

Influence of L-arginine during bovine *in vitro* fertilization

Thiago Velasco Guimarães Silva^{1*}, Bruno Baraúna da Silva¹, André Luiz Alves de Sá¹, Nathalia Nogueira da Costa¹, Rafael Vilar Sampaio², Marcela da Silva Cordeiro³, Priscila Di Paula Bessa Santana¹, Paulo Roberto Adona⁴, Simone do Socorro Damasceno Santos¹, Moisés dos Santos Miranda¹ & Otávio Mitio Ohashi¹

¹Institute of Biological Sciences, *In vitro* Fertilization Laboratory, Federal University of Pará, Rua Augusto Corrêa 66075110, Brazil

²Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

³Federal Institute of Pará, Abaetetuba, PA, Brazil

⁴Paraná North University, Tamarana, PR, Brazil

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The objective of this work was to evaluate the effect of using L-arginine during *in vitro* fertilization (IVF) on *in vitro* embryonic development using *Bos taurus* and *Bos indicus* semen. Effect of different concentrations (0, 1, 10 and 50 mM) of L-arginine, added to the IVF medium, was evaluated on the fertilization rate at 18 h post-fertilization (hpf), NO₃⁻/NO₂⁻ production during IVF by the Griess colorimetric method (30 hpf), cleavage and blastocyst rates (on Day 2 and Day 7 of culture, respectively) and total blastocyst cell number (Day 7 of culture). The results reveal that the addition of 50 mM L-arginine to IVF medium, with either *Bos taurus* or *Bos indicus* spermatozoa, decreased the cleavage rate and blastocyst rate compared to the control group. Other concentrations did not affect embryo production. However, 1 mM L-arginine with *Bos indicus* semen increased the proportion of hatched blastocysts. These results indicate that high L-arginine concentrations may exhibit toxic effects on bovine gametes during *in vitro* fertilization.

Keywords: Blastocysts, *Bos indicus*, *Bos taurus*, Cleavage, Embryo, IVF, Oocytes, Semen, Sperm capacitation, Spermatozoa

Sperm capacitation *in vivo* occurs in the female genital tract, but capacitation can be induced *in vitro* by incubating spermatozoa in oviduct fluid or, in some species, in a defined medium containing certain components such as heparin, albumin, calcium (Ca²⁺) and bicarbonate (HCO₃⁻)¹. In addition, other elements, such as geldanamycin², osteopontin³ and L-arginine, have been used in pursuit of improving sperm capacitation *in vitro*⁴⁻⁶.

L-arginine plays an important role in sperm motility in addition to inducing capacitation and acrosome reaction in several mammalian species, including cattle. Such effects have been associated with nitric oxide (NO), where L-arginine serves as a substrate for nitric oxide synthase (NOS)⁵.

The identification of NO as an intra- and intercellular messenger has garnered high levels of research interest, which is due to the observation that NO synthesis occurs in a wide variety of cell types, including those involved in reproduction^{4,7,8}.

O'Flaherty *et al.*⁵ showed that the addition of 10 mM L-arginine for 45 min to sperm capacitation medium improved sperm motility, capacitation and the acrosome reaction in bovine spermatozoa⁵. However, higher concentrations inhibited motility but did not exhibit any significant difference compared to the control group when acrosomal integrity was evaluated. In buffaloes, Roy and Atreja⁶ observed that the addition of 15 and 20 mM L-arginine to sperm capacitation medium decreased sperm motility but increased sperm capacitation when used at lower concentrations (5 and 10 mM).

Sperms are capable of fertilization only after the capacitation process and acrosome reaction, which promote an improvement in sperm quality variables^{9,10}. A possible mechanism of activation of these events may be correlated with NO action during fertilization⁵. Leal *et al.*⁴ observed that the addition of 10 mM L-arginine increased sperm quality variables, such as sperm motility, vigor, membrane integrity and mitochondrial activity. Additionally, the fertilization potential of sperm may be related to their quality variables. Certain concentrations of NO have been shown to improve sperm quality but

*Correspondence:

Phone: +55 (91) 32017773

E-mail: velascotvgs@hotmail.com

exhibit deleterious effects in several species at higher concentrations^{5,11–16}.

Effects on sperm quality may correlate with an increased induction of sperm capacitation and the acrosome reaction, which could positively influence fertilization rates and embryonic development. However, most studies using L-arginine during sperm capacitation in mammals use it exclusively in *in vitro* fertilization (IVF). Therefore, this study has been aimed to evaluate the effects of L-arginine during *in vitro* fertilization and on subsequent embryonic development.

Material and Methods

Reagents and media—All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and growth media were obtained from Gibco (Grand Island, NY, USA) unless otherwise stated.

In vitro embryo production

Oocyte collection and *in vitro* maturation (IVM)—Ovaries were collected from a slaughterhouse and transported at 30 °C within two hours. Cumulus oophorus complex (COCs) were collected by aspirating 2–8 mm follicles and washed in a HEPES-buffered TCM 199 supplemented with 50 µg/mL gentamicin and 10% fetal calf serum (FCS) (Gibco). Only COCs with at least three layers of compact cumulus cells and homogeneous cytoplasm were used for the experiments. Groups of 10 COCs were placed into 100 µL maturation medium droplets, covered with mineral oil and allowed to undergo maturation for 18 h at 38.5 °C under a humidified atmosphere of 5% CO₂. Maturation medium comprised TCM 199 supplemented with 10% FCS, 50 µg/mL gentamicin, 5.0 µg/mL FSH, 5.0 µg/mL LH and 11 mg/mL pyruvate.

Sperm treatment with L-arginine and *in vitro* fertilization (IVF)—IVF was performed using frozen-thawed semen from two bulls of different breeds (*Bos taurus* (Simmental) and *Bos indicus* (Nellore)). Motile sperm was separated by a discontinuous Percoll gradient and washed in IVF medium consisting of TALP supplemented with heparin, hypotaurine, penicillamine, epinephrine, BSA¹⁷ and L-arginine at four different concentrations (0, 1, 10 or 50 mM), according to the experimental group. Following maturation, COCs were washed thrice in IVF medium and randomly transferred to 80 µL droplets for each experimental group at a concentration of 20 COCs droplet. IVF medium was

supplemented with 1, 10 or 50 mM L-arginine, whereas the control group was not supplemented with L-arginine (0 mM). Sperm and COCs were co-incubated in a final concentration of 2×10^6 cells/mL for approximately 30 h in the same conditions as in IVM. The co-incubation time was based on a protocol developed by Cordeiro *et al*¹⁸.

***In vitro* culture (IVC)**—Approximately after 30 h of fertilization, cumulus cells were removed by successive pipetting, and presumptive zygotes were washed in IVC medium and transferred to 100 µL IVC droplets, corresponding to each experimental group, in a co-culture system using a monolayer of cumulus cells. IVC medium comprised synthetic oviductal fluid (SOF) supplemented with 6 mg/mL BSA, 10% FCS and 50 µg/mL gentamicin¹⁹. Culture conditions were the same as in IVM and IVF.

Fertilization rate—To assess the fertilization rate 18 h post-fertilization (hpf), presumptive zygotes were denuded by successive pipetting in hyaluronidase droplets, fixed with acetic acid:ethanol (1:3) and stained with 1% acetic orcein to determine the pronuclear formation. The presence of two pronuclei, a metaphase spindle or more than two pronuclei was considered to indicate monospermic fertilization, lack of fertilization or polyspermic fertilization, respectively.

NO₃⁻/NO₂⁻ production assay—Due to the short half-life of NO, its production was quantified indirectly by nitrite and nitrate (NO₃⁻/NO₂⁻) measurements²⁰. NO production during IVF was evaluated indirectly by determining the NO₃⁻/NO₂⁻ concentration in IVF droplets, where the fertilization occurred. For this purpose, 30 hpf, the IVF medium was stored at -20 °C and subjected to the Griess colorimetric method according to Ricart-Jané *et al*²¹. Griess reagent is composed of 2% (w/v) sulphanilamide and 0.2% (w/v) N-(1-naphthyl) ethylene-diamine in deionized water. These components react with NO metabolites in a test solution, yielding a purple azo-dye product with a peak absorbance at 540 nm. Readings were performed using a microplate reader (BIO-RAD 450, Bio-Rad Laboratories, Hercules, CA).

Embryonic development analysis—Cleavage and blastocyst development were assessed at Day 2 and Day 7 after fertilization, respectively. The blastocyst rate was based on the number of COCs subjected to maturation. Embryonic development kinetics were evaluated by the proportion of early blastocysts, blastocysts, advanced blastocysts and hatched

blastocysts on Day 7. For total cell counting, blastocysts were fixed with formol-saline, stained with 10 µg/mL Hoechst 33342 and analyzed by fluorescence microscopy.

Statistical analysis—Data were analyzed by ANOVA, using Tukey's post hoc test, with a level of significance at 5% ($P < 0.05$) using BioEstat 5.0 software²². For NO dosage analysis, the results were normalized with the 0 mM group, depicted as $\text{NO}_3^-/\text{NO}_2^-$ n-fold concentration and analyzed by ANOVA using SigmaPlot 11.0.

Results

Fertilization rate—The fertilization rate of 302 presumptive zygotes from all treatment groups was assessed. There was no significant difference ($P > 0.05$) in fertilization and monospermic fertilization rates among the different experimental groups (0, 1, 10 and 50 mM) or in the different semen used (data not shown).

Nitrate/Nitrite ($\text{NO}_3^-/\text{NO}_2^-$) dosage on IVF medium—No difference was observed in $\text{NO}_3^-/\text{NO}_2^-$ production in 30 hpf IVF medium among different treatment groups or between the bull breeds ($P > 0.05$) (Fig. 1).

Embryo production—COCs 792 were divided between IVF treatments. Cleavage rates decreased significantly ($P < 0.05$) when IVF medium was supplemented with 50 mM L-arginine in both bull breeds. There was no change in the rate of oocytes that developed to blastocysts among 0, 1 and 10 mM L-arginine treatment during IVF ($P > 0.05$) (Table 1).

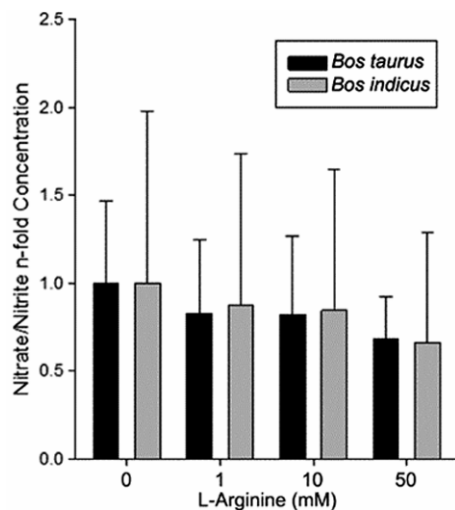


Fig 1—Effect of different concentrations of L-arginine and breed on production of $\text{NO}_3^-/\text{NO}_2^-$ during in vitro fertilization in bovines.

Similar kinetics of embryonic development, including the distribution of early blastocysts (EB), blastocysts (BL), advanced blastocysts (AB) and hatched blastocysts (HB) were observed among all treatment groups and between both bull breeds ($P > 0.05$). However, blastocysts and advanced blastocysts were most prevalent, except at 1 mM L-arginine using *Bos indicus* semen, which exhibited a greater proportion of hatched blastocysts ($P < 0.05$) (Table 2).

Total cell number counting—To evaluate the total embryo cell number, 170 embryos were fixed on Day 7. Treatments did not reveal any influence on total cell numbers when counted at different L-arginine concentrations (0, 1, 10 and 50 mM) or on the semen used for IVF (102.3 ± 32.3 ; 101.2 ± 32.1 ; 112.8 ± 41.6 ; 90.5 ± 25.9 for *Bos taurus* and 102.8 ± 33.5 ; 111.4 ± 28.4 ; 107.4 ± 32.7 ; 96.1 ± 34.4 for *Bos indicus*) ($P > 0.05$).

Discussion

Beneficial effects of L-arginine on sperm function, where it serves as a substrate for the NOS enzyme during NO production has been reported. NO is a potent cellular messenger that participates crucially in capacitation and the acrosome reaction⁴. Such behaviour was confirmed by a decrease in the acrosome reaction rate after the use of NOS and NO inhibitors^{5,23–25}.

Leal *et al.*⁴ have observed that the addition of 10 mM L-arginine increased sperm quality variables (sperm motility, vigor, membrane integrity and mitochondrial activity). Moreover, Kim *et al.*²⁶ have

Table 1—*In vitro* developmental potential of oocytes fertilized with semen (*Bos taurus* × *Bos indicus*) treated with L-arginine. [Values are mean ± SD of fertilized oocytes]

L-arginine concentration (mM)	n	Cleavage rate	Blastocyst rate
<i>Bos taurus</i>			
0	99	78.7 ± 2.17^a	39.4 ± 3.78^a
1	98	77.6 ± 2.88^a	34.7 ± 4.55^a
10	98	74.5 ± 3.78^{ab}	36.7 ± 4.32^a
50	99	65.7 ± 9.32^b	15.2 ± 6.12^b
<i>Bos indicus</i>			
0	99	72.7 ± 3.36^{ab}	39.4 ± 4.39^a
1	100	74 ± 4.18^{ab}	40 ± 6.12^a
10	99	67.7 ± 2.51^b	37.4 ± 3.36^a
50	100	45 ± 7.12^c	16 ± 8.54^b

Values within the same column with different superscript differ significantly ($P < 0.05$).

Table 2— Embryonic developmental kinetics of day-7 bovine embryos produced by IVF with semen (*Bos taurus* × *Bos indicus*) treated with L-arginine. [Values are mean ± SD of fertilized oocytes]

L-arginine concentration (mM)	n	EB	BL	AB	HB
<i>Bos taurus</i>					
0	39	15.5±10.8	31.5±19	40.4±9.9	12.5±8.9 ^b
1	34	21.4±11.8	24.4±13.4	39.6±19.5	12.5±13.8 ^b
10	36	14.4±10.2	33.4±14.3	30.2±17.2	21.9±10.7 ^{ab}
50	15	13.3±18.2	38.3±37	26.6±25.2	21.6±21.7 ^{ab}
<i>Bos indicus</i>					
0	39	12.5±8.9	24.3±19.6	38.1±10.8	27.3±15.7 ^{ab}
1	40	13±9	15±9.9	39.1±11.5	32.7±10.8 ^a
10	37	16.2±5	30±7.4	40.7±9.3	13.2±8.9 ^b
50	16	13.3±18.2	23.3±27.8	56.6±27.9	6.6±14.9 ^b

Values within the same column with different superscript differ significantly ($P<0.05$). EB=early blastocyst; BL=blastocyst; AB=advanced blastocyst; HB=hatched blastocyst

shown that sperm treated with low doses of L-arginine exhibited a significant increase in fertilization rates. The effects on sperm variables may be linked to an increased induction of sperm capacitation and the acrosome reaction, which could positively influence fertilization and embryonic development rates. In the present study, 10 mM L-arginine did not differ from the 0 mM group when blastocyst formation and quality were assessed.

An increased production of $\text{NO}_3^-/\text{NO}_2^-$, depending on the L-arginine concentration added to the capacitation medium was observed for a period of 5 h^{4,5}. However, the measure of $\text{NO}_3^-/\text{NO}_2^-$ production in the present study did not reveal a difference between the experimental groups for both bulls. Notably, this assessment was performed in IVF medium (30 hpf) under the influence of other factors, such as the presence of cumulus cells and exposure time.

L-NAME, a NOS inhibitor, inhibited fertilization and embryonic development in mice²⁶. It was proposed that a moderate amount of NO production was essential for fertilization and embryonic development and that excessive NO generation was inhibitory²⁶.

Using 50 mM L-arginine in IVF medium led to the same detrimental effects, impairing embryonic development, which was indicated by its low rate of embryo production, suggesting that L-arginine supplementation at high concentrations is toxic. This effect may be attributed to a greater production of NO during IVF via L-arginine's role in NO production. However, the present results did not show increased NO concentrations in IVF medium when L-arginine

was added. Therefore, L-arginine may be affecting embryonic development by other routes or exhibiting toxic effects under these culture systems.

Under the conditions established in the present study, significant improvements on *in vitro* fertilization rates were not observed, unlike the results obtained by Leal *et al.*⁴, who observed an increase of 21% in the penetration rate when 10 mM L-arginine was added to the sperm capacitation medium for 5 h before co-incubating the spermatozoa with oocytes matured *in vitro*. However, differences in the capacitation process may have influenced these results. In the present study, sperm were capacitated in the same droplet in which fertilization occurred in the presence of COCs. Moreover, the spermatozoa used in this study performed well in IVP. Therefore, L-arginine may exhibit stronger effects when used in semen with a low fertilization rate.

Most L-arginine mammalian studies do not investigate the effects of L-arginine on subsequent processes such as *in vitro* embryonic development. To our knowledge, the present study is the first to describe the use of L-arginine during the fertilization process and its effect on embryonic development in cattle. In this context, a significant reduction was observed in the rates of cleavage and blastocyst formation, as well as several irregular and lagging cleavages (data not shown), when 50 mM L-arginine was added to the IVF medium.

Other L-arginine concentrations (1 and 10 mM) tested in the present study did not exhibit effects on the fertilization rate, blastocyst formation or embryo quality analyzed by total cell number. In the present study, L-arginine was added to IVF medium

supplemented with heparin, a capacitation agent. The presence of heparin may have masked putative beneficial L-arginine effects on fertilization and embryo production. This proposed antagonism by heparin against L-arginine as a capacitation agent may be responsible for blocking L-arginine's mechanism of action. Other routes by which L-arginine may be acting in the present system remain unexplained. More studies using only L-arginine as a capacitation agent during bovine embryo production are necessary to further elucidate its role during IVF and its potential for replacing heparin.

The addition of 1 mM L-arginine in the *Bos indicus* experimental group increased the proportion of HB compared to 0 and 1 mM L-arginine using *Bos taurus* semen, and 10 and 50 mM L-arginine *Bos indicus* groups, possibly indicating that L-arginine's influence on the kinetics of embryonic development may be dose-dependent and may respond to bull-to-bull variations, including species variations.

Further studies are necessary to elucidate the dose-dependent responses to L-arginine treatment during IVF, including exposure time and capacitation conditions. Perhaps the directly co-incubating L-arginine with the female gamete, as performed in the present work, may have been detrimental to the oocyte and subsequently influenced embryonic development. Experiments using different bulls and different breeds are needed to evaluate any breed- or bull-specific effects on such treatment and on NO production. Thus, evaluating the precise mechanisms that influence the L-arginine/NO system implicated in *in vitro* fertilization and early embryonic development in bovine is imperative.

This study showed that L-arginine supplementation during IVF did not increase fertilization rates or embryo production. When higher concentrations were used, L-arginine exhibited toxic effects under the present culture system. More studies addressing bull-to-bull variations and other routes where L-arginine may affect embryo development will help to elucidate its real potential on IVF.

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