to proceed with the dosages needed, and only 100 μL of medium remained. Thus, 10 μL of MTT (5 mg/mL) were added to each well, and next the sperm were incubated inside the 37 °C chamber for 3 h. Thereafter, to solubilize the crystals formed, 110 μL acidified isopropanol (0.04N HCl) containing 10% Triton X-100 was added and remained for 14–16 h at room temperature. After that period, reading took place of a spectrophotometer (Multiskan EX Primary EIA V 2.1-0) with 570 nm wave length and 670 nm background subtraction. The relationship between absorbance and the number of cells was determined by previous incubation with MTT of known quantity of cells, thus creating a standard curve.

2.5. Assessment of sperm capacitation by penetration test in homologue oocytes

2.5.1. Selection and in vitro maturation of oocytes

Oocytes grade 1 and 2 were selected in agreement with quality rating reported by de Loos et al. (1989). The oocytes selected were washed 3–4 times in a washing medium (tissue culture medium—TCM 199 with HEPES, supplemented with 5% FCS, 10 IU/mL penicillin and 100 μg/mL streptomycin), and thereafter transferred to the maturation medium (TCM 199 with 10% FCS, 5 μg LH and 0.5 μg FSH, plus the above mentioned antibiotics). Maturation was performed in 100 μL drops under mineral oil within a chamber at 38.5 °C with an atmosphere of 5% CO_2, for 22 h. After this period, removal of cumulus cells was performed by mechanical pipetting.

2.5.2. In vitro fertilization

The previously capacitated sperm were washed in sp-TALP + BSA for removal of the treatments. A concentration at 1.6×10^6 sperm/mL was set. Such sperm were co-incubated during 5 h (Dominko and First, 1997) at 38.5 °C under a humidified atmosphere (95% air and 5% CO_2), together with the oocytes ($n = 22$) in fertilization drops (110 μL—TALP medium with 6 mg/mL fatty acid-free BSA, 2 mM penicillamin, 1 mM hipotaurine, 250 mM epinefrine, 100 IU/mL penicillin and 100 μg streptomycin), in agreement with Parrish et al. (1988).

2.5.3. Assessment of penetration percentage

After the 5 h period, oocytes were mounted between a slide and coverslip, fixed in acetic acid:ethanol (1:3), for 24–72 h and stained with orcein at 2% in acetic acid (40%).

The sperm penetration rate was determined in agreement with Rosenkranz and Holzmann (1997), with oocytes classified either as non-penetrated or penetrated, where sperm head was observed, as well as descondensation of chromosomes or formation of the pronuclei male and female. This test indirectly evaluates sperm capacitation and acrosome reaction (Brackett et al., 1982), because only fully capacitated sperm can bind to the zona pellucida, undergo acrosome reaction and penetrate the oocyte plasma membrane.

2.6. Experiments

2.6.1. Experiment I: Kinetics of L-NAME and L-Arg action during capacitation

Assessment has been performed of the effect of the addition of different concentrations of L-NAME (0.1, 1 and 10 mM) and 10 mM L-Arg (O'Flaherty et al., 2004) to the heparin-induced *in vitro* capacitation medium (T-H) for time periods 0.25, 1, 2, 3, 4 and 5 h. The control consisted of sperm cultured both in TALP plus heparin and in 10 mM D-NAME, an enantiomer for L-NAME. To evaluate if the effects observed after the treatment with L-NAME were caused by the NO synthesis inhibition, 0.6 mM L-Arg was added (Francavilla et al., 2000) to the treatment with the concentration that decreased the variables evaluated.

The following variables were analyzed: motility, vigor, and membrane integrity of the sperm. For all time periods evaluated, the capacitation medium was collected and stored at −20 °C for posterior dosing of nitrate/nitrite (NO_3^-/NO_2). The experiment was completed with 6 iterations.

In Experiment I, the results respective to the addition of different L-NAME and L-Arg concentrations were assessed in relation to concentration and time as affecting sperm motility, sperm vigor, and sperm membrane integrity, and the concentration of nitrate/nitrite.

2.6.2. Experiment II: Role of NO in sperm capacitation and mitochondrial activity in vitro

Mitochondrial activity and sperm capacitation were evaluated after the 5 h culture period, only for those L-NAME (10 mM) and L-Arg (10 mM) concentrations that showed significantly different results from the control in Experiment 1. The control consisted of T + H, 10 mM D-NAME and 0.01 M L-NAME + 0.6 mM L-Arg. The experiment was repeated six times.

2.7. Statistical analysis

The data were first analyzed by using the SAS (Statistical Analysis System, Cary, NC, USA) and then submitted to analysis of variance (PROC GLM) for verification of the effects of concentration and time on the characteristics studied, as well as verification of averages as compared by the Tukey test, at a 5% probability.

3. Results

3.1. Experiment I: Kinetics of L-NAME and L-Arg action during capacitation

3.1.1. Assessment of sperm motility

There was interaction between treatment and specific time when sperm motility was analyzed during the 5 h-capacitation period ($P < 0.05$, Table 1). The addition of 10 mM L-NAME caused the per-

Table 1
Effect of progressive sperm motility from addition of Nω-nitro-L-arginine methyl ester (L-NAME) and L-arginine (L-Arg) at different concentrations during *in vitro* capacitation of bull sperm (5 h).

Treatment	Time (h)					
	0.25	1	2	3	4	5
Control	80 ± 0Aa	77 ± 5Aab	73 ± 5ABCb	65 ± 5Abc	55 ± 5Bc	37 ± 8Bd
L-NAME						
0.1 mM	80 ± 0Aa	78 ± 4Aab	73 ± 5ABCbc	67 ± 5Ac	50 ± 6BCd	35 ± 4Be
1.0 mM	80 ± 0Aa	78 ± 4Aa	75 ± 5ABCab	67 ± 5Ab	50 ± 6BCc	33 ± 4Bd
10 mM	80 ± 0Aa	75 ± 5Aa	68 ± 4Cb	55 ± 8Bc	42 ± 10Cc	25 ± 2Cd
10 mM D-NAME	80 ± 0Aa	73 ± 5Ab	70 ± 0BCbc	67 ± 5Ac	55 ± 5Bd	38 ± 7Be
10 mM L-NAME + 0.6 mM L-Arg	80 ± 0Aa	78 ± 4Aa	77 ± 5ABa	70 ± 0Ab	60 ± 1Bc	40 ± 5Bd
10 mM L-Arg	80 ± 0Aa	80 ± 0Aa	80 ± 0Aa	73 ± 5Ab	67 ± 5Abc	60 ± 6Ac

Averages followed by different upper case letters (A–C) in same column show differences ($P < 0.05$) for each time, among the different concentrations. Averages followed by different lower case letters (a–d) in same row show differences ($P < 0.05$) for each concentration, at the different times. Data are presented as average ± SD of six replications in percentage.

centage of bull sperm with progressive motility to decrease after 3 h during the *in vitro* capacitation, as compared to the control and to lesser concentrations (0.1 and 1.0 mM L-NAME, Table 1). The addition of 0.6 mM L-Arg to the 10 mM L-NAME in capacitation medium reversed the inhibitory effect at every assessment time point. The addition of 10 mM L-Arg into capacitation medium increased progressive sperm motility as compared with the control and other concentrations, from 4 h onwards (Table 1).

3.1.2. Assessment of vigor

There was no interaction between the treatment and the time ($P < 0.05$) at which vigor in sperm was analyzed during capacitation. There were differences ($P < 0.05$) between treatments and, particularly between specific times (Fig. 1, panel a).

The addition of 10 mM L-NAME caused vigor of bull sperm to decrease during *in vitro* capacitation, as compared with the control. However, at the greater concentration, 10 mM, the vigor of the sperm did not differ from that seen at 0.1 and 1.0 mM L-NAME. The addition of 0.6 mM L-Arg to the 10 mM L-NAME in capacitation medium reversed the inhibitory effect of 10 mM L-NAME. The addition of 10 mM L-Arg into capacitation medium improved vigor of bull sperm as compared with the control and the other concentrations (Fig. 1, panel a).

3.1.3. Assessment of sperm membrane integrity

There was an interaction between the treatment and the specific time when sperm membrane integrities were determined during the 5 h-capacitation period ($P < 0.05$; Table 2). The addition of 10 mM L-NAME caused sperm membrane integrity to decrease after 3 h of capacitation, when compared with controls and other concentrations. The addition of 0.6 mM L-Arg to the 10 mM L-NAME in the capacitation medium, reversed the inhibitory effect of 10 mM L-NAME, having greater values than those of control after 1 h of capacitation. The addition of 10 mM L-Arg increased sperm membrane integrity, as compared with the control and other concentrations, after 0.25 h of capacitation (Table 2).

3.1.4. NO_3^-/NO_2^- concentration in the capacitation medium

There was a significant difference ($P < 0.05$) in the NO_3^-/NO_2^- concentrations during the capacitation period (5 h) (Fig. 1, panel b). There was a dose–response effect on the concentration of nitrate/nitrite after addition of different concentrations of L-NAME, as compared with the control and its inactive isomer (D-NAME). The addition of 0.6 mM L-Arg to the 10 mM L-NAME, into the capacitation medium, reversed the inhibitory effect of L-NAME in the NO synthesis, with greater concentrations (5%) resulting than those of control ($P < 0.05$). The addition of 10 mM L-Arg increased NO_3^-/NO_2^- concentration (21%), as compared with the control and other concentrations ($P < 0.05$) (Fig. 1, panel b).

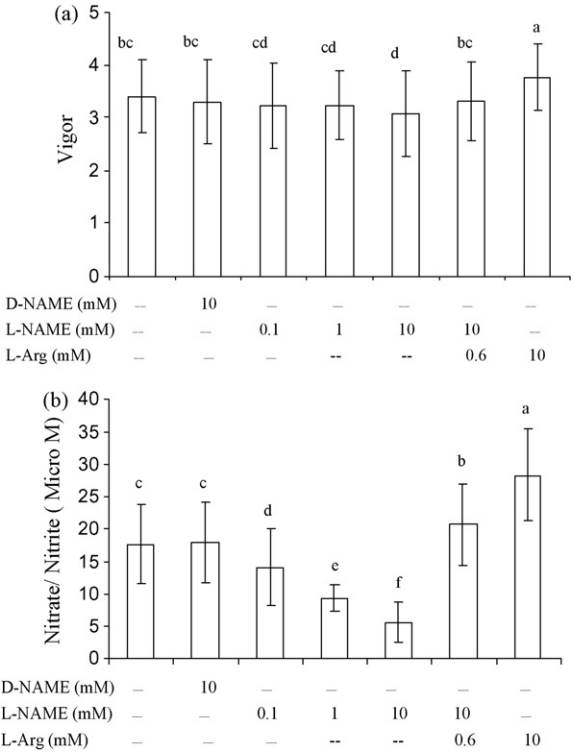

Fig. 1. Vigor (panel a) and concentration of nitrate/nitrite in the culture medium (panel b) of bull sperm treated with different concentrations of Nω-nitro-L-arginine methyl ester (L-NAME) and L-arginine (L-Arg), during the capacitation *in vitro* (5 h). Different letters indicate differences ($P < 0.05$). Data are presented as average ± SD of six replications in percentage.

3.2. Experiment 2: Role of NO in sperm capacitation and mitochondrial activity in vitro

3.2.1. Mitochondrial activity

There was a difference ($P < 0.05$) in the mitochondrial activity evaluated by MTT method after the *in vitro* capacitation (5 h) (Fig. 2, panel a). Sperm treated with 10 mM L-NAME had lesser absorbance

Table 2
Percentages of membrane intact bull sperm after addition of Nω-nitro-L-arginine methyl ester (L-NAME) and/or L-arginine (L-Arg) during *in vitro* capacitation (5 h).

Treatment	Time (h)					
	0.25	1	2	3	4	5
Control	76 ± 9BCa	70 ± 3Ba	57 ± 6Cb	48 ± 5Cb	39 ± 4Cc	35 ± 6Bc
L-NAME						
0.1 mM	71 ± 9Ca	67.6 ± 9Bab	55.5 ± 8Cb	51 ± 9Cbc	39 ± 4Cc	33 ± 5Bc
1.0 mM	73 ± 11BCa	65 ± 11Bab	58 ± 11Cbc	51 ± 10Ccd	40 ± 4Cde	34 ± 5Be
10 mM	73 ± 9BCa	62 ± 8Ba	54 ± 9Cab	44 ± 9Dbc	30 ± 5Dcd	23 ± 3Cd
10 mM L-NAME + 0.6 mM L-Arg	85 ± 3ABa	62 ± 2Aa	74 ± 3Bb	65 ± 5Bcd	55 ± 5Bd	42 ± 3Be
10 mM D-NAME	76 ± 5BCa	72 ± 3Ba	58 ± 3Cb	51 ± 5Cb	40 ± 3Cc	33 ± 5Bc
10 mM L-Arg	88 ± 2Aa	88 ± 2Aa	86 ± 2Aab	8 ± 2Ab	81 ± 3Ab	78 ± 2Ab

Averages followed by different upper case letters (A–C) in same column show differences ($P < 0.05$) for each time, among the different concentrations. Averages followed by different lower case letters (a–d) in same row show differences ($P < 0.05$) for each concentration, at the different times. Data are presented as average ± SD of six replications in percentage.

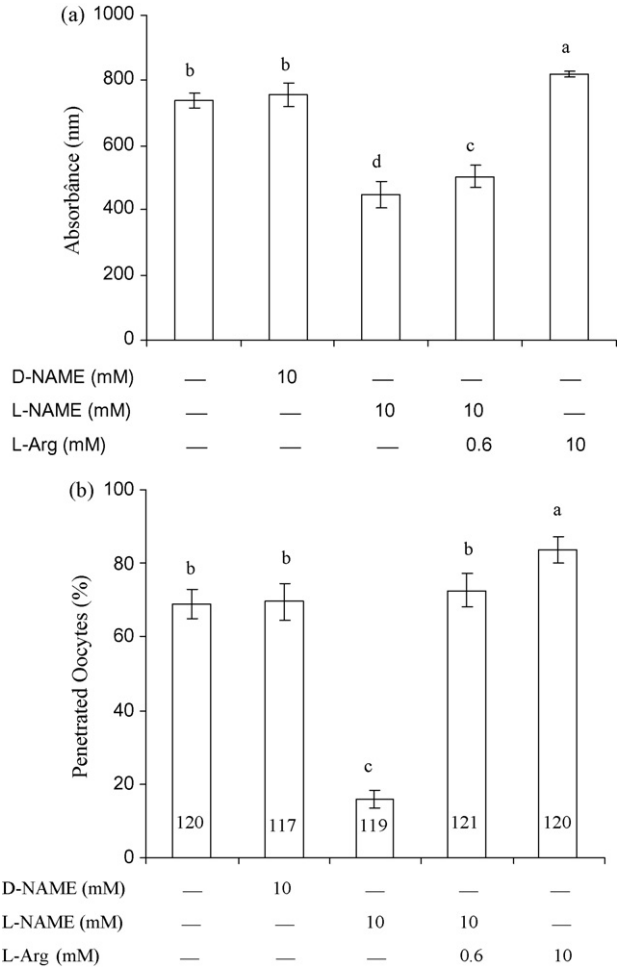

Fig. 2. (a) Mitochondrial activity of bull sperm treated with different concentrations of Nω-nitro-L-arginine methyl ester (L-NAME) and L-arginine (L-Arg) after the capacitation *in vitro* (5 h) and (b) percentage of penetrated oocytes in result of IVF with previously capacitated sperm (5 h) at different concentrations of L-NAME and L-Arg. The number of oocytes examined in each treatment is shown at the base of each bar. Data are presented as average ± SD of six replications in percentage. Different letters indicate statistical difference between groups ($P < 0.05$).

(40%) when compared with the control and D-NAME. The association of 0.6 mM L-Arg with 10 mM L-NAME in the capacitation medium had greater absorbance (20%) than that of sperm treated with 10 mM L-NAME, but this was not sufficient to reverse the inhibitory effect (Fig. 2, panel a). The addition of 10 mM L-Arg caused mitochondrial activity to increase (11%) as compared with the control ($P < 0.05$).

3.2.2. Sperm capacitation

There was a significant difference in the treatments when of the assessment of the NO role after the *in vitro* capacitation (5 h) (Fig. 2, panel b). Oocytes co-incubated with sperm treated with 10 mM L-NAME had less oocyte penetration (23%) when compared to oocytes co-incubated with sperm without and with D-NAME (controls). The addition of 0.6 mM L-Arg reversed the inhibitory effect that resulted from addition the of L-NAME (10 mM) (Fig. 2, panel b). Oocytes co-incubated with sperm treated with 10 mM L-Arg had greater oocyte penetration (21%) than those observed in the control ($P < 0.05$) (Fig. 2, panel b).

4. Discussion

The present study demonstrated that inhibition of NO synthesis by L-NAME (10 mM) in culture medium for fresh bull sperm capacitation caused, from 3 h onwards, a decrease in sperm motility, vigor, membrane integrity, mitochondrial activity, and heparin-induced capacitation *in vitro*. Decrease of sperm motility after addition of L-NAME has been described, in the literature, for hamster sperm (Kameshwari et al., 2003). However, Francavilla et al. (2000) observed no change in human sperm motility upon adding L-NAME at varying concentrations (0.1, 0.6 and 1.2 mM) to the capacitation medium after 30 min of incubation. Neither did Rodriguez et al. (2005b) observe any change in bull sperm upon adding L-NAME (0.001, 0.01, 0.1 and 0.5 mM) after 45 min of culture.

Decrease of sperm membrane integrity after addition of NO-synthesis inhibitors has been described in humans (Rosselli et al., 1995). Sperm are protected by antioxidant substances and antioxidant enzymes that are present in seminal plasma and in sperm (Kim and Parthasarathy, 1998). When cultured *in vitro*, sperm become susceptible to oxidative damage due to an imbalance in redox status (Kim and Parthasarathy, 1998). The NOS/NO pathway provides an important antioxidant action that protects cells from lipid peroxidation, that is, from the reaction of free radicals with lipids in cell membranes (Srivastava et al., 2006), resulting in destruction/modification of numerous lipid molecules, and leading to loss of the sperm membrane integrity. A decrease in sperm membrane integrity was observed after 3 h incubation with the 10 mM L-NAME concentration, suggesting that the decrease in NO concentration after the addition of L-NAME may lead to a increase in lipid peroxidation. For cattle, this is the first report to demonstrate that the addition of L-NAME to sperm capacitation medium decreases sperm membrane integrity.

In the present study, addition of L-NAME induced a dose–response effect on the decrease of NO synthesis, demonstrating that the L-NAME effect on control of sperm motility, vigor, membrane integrity and capacitation of bull sperm was due to a decrease in the NO synthesis. This hypothesis reinforced by the fact that the addition of its inactive isomer (D-NAME) did not show to have any effect on any of the variables that were appraised, and addition of 0.6 mM L-Arg reversed all inhibitory effects promoted by L-NAME.

A decrease in cellular NO concentration affects the mitochondrial activity (Brookes et al., 2003), because it causes an imbalance in the NO/O_2 ratio, because NO and O_2 compete for the center of reaction of the cytochrome oxidase enzyme (Boveris et al., 1999), suggesting less ATP production by the respiratory chain (Brookes et al., 2003). This reaction may lead not only to alterations in sperm motility and vigor but also to biochemical reactions that result in less fertilization potential of bull sperm.

The assessment of NO role in sperm capacitation *in vitro* has been performed by penetration test after the addition of 10 mM L-NAME. There was a decrease in percentage (77%) of the bull sperm penetrating homologous oocytes, suggesting that this may have occurred due to the decrease in NO synthesis. These results corroborate those observed by Francavilla et al. (2000) in humans, which have demonstrated a decrease in the percentage of L-NAME-treated sperm penetrating the zona pellucida after 5 h in culture, although with lesser L-NAME concentrations (0.1, 0.6 and 1.2 mM). Rodriguez et al. (2005b) demonstrated in cattle that capacitation was inhibited by addition of L-NAME (0.001, 0.01 and 0.1 mM) to the culture medium, as assessed by chlortetracycline (CTC) assessment of acrosomal status during 45-min of incubation. Recently, inhibition of capacitation has been also demonstrated in buffalo sperm and assessed by induction of the acrosome reaction after 6 h capacitation by addition of 0.5 mM L-NAME (Roy and Atreja, 2008). These differences in sperm capacitation-inhibiting concentrations among studies can be explained by three factors: (1) in this present research, fresh semen was used rather than cryopreserved (Rodriguez et al., 2005b); (2) in previous research where fresh semen was used, physiological differences were observed between the human (Francavilla et al., 2000) and buffalo (Roy and Atreja, 2008) sperm in regard to sensitivity to a decrease in NO concentration in the culture medium; (3) the length of assessment period.

In the present experiment, a decrease in mitochondrial activity (40%) of sperm after a 5 h culture in the capacitation medium was observed with 10 mM L-NAME. However, after the addition of 0.6 mM L-Arg to the 10 mM L-NAME, restoration of only 20% of the mitochondrial activity took place, different from the total restoration observed for the other variables assessed (sperm motility, vigor,

membrane integrity, NO concentration, and capacitation). Besides, NO offers additional mechanisms of action those benefit mitochondrial activity. Nevertheless, the hypothesis that this partial recovery of mitochondrial activity is reflected in the further development of the embryo cannot be negated.

Another possible mechanism of action of NO during capacitation is in cytoskeleton dynamics (Brenner et al., 2003; Breitbart et al., 2005). Thus, a decrease in NO concentration after treatment with 10 mM L-NAME may have prevented polymerization of the actin filaments that occurs during this process in the acrosome (Brenner et al., 2003; Cohen et al., 2004), also suggesting a decrease in sperm motility and vigor, because this filament is also present in the sperm tail, and thus involved in sperm motility (Virtanen et al., 1984).

The addition of 10 mM L-Arg improved all of the sperm quality variables appraised in the present research: sperm motility, vigor, membrane integrity, mitochondrial activity and heparin-induced capacitation in bull sperm. Sperm motility improvement after addition of L-Arg has been described for human sperm (Keller and Polakoski, 1975; Mendez and Hernandez, 1993) and has been successfully used in treatments for some fertility problems (Mendez and Hernandez, 1993). In the present research, the increase in sperm motility and sperm membrane integrity observed after addition of 10 mM L-Arg may result from amino acid serving as substrates for the NO synthesis, allowing sperm to spare L-Arg from their own reserves. Roy and Atreja (2008) and O'Flaherty et al. (2004) did not note any significant difference in sperm motility or integrity after addition of L-Arg (10 mM) to the capacitation medium for buffalo and bull sperm cultured for 6 h and 45 min, respectively. The difference between these previous findings and the present study may be accounted by the fact that in buffalo there is enough L-Arg to promote capacitation (Roy and Atreja, 2008) and also by the much shorter period of culture used by O'Flaherty et al. (2004) for cattle. In the present research, there was a significant increase both in sperm motility and integrity only from 4 h of incubation onwards.

In the present experiment, the effect of adding 10 mM L-Arg to capacitation was evaluated, by using the oocyte penetration test. The increase of NO concentration and percentage of oocytes penetrated by the sperm treated (21%) suggest that L-Arg has promoted an increase in the percentage of sperm capacitated through increased biosynthesis of nitric oxide, be it by increasing the glycolytic metabolism or by decreasing the lipid peroxidation in the sperm (Srivastava et al., 2006). These results corroborate those observed by Funahashi (2002) (pig), Roy and Atreja (2008) (buffalo) and O'Flaherty et al. (2004) (cattle), where there was an increase in capacitation rate after addition to the culture medium of 2 mmol; 5 and 10 mM and 10–30 mM L-Arg, respectively. Differences in concentration that starts to stimulate capacitation are due to physiological differences among the animal species and to the time of the assessment of *in vitro* capacitation. In the present study sperm L-Arg and heparin were both used in the incubation medium, while L-Arg was used alone in the capacitating medium in previous studies, a fact that suggests that L-Arg may be used together with heparin for potentiating its capacitating action on bull sperm.

After adding 10 mM L-Arg in the present study, an increase in sperm membrane integrity in relation to control from the first 15 min onwards was observed. Such an increase was observed as having been sustained for the remaining part of the 5-h culture period, with no significant difference from the control at 0.25 h. In spite of having occurred, alterations in the sperm plasma/acrosome membrane during capacitation (Didion et al., 1989), maintenance of sperm membrane integrity during the capacitation process as observed in the present experiment is not related to a capacitation decrease, because there was a 21% increase in number of oocytes penetrated by sperm treated with 10 mM L-Arg. This, together with the NO protective action described with evaluation of L-NAME addition, may explain the results of Srivastava et al. (2006), when addition of L-Arg to capacitation medium increased protection of ram sperm against damage from lipid peroxidation *in vitro*, preserving sperm integrity.

Besides an increase in sperm motility, vigor, membrane integrity and capacitation after the addition of 10 mM L-Arg, there was also an increase in sperm mitochondrial activity (11%) after 5-h culture in capacitating medium (TALP + heparin), as compared with the control. These data, together with the data observed after the addition of 10 mM L-NAME, support the hypothesis that one of the NO mechanisms of action in sperm capacitation is control of mitochondrial respiration (Brookes et al., 2003).

These results indicate that (1) NO is involved in control of progressive sperm motility, vigor, membrane integrity, and sperm mitochondrial activity along the period of heparin-induced capacitation

of fresh bull sperm via NOS/NO; (2) adequate NO concentrations into the capacitation medium may potentiate heparin action or may act independently in increasing number or quality of capacitated sperm; (3) L-Arg/NO modulating mitochondrial activity during capacitation. Other mechanisms of the NO/L-Arg action during sperm capacitation should be assessed to verify if the increase in sperm motility, vigor, membrane integrity, mitochondrial activity and capacitation promoted by addition of L-Arg are associated with fertilization potential of the sperm and, consequently, with an increase in the production of cattle embryos, *in vitro*.

Acknowledgments

Authors thank Márcia Resende Faes, Janaína Barcelos Porto Ferreira-Berbari, João Gomes Siqueira and Fausto Paes de Carvalho for their technical support, Professora Elena Lassovnskaya for her collaboration (LRBCT, CBB, UENF). This work was supported by CAPES and FAPERJ. A.C. M.S. Leal—recipient of a fellowship from CAPES.

References

Boveris, A.A., Costa, L., Cadenas, E., Poderoso, J.J., 1999. Regulation of mitochondrial respiration by adenosine diphosphate, oxygen, and nitric oxide. Methods Enzymol. 301, 188–198.
Brackett, B.G., Core, M.A., Boice, M.L., Bousquet, D., 1982. Use of zona-free hamster ova to assess the sperm fertilizing ability of bull and stallion. Gamete Res. 5, 217–227.
Bredt, D.S., Glatt, C.E., Hwang, P.M., Fotouhi, M., Dawson, T.M., Snyder, S.H., 1991. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuronal 7, 615–624.
Breitbart, H., Cohen, G., Rubinstein, S., 2005. Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. Soc. Reprod. Fertil. 129, 263–268.
Brenner, E., Rubinstein, S., Cohen, G., Shternall, K., Rivlin, R., Breitbart, H., 2003. Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. Biol. Reprod. 68, 837–845.
Brookes, P.S., Kraus, D.W., Shiva, S., Doeller, J.E., Barone, M.C., Patel, R.P., Lancaster, J.R., Darley-Usmar, V., 2003. Control of mitochondrial respiration by NO, effects of low oxygen and respiratory state. J. Biol. Chem. 278, 31603–31609.
Chamberland, A., Fournier, V., Tardif, S., Sirard, M., Sullivan, R., Bailey, J., 2001. The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. Theriogenology 55, 823–835.
Cohen, G., Rubinstein, S., Gur, Y., Breitbart, H., 2004. Crosstalk between protein kinase A and C regulates phospholipases D and F-actin formation during sperm capacitation. Dev. Biol. 267, 230–241.
Colégio Brasileiro de Reprodução Animal, 1998. Manual para exame andrológico e avaliação de sêmen animal, 2ª ed, Belo Horizonte, 49 pp.
de Loos, F., Van Vliet, C., Van Maurik, P., 1989. Morphology of immature bovine oocytes. Gamete Res. 24, 197–204.
Didion, B.A., Dobrinsky, J.R., Giles, J.R., 1989. Staining procedure to detect viability and true acrosome reaction in spermatozoa of various species. Gamete Res. 22, 51–57.
Dixit, V.D., Parvizi, N., 2001. Nitric oxide and the control of reproduction. Anim. Reprod. Sci. 65, 1–6.
Dominko, T., First, N., 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. Mol. Reprod. Dev. 47, 456–467.
Donnelly, E.T., Lewis, S.E.M., Thompson, W., Chakravarthy, U., 1997. Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition. Mol. Hum. Reprod. 3, 755–762.
Francavilla, F., Santucci, R., Acerola, B., Ruvolo, G., Romano, R., 2000. Nitric Oxide syntase inhibition in human sperm affects sperm affects sperm-oocyte fusion but not zona pellucida binding. Biol. Reprod. 63, 425–429.
Funahashi, H., 2002. Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. Reproduction 124, 857–864.
Gath, I., Closs, E.I., Godtel-Armbrust, U., Schmitt, S., Nakane, M., Wessler, I., Forstermann, 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.
Guzman-Grenfell, A.M., Hernandez, S.R., Gonzalez-Martinez, M.T., Hicks, J.J., 1999. Effect of nitric oxide releasers on some metabolic process of rabbit spermatozoa. Arch. Androl. 42, 119–123.
Herrero, M.B., Cebral, E., Boquet, M., Viggiano, J.M., Vitullo, A., Gimeno, M.A.F., 1994. Effect of nitric oxide on mouse sperm hyperactivation. Apptla 44, 65–69.
Herrero, M.B., Perez-Martinez, S., Viggiano, J.M., Polak, J.M., Gimeno, M.F., 1996. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod. Fertil. Dev. 8, 931–934.
Herrero, M.B., Chatterjee, S., Lefievre, L., de Lamirande, E., Gagnon, C., 2000. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Rad. Biol. Med. 29, 522–536.
Kameshwari, D.B., Siva, A.B., Shivaji, S., 2003. Inhibition of in vitro capacitation of hamster spermatozoa by nitric oxide synthase inhibitors. Cell Mol. Biol. 49, 421–428.
Keller, D.W., Polakoski, F.L., 1975. L-arginine stimulation of human sperm motility in vitro. Biol. Reprod. 13, 154–157.
Kim, J.G., Parthasarathy, S., 1998. Oxidation and the spermatozoa. Sem. Reprod. Androl. 16, 235–239.
Lowenstein, C.J., Dinerman, J., Snyder, S.H., 1994. Nitric oxide a physiologic messenger. Ann. Int. Med. 120, 227–237.
Meiser, H., Schulz, R., 2003. Detection and localization of two constitutive NOS isoforms in bull spermatozoa. Anat. Histol. Embriol. 32, 321–325.

Mendez, J.D., Hernandez, M.P., 1993. Efecto de L-arginina y poliaminas sobre la movilidad espermática. Ginecol. Obstet. Mex. 61, 229–234.
Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.
Moncada, S., Higgs, E.A., 1993. The L-arginine-nitric oxide pathway. New Engl. J. Med. 329, 2002–2012.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
O'Bryan, M.K., Zini, A., Yan Scheng, C., Schlegel, P.N., 1998. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. Fertil. Steril. 70, 1143–1147.
O'Flaherty, C., Rodriguez, P., Srivastava, S., 2004. L-arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. Biochem. Biophys. Acta 1674, 215–221.
Parrish, J.J., Eid, L., 1994. Paternal influence on S-phase in the first cell cycle of the bovine embryo. Biol. Reprod. 51, 1232–1237.
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.
Ricart-Jane, D., Lobera, M., Lopez-Tejero, M.D., 2002. Anticoagulants and other preanalytical factors interfere in plasma nitrate/nitrite quantification by the Griess method. Nitric Oxide 6, 178–185.
Rodriguez, P.C., O'Flaherty, C.M., Beconi, M.T., Beorlegui, N.B., 2005a. Nitric oxide-induced capacitation of cryopreserved bull spermatozoa and assessment of participating regulatory pathways. Anim. Reprod. Sci. 85, 231–242.
Rodriguez, P.C., O'Flaherty, C.M., Beconi, M.T., Beorlegui, N.B., 2005b. Nitric oxide induces acrosome reaction in cryopreserved bovine spermatozoa. Andrologia 37, 166–172.
Rosenkranz, C., Holzmann, A., 1997. The effect of sperm preparation on the timing of penetration in bovine in vitro fertilization. Anim. Reprod. Sci. 46, 47–53.
Rosselli, M., Dubey, R.K., Imthurn, B., Macas, E., Keller, P.J., 1995. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum. Reprod. 10, 1786–1790.
Roy, S.C., Atreja, S.K., 2008. Tyrosine phosphorylation of a 38-kDa capacitation-associated buffalo (*Bubalus bubalis*) sperm protein is induced by L-arginine and regulated through a cAMP/PKA-independent pathway. Int. J. Androl. 31, 12–24.
Srivastava, S., Desai, P., Coutinho, E., Govil, G., 2006. Mechanism of action of L-arginine on the vitality of spermatozoa is primarily through increased biosynthesis of nitric oxide. Biol. Reprod. 74, 954–958.
Virtanen, I., Badley, R.A., Paasivuo, R., Lehto, V.P., 1984. Distinct cytoskeletal domains revealed in sperm cell. J. Cell Biol. 99, 1083–1091.