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L-arginine affects the IVM of cattle cumulus-oocyte complexes

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ABSTRACT

Nitric oxide (NO) is identified as a signaling molecule involved in many cellular or physiological functions, including meiotic maturation of cattle oocytes. This study aimed to evaluate the effect of supplementation of culture medium with the L-arginine (L-arg, NO synthesis precursor) in nuclear maturation of oocytes, concentrations of nitrate/nitrite, progesterone (P_4), and 17β-estradiol (E_2) in the culture medium; and the cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) intracellular concentrations in the cumulus-oocyte complexes (COCs) during the first hours of maturation in the presence of hemisections (HSs) of the follicular wall (control −ve). The addition of 5.0-mM L-arg increased ($P < 0.05$) the percentage of oocytes at the germinal vesicle breakdown stage after 7 hours of cultivation compared with control −ve. All concentrations of L-arg (2.5, 5.0, and 10.0 mM) increased the percentage of oocytes that reached the metaphase I (MI) at 15 hours ($P < 0.05$) but do not affect the progression from MI to metaphase II ($P > 0.05$) at 22 hours. All concentrations of L-arg tested increased ($P < 0.05$) the percentage of cumulus cells with plasma membrane integrity at 22 hours of cultivation. L-arginine did not change ($P > 0.05$) the nitrate/nitrite, P_4, and E_2 concentrations in relation to control −ve at any of the times tested. In immature COCs, immediately after being removed from the follicles (0 hours), the intracellular concentration of cGMP in the control −ve and treatment with 5-mM L-arg progressively decreased ($P < 0.05$) after the first hour of cultivation; however, COCs treated with 5.0-mM L-arg had higher concentrations of cGMP at 1 hour of cultivation ($P < 0.05$). The cAMP concentration of COCs supplemented or not with 5.0-mM L-arg progressively increased until 3 hours of cultivation and at, 6 hours, decreased ($P < 0.05$). The results show, in using this system, that (1) the mechanisms that give the oocyte the ability to restart the meiosis until MI after adding 5.0-mM L-arg do not involve changes in the concentration of nitrate/nitrite, P_4, and E_2 in the culture medium and (2) L-arg acts on a pathway that involves changing the cGMP concentration but does not involve changing cAMP concentration. More studies are needed to assess whether the observed effects of L-arg during IVM using this system are via NO or not and what the role is in increasing the viability of cumulus cells in the resumption and progression of meiosis until MI.

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1. Introduction

The signaling pathways and associated substances that promote the resumption and progression of meiosis of bovine oocytes *in vivo* after the LH peak are not completely understood [1]. One of the substances whose involvement in oocyte maturation has been studied is nitric oxide (NO). It is produced by the activity of the nitric oxide synthase (NOS) enzyme, which catalyzes the reaction that converts the amino acid L-arginine (L-arg) to L-citrulline and NO [2,3]. Availability of L-arg for NO synthesis in the medium used to culture cells is one of the key points in controlling the synthesis of NO *in vitro* [3].

Most studies use artificial NO donors compounds (sodium nitroprusside [SNP] and S-nitroso-*N*-acetylpenicillamine) rather than natural precursor, L-arginine, to ascertain the role of this signaling molecule on *in vitro* oocyte maturation in rodents [4,5], pigs [6,7], sheep [8], buffalo [9], and cattle [10,11]. Supplementation of the medium with L-arg instead of NO donors to assess their role in the IVM of oocytes may have the advantage of controlling the amount of NO synthesized by the cells themselves in culture, whereas the L-arg availability can play important roles in determining rates of NO synthesis [3]. The L-arg is a semi-essential amino acid, consumed by metabolic pathways other than NO synthases—for instance, the synthesis of protein, urea, creatine, agmatine, and L-ornithine [12].

The importance of NO in oocyte maturation in cattle has been demonstrated by the presence of different isoforms of NOS, especially endothelial (eNOS) and inducible NOS (iNOS), in oocytes and in ovarian somatic cells [13,14]. Likewise, changes in NO concentration in the maturation medium due to supplementation with NO donor [10,15,16] or blocking NOS activity [17,18] affect the resumption and progression of meiosis.

In turn, NO is involved in various events affecting the IVM of bovine oocytes as the regulation of the integrity, viability, and activity of follicular cells [19]. The NO can protect follicular cells from apoptosis [20,21] and, in suitable *in vitro* concentrations, NO improves the integrity of the plasma membrane of granulosa cells [22], cumulus cells (CCs) [15,17], and oocytes [15]. There is evidence that NO may participate in the regulation of steroidogenic activity of the follicular cells [20,22]. Depending on the concentration in the culture medium, NO may promote or inhibit the synthesis of progesterone (P_4) and 17β-estradiol (E_2) by the granulosa cells [9,22], these being involved in IVM [23,24].

Nitric oxide exerts its effects mainly by the activation of the soluble isoform of guanylate cyclase enzyme (sGC), resulting in the production of cyclic guanosine monophosphate (cGMP) [3]. This nucleotide inhibits the phosphodiesterase 3A in rodent oocytes [25], an enzyme that degrades cAMP, keeping high levels intraoocyte of cAMP [26]. cAMP produced by adenylate cyclase present in the CCs and oocyte has been described as a major modulator of the resumption and progression of meiosis in oocytes [27]. Thus, NO participates in oocyte maturation, regulating the levels of cyclic nucleotides intraoocyte [25,28]. The role of cGMP in the modulation of cAMP levels has been well described in rodents [25,28], but there are few studies on the role of NO/cGMP pathway in the meiosis and this pathway in the modulation of cAMP concentration in cattle during this process [11].

The events that modulate the nuclear maturation of meiotically competent bovine oocytes have been difficult to study in conditions outside a physiological environment because the oocytes spontaneously resume meiosis as soon as they are removed from the follicles and cultured in a suitable medium [29]. The IVM of oocytes in coculture with hemisections (HSs) of the follicular wall is an attempt to mimic the conditions of the follicular environment *in vivo*. The cultured cells of the follicular wall, especially the theca cells, produce a factor of an unknown nature that is soluble in the culture medium and has the ability to partially block the nuclear maturation of oocytes [30,31].

Considering that NO can influence oocyte maturation and the enzymatic activity of NOS is tightly controlled and dependent on the availability of substrate, we therefore used L-arg supplementation to investigate the role of NO on nuclear maturation, on CC plasma membrane integrity, on nitrate/nitrite (NO_3^-)/(NO_2^-), and steroid (P_4 and E_2) production during the IVM of cumulus-oocyte complexes (COCs) in coculture with follicular wall HSs in cattle.

2. Materials and methods

All reagents used in these experiments were obtained from Sigma–Aldrich BrasilLtda (São Paulo, Brazil), unless otherwise indicated.

2.1. Obtaining the follicular wall HSs

Bovine ovaries were collected from adult cows, irrespective of stage of the estrous cycle, at a local abattoir immediately after slaughter and carried to the laboratory within 2 hours in sterile saline solution with antibiotics (100-IU/mL potassium penicillin G, and 50-μg/mL streptomycin sulfate) at 30 °C. In the laboratory, the ovaries were washed three times with saline. Follicles were isolated from ovary, dissected from the stromal tissue, and were selected based on size (3–5 mm) and transparency [32]. These follicles were cut into equal halves with a scalpel, and their respective oocytes were discarded [30]. The follicular walls (HSs) were washed three times in washing medium (tissue culture medium 199 [TCM 199—HEPES], in addition to 0.4% fatty acid–free BSA, 0.2-mM pyruvate, 100-IU/mL potassium penicillin G, and 50-μg/mL streptomycin sulfate). Two hours before the addition of COCs, the HSs were transferred to four-well plates (NUNC, Rochester, NY, USA) containing 200 μL of maturation medium (TCM 199 supplemented with 0.4% BSA [fatty acid free], 0.5-μg/mL FSH [Folltropin-V, Bioniche Life Science Inc., Canada], 5-μg/mL LH [Lutropin-V, Bioniche Life Science Inc., Canada], and 0.2-mM pyruvate, and antibiotics) with the desired treatment [31].

2.2. Collection and cultivation of COCs

Three- to 8-mm follicles were aspirated, and the collected COCs were immediately placed in TCM washing medium supplemented with 3-isobutyl-1-methylxanthine

(IBMX, 0.5 mM) [33]. The IBMX, a reversible meiotic inhibitor, has been used to inhibit spontaneous resumption of meiosis in bovine oocytes during collection and handling of COCs *in vitro* [10,15]. After selection, the diplotene-arrested oocytes were washed quickly with TCM 199 medium to remove IBMX from culture medium and were used for *in vitro* studies. The average time for collection and preparation of culture was 30 minutes for all *in vitro* studies. Only COCs with more than three layers of CCs and a homogeneous ooplasm [32] were selected and transferred to the plates containing the follicular wall HSs. The cultivation was performed in an incubator at 38 °C in an atmosphere containing 5% CO_2 in air, at 95% relative humidity for 7, 15, and 22 hours.

2.3. Evaluation of the nuclear maturation

The oocytes were denuded by repeated pipetting in a solution of phosphate-buffered saline (PBS) with 0.1% polyvinyl alcohol, subsequently mounted between a slide and a cover slip, fixed in a 3:1 solution of ethanol:acetic acid (Merck Industries SA, Rio de Janeiro, Brazil) for at least 22 hours, and finally stained with 2% acetic orcein. The nuclear maturation stage of each oocyte was assessed with the aid of an inverted microscope (× 400, NIKON–Eclipse TE300, Melville, Japan). The oocytes were classified according to the characteristics of the chromatin as follows: germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI), and metaphase II (MII).

2.4. Evaluation of the plasma membrane integrity of CCs

After the period of maturation, COCs were mechanically denuded, the oocytes were removed from the medium, and the CC centrifuged (700 × *g*) for 10 minutes and resuspended in PBS with 5% fetal calf serum–containing stains. Double staining with the fluorescent markers Hoechst 33342, which labels cells with intact membranes (blue staining), and propidium iodide, which labels cells with damaged membranes (red staining), was used to observe the plasma membrane integrity of CC [15]. The cells (200/treatment) were exposed to each of the stains for 5 minutes and placed between a slide and a cover slip. Then the slides were analyzed under an epifluorescence microscope (× 400, NIKON–Eclipse TE300, Melville, NY, USA). The results are expressed as the proportion of cells with an intact or a damaged membrane.

2.5. Measurement of the concentrations of NO

Nitric oxide concentration was evaluated by measuring in the culture medium of nitrate/nitrite concentrations, stable NO metabolites, as an indirect indicator of the concentration NO, since this has a very short half-life. The maturation medium containing the different treatments was centrifuged (2700 × *g* for 5 minutes), stored (50 μL), and maintained at −20 °C for later measurement using the Griess colorimetric reaction [34]. To transform NO_3^- into NO_2^-, the samples were incubated in 96-well plates with the reducing solution (10-IU of the NO_3^- reductase enzyme diluted in ultrapure water + 900 μL of ultrapure water, 1000 μL of the NADPH cofactor [5 mg/mL] diluted in ultrapure water, 1000 μL of potassium phosphate buffer solution [0.5 M]) at 37 °C for 16 hours. The standard curve of $NaNO_3$was diluted in TCM 199 medium at concentrations ranging from 0.5 to 100 μM. The reading was performed in a spectrophotometer (ELISA reader, BioTek, Winooski, VT, USA) at 540 nm. A scatterplot was plotted with the absorbance values. The results are expressed in micromolar units.

2.6. Measurement of the concentrations of steroids

The culture media of the different treatments were centrifuged (2700 × *g* for 5 minutes), and the supernatant was stored and maintained at −20 °C for later measurement of the concentration of P_4 and E_2. The samples were diluted 1:20 or 1:40 to measure the concentration of P_4 and 1:200 to measure the concentration of E_2, both in distilled water. The chemiluminescence-automated system (Listed Laboratory Equipment 7 C20 UL, MH 15423, series 3975, DPC Immulite I) and commercial diagnostic set were used for quantitative measurement of P_4 and E_2 (Immulite 1000 Systems, Siemens Healthcare Diagnostics Products Ltd, Gwynedd, UK). The method was based on a competitive solid-phase immunoassay that used enzyme chemiluminescence technology with a detection limit of 15 to 20 ng/mL [22]. A high and low control provided by the manufacturer was measured along with the samples in each experiment. The P_4 and E_2 concentrations are expressed in nanograms/milliliter.

2.7. Measurement of the concentrations of cGMP and cAMP nucleotides

The intracellular concentrations of cGMP and cAMP were determined by enzyme immunoassay using commercial kits (Cayman's cGMP and cAMP EIA kits, Ann Arbor, MI, USA). Groups of 30 and 10 COCs with similar sizes were used for the measurement of cGMP and cAMP, respectively [35]. After the cultivation, the COCs were washed in 199-HEPES medium supplemented with 0.4% of BSA. To induce cell lysis, the COCs were transferred to a solution of 0.1-M HCl (200 μL), where they were kept for 20 minutes, and then vortexed for 1 minute every 5 minutes. The samples were then centrifuged at 12,000 × *g* for 5 minutes and the supernatant stored at −20 °C until the evaluations were performed according to the instructions of the respective kits. All samples were acetylated to increase the sensitivity of the assay, according to the manufacturer's recommendation. The concentration of the nucleotides was determined using a spectrophotometer (ELISA reader, BioTek) at 405 nm. The results from the spectrophotometer readings were transformed into fmol/COC.

2.8. Experimental design

2.8.1. Experiment 1: effects of L-arg supplementation on oocyte nuclear maturation, on the integrity of the plasma membrane of CCs, on the NO_3^-/NO_2^-, and steroids production

This experiment was conducted to evaluate the effect of L-arg supplementation of the maturation medium on

nuclear maturation, on the integrity of the plasma membrane of CC, and concentration of NO_3^-/NO_2^- and steroids in the culture medium. Groups of 20 COCs were randomly cultured with eight follicular wall HSs in 200 μL of maturation medium [30] supplemented with 2.5-, 5.0-, or 10.0-mM L-arg. These L-arg concentrations were defined after previous experiments in our laboratory testing different L-arg concentrations in the IVM in the presence of HSs based on concentrations used by Leal et al. [36]. Two control groups were evaluated along with the treatments: negative control (control −ve), COCs cultured in the presence of HSs (1 HSs/25 μL of medium); and positive control (control +ve), COCs cultured in the absence of HSs. The percentage of oocytes that reached GVBD at 7 hours of culture, MI at 15 hours, and MII at 22 hours was determined. The plasma membrane integrity of CC was evaluated at 22 hours after cultivation to verify whether the treatment would interfere with the quality of these cells, since the communication between oocytes and viable CC is required for maturation of cattle oocytes [37].

The reversibility of the inhibitory effect occasioned by the presence of the eight follicular wall HSs (control −ve) and the supplementation with 10.0-mM L-arg on the meiotic progression of oocytes matured *in vitro* for 22 hours was tested based on the results of the experiment 1. The COCs were then cultured for more 16 hours in a medium without HSs (control −ve) and with L-arg supplementation and without follicular wall HSs. The nuclear maturation stage was evaluated at the end of the additional period of cultivation.

Finally, the effect of the supplementation with L-arg on the production of the nitrate/nitrite and steroids (P_4 and E_2) by the CCs of the COCs and by the HSs cells of the follicular wall was assessed. The culture medium of each of the replicates was used to determine the concentrations of these metabolites and steroids at 7, 15, and 22 hours of the culture.

2.8.2. Experiment 2: effect of the supplementation of L-arg on the intracellular concentration of cGMP and cAMP nucleotides in COCs

The effect of the addition of L-arg in COCs on the intracellular concentration of cGMP and cAMP, which are nucleotides related to the events of meiosis resumption, was evaluated. Based on the results of the experiment 1, the COCs were cultured for 1, 3, or 6 hours without supplementation (control −ve) or supplemented with 5.0-mM L-arg. The nucleotide concentrations were also measured in COCs immediately after being removed from the follicles (0 hours of cultivation, immature oocytes) and were used both in the control group and in the group treated with L-arg.

2.9. Statistical analysis

Data consistency analysis and descriptive statistics (PROC UNIVARIATE, PROC MEANS) [38] were performed. Data transformation (log +1) and arcsin were performed for the variables that showed a coefficient of variation greater than 30% [39]. The effects of the different treatments on the variables studied were evaluated by analysis of variance. Means were compared by the SNK test at a 5% probability (PROC GLM) [38].

3. Results

3.1. Experiment 1: effects of L-arg supplementation on oocyte nuclear maturation, on the integrity of the plasma membrane of CCs, and on the NO_3^-/NO_2^- and steroids production

The addition of 5.0-mM L-arg increased ($P < 0.05$) the percentage of oocytes at the GVBD stage after 7 hours of cultivation compared with control −ve (Fig. 1A). All concentrations of L-arg (2.5, 5.0, and 10.0 mM) increased ($P < 0.05$) the percentage of oocytes that reached the MI at 15 hours. The percentage of oocytes that reached the MI was greater ($P < 0.05$) after supplementation of the medium with 10 mM L-arg compared with other concentrations and with control −ve (Fig. 1B). L-arg had no effect on the progression of meiosis until MII ($P > 0.05$) when oocytes were observed after 22 hours of the cultivation (Fig. 1C).

There was no difference between the nuclear maturation of oocytes in control +ve (87.67 ± 10.03%) and control −ve (82.52 ± 6.72%) cultured for an additional period of 16 hours without the presence of HSs ($P > 0.05$); however, oocytes cultured in presence of 10-mM L-arg without HSs showed a lower percentage of MII (66.32 ± 6.20%) than that observed in two controls ($P < 0.05$) after 16 hours of additional cultivation (Fig. 2).

At all concentrations tested (2.5, 5.0, and 10.0 mM), L-arg increased ($P < 0.05$) the percentage of CC with plasma membrane integrity with 22 hours of cultivation. Cumulus-oocyte complexes treated with 10.0-mM L-arg decreased ($P < 0.05$) the CC plasma membrane integrity compared with 2.5-mM L-arg (Fig. 3A).

The NO_3^-/NO_2^- concentrations were not affected ($P > 0.05$) by any of the concentrations of L-arg at any of the times assessed; however, there was a time-dependent increase in NO_3^-/NO_2^- concentration in the culture medium after the addition of the follicular wall HSs ($P < 0.05$) (Table 1).

L-arg did not change ($P > 0.05$) the P_4 concentration in relation to control −ve at any of the times tested (Table 2). The P_4 concentration increased approximately 20.1-fold in control −ve compared with the concentration observed in control +ve ($P < 0.05$). There was a time-dependent increase in P_4 concentration in the culture medium after the addition of the follicular wall HSs ($P < 0.05$).

There was no difference in E_2 concentration over time after the addition of the follicular wall HSs ($P > 0.05$) and different concentrations of L-arg, except between the control −ve and control +ve (1.3-fold) at 22 hours ($P < 0.05$) (Table 3).

3.2. Experiment 2: effects of L-arg on intracellular concentrations of cGMP and cAMP

In immature COCs, immediately after being removed from the follicles (0 hours), the intracellular concentration of cGMP in COCs with and without supplementation with L-arg progressively decreased after the first hour of cultivation ($P < 0.05$). COCs treated with L-arg had higher

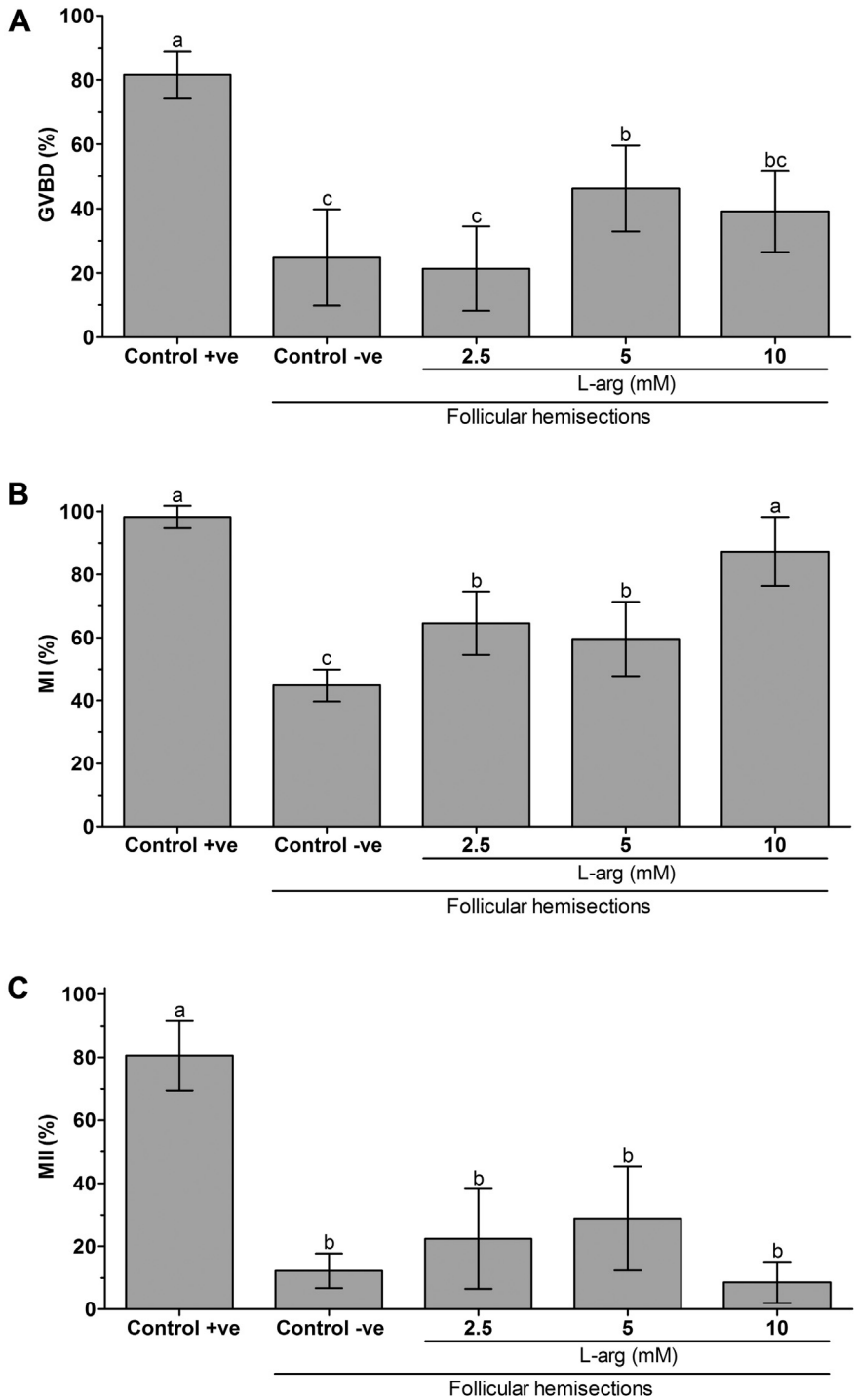

Fig. 1. Effect of L-arg on the nuclear maturation of bovine oocytes cocultured with eight follicular wall HSs. Data shown (A) germinal vesicle breakdown (GVBD) at 7 hours of cultivation, (B) metaphase I (MI) at 15 hours of cultivation, and (C) metaphase II (MII) at 22 hours of cultivation. Values are expressed as mean ± standard deviation for seven replicates. Twenty COCs per treatment (n = 140) were analyzed from a total of 980 COCs. Different letters within each maturation stage denote significant difference ($P < 0.05$). HSs, hemisections; L-arg, L-arginine.

concentrations of cGMP at 1 hour of cultivation compared to COCs from the control group ($P < 0.05$) (Fig. 4).

Compared with immature COCs (0 hours), the cAMP concentration of COCs supplemented or not supplemented with 5.0-mM L-arg increased until 3 hours of cultivation and decreased after this period ($P < 0.05$) (Fig. 5). There was no difference between control −ve and treatment ($P > 0.05$).

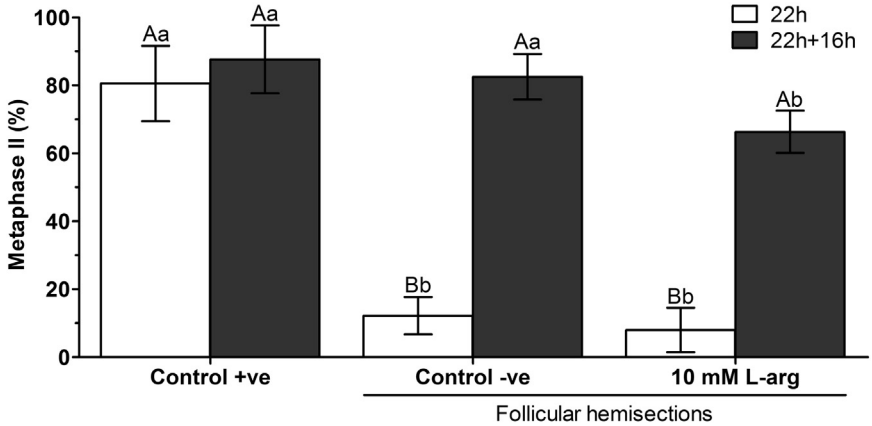

Fig. 2. Effect of additional cultivation for 16 hours in a medium with 10-mM L-arg and without follicular wall HSs during nuclear maturation of oocytes for 22 hours cocultured with eight follicular wall HSs. Values are expressed as mean ± standard deviation for seven replicates. Twenty COCs per treatment (n = 140) were analyzed from a total of 560 COCs analyzed. Different lowercase letters in columns of the same color denote significant differences ($P < 0.05$). Different uppercase letters in columns with different colors within the same treatment denote significant difference ($P < 0.05$). HSs, hemisections; L-arg, L-arginine.

4. Discussion

This study evaluated the effect of L-arg supplementation in the events of IVM of bovine oocytes in a cultivation system that inhibited nuclear maturation through the presence of follicular wall HSs.

Follicular cells, especially theca cells, produce some soluble factor(s) in the medium that inhibit the meiosis of oocytes in coculture [30,31]. Confirming the previously reported data, the follicular wall HSs in the present study partially blocked the resumption and spontaneous progression of meiosis. The mechanism of action of the inhibitory factor(s) is not completely understood. However, this mechanism could include the effects on intercellular communication between the CC and the oocyte, given that in the presence of HSs, there was no expansion of CCs (data not shown) and there was an increase in the percentage of CC with plasma membrane integrity.

Ovarian follicles are multicellular complexes that consist of oocytes surrounded by somatic cells. All these cells are interconnected by dynamically regulated gap junctions and function together to regulate meiosis in the oocyte [40]. In this culture system used, the HS follicular wall was added, but these follicular HSs are not connected with the CC by gap junctions. Partial inhibition of the resumption of meiosis occurs by substances that are synthesized by cells from the follicular wall and pass into the culture medium, reaching the CC by a different pathway of gap junctions, or with different features from those observed in intact follicles. Since the inhibition of the resumption of meiosis was around 30%, these data suggest that the other 70% of meiosis blockage remaining as the oocyte is in the follicle *in vivo* occurs through substances secreted by the wall that reach the oocyte via gap junctions between granulosa cells/CCs/oocyte.

Nitric oxide is one of the main intercellular and intracellular signaling molecules involved in many physiological processes. The role of NO in reproductive events shows a biphasic pattern. At suitable concentrations, NO stimulates these processes, but a decrease or increase in NO leads to deleterious effects [10,15,41]. The concentration of 5.0 mM stimulated the GV disruption, whereas all concentrations of L-arg stimulated the progression to MI with 15 hours of the culture but did not affect the progression of meiosis until MII with 22 hours of the culture.

Bu et al. [41] also observed a stimulatory effect of suitable concentrations of SNP, an NO donor, on the nuclear maturation of mice oocytes, in a system with meiosis block. At low concentrations (0.1, 1.0, 10 μM), the SNP reversed the inhibitory effect of hypoxanthine. Likewise, when Bilodeau-Goeseels [35] used a low concentration of SNP (0.01 μM) in bovine oocytes, it was observed an increase in the spontaneous resumption of meiosis at 7 hours of cultivation, when there were higher rates of rupture of the GV. However, when higher concentrations of SNP were used (100 μM), there was a decrease in the GVBD. These data suggest that low concentrations of NO are required to enable the stimulatory pathway of bovine oocyte maturation, confirming the results obtained in the present experiment.

Other studies in cattle have observed a stimulatory effect of low concentrations of SNP on cytoplasmic maturation [10,15], but without an effect on nuclear maturation. This result can be explained by the fact that these studies evaluated the meiotic stage in a cultivation system with spontaneous maturation only at 22 hours of cultivation. The difficulty of determining the stimulatory effect of any substance on nuclear maturation under conditions of spontaneous maturation when evaluating the meiotic stage at 22 or 24 hours of cultivation should be considered, given that oocytes that have completed the growth phase and are cultured *in vitro* in a suitable medium usually reach MII at a high rate [42]. In the present experiment, we also did not observe any effect of medium supplementation with L-arg in MII stage observed at 22 hours of cultivation.

All L-arg concentrations stimulate the progression of meiosis until the MI with 15 hours of culture, and supplementation of the medium with 10-mM L-arg increased the

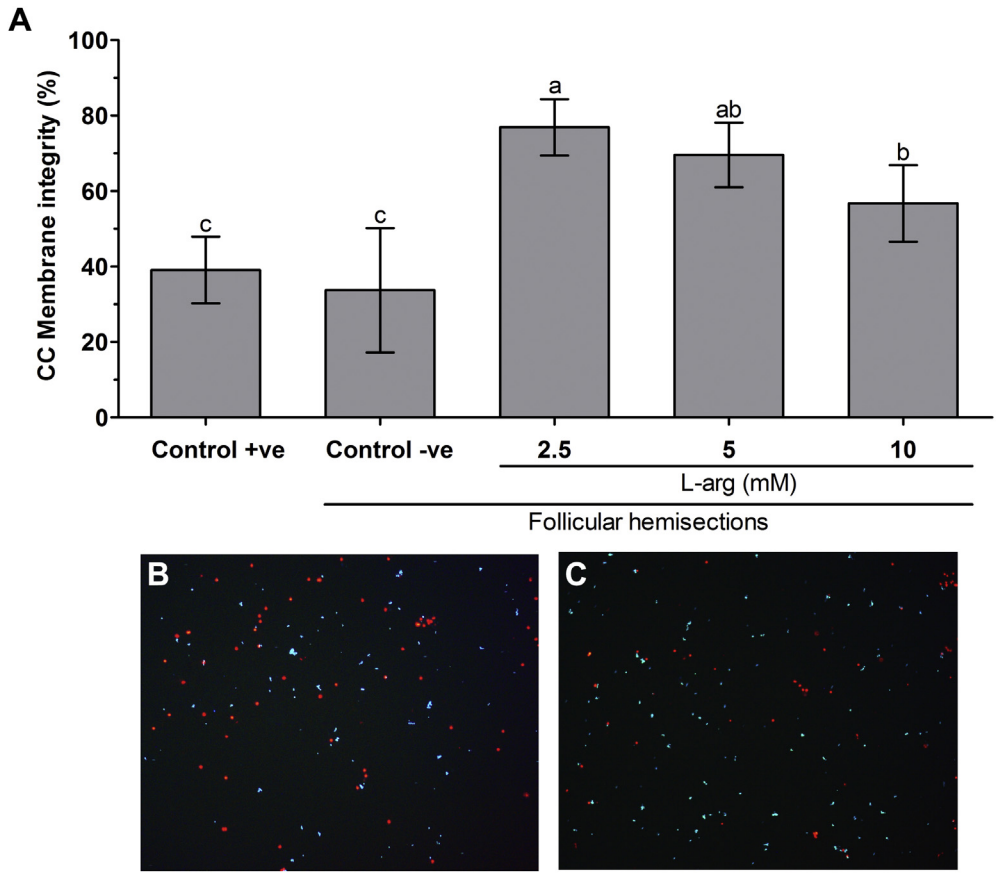

Fig. 3. Effects of different concentrations of L-arg on the plasma membrane integrity of the cumulus cells (CCs) of cumulus-oocyte complexes (n = 20) cocultured with eight follicular wall HSs (A) were counted for 200 cells for each repetition. Values represent the mean ± standard deviation of six replicates (n = 6000). Different letters within the columns indicate significant differences ($P < 0.05$). Integrity of the plasma membrane–derived cumulus cells of control −ve (B) and the group treated with 10-mM L-arg (C). Double staining with the fluorescent markers Hoechst 33342, which labels cells with intact membranes (blue staining), and propidium iodide (PI), which labels cells with damaged membranes (red staining), was used to observe the plasma membrane integrity of cumulus cells. HSs, hemisections; L-arg, L-arginine. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

percentage of oocytes in MI compared with other concentrations. There is no report in the literature evaluating the effect of L-arg/NO on this stage of meiosis.

To assess whether the effect of follicular HSs and HSs/L-arg was reversible, additional cultivation for 16 hours in medium without follicular HSs and HSs/L-arg allowed the oocytes to progress through meiosis and reach MII. These data demonstrate that the inhibitory effect on resumption and progression of meiosis exerted by the follicular wall HSs is reversible. However, the group treated with 10-mM L-arg had a lower percentage of oocytes in MII after an additional 16 hours in cultivation. Also, compared with the group treated with 5-mM L-arg, this group reached a lower proportion of oocytes in MII after the first 22 hours of cultivation, suggesting that this concentration of L-arg can cause deleterious effects on oocytes, since 5.8% of oocytes had high chromatin condensation, which was not observed in oocytes treated with lower concentrations of L-arg (data not shown). The occurrence of chromatin condensation is a specific morphologic aspect of cell death by apoptosis [43]. Moreover, further studies are necessary to assess whether a period longer than 16 hours of culture without the presence of HSs would be required for the group treated with L-arg reach the MII because there was a higher percentage of oocytes in MI and A/T, when comparing to the control +ve and control −ve (data not shown).

All the concentrations of L-arg studied improved the integrity of CC compared with the control −ve. Other studies have demonstrated a protective effect of NO on the integrity of the plasma membrane in cattle. Zamberlam et al. [21] demonstrated the role of NO in inhibiting the expression of genes responsible for triggering the apoptotic cascade, indicating its involvement in protecting cells from follicular atresia. Matta et al. [17] observed that the inhibition of iNOS activity reduces the viability of CC, indicating that a particular concentration of NO derived from iNOS activity is necessary to maintain the cellular integrity and/or that iNOS is involved in a signaling pathway linked to cell membrane integrity.

In turn, L-arg is also the substrate for the synthesis of polyamines, via ornithine decarboxylase [44]. Polyamides (putrescine, spermidine, and spermine) are essential substances involved in regulating gene expression, membrane stabilization, and synthesis of proteins and DNA;

Table 1
Effects of different concentrations of L-arg on the kinetics of nitrate and nitrite (μM) production in the presence of bovine COCs (n = 20) and eight follicular wall HSs.

Treatments	7 h	15 h	22 h
Control +ve	$10.25 \pm 2.17^{A,b}$	$14.75 \pm 4.38^{A,c}$	$10.67 \pm 1.44^{A,b}$
Control −ve	$31.50 \pm 8.75^{B,a}$	$62.75 \pm 9.92^{A,b}$	$62.00 \pm 5.00^{A,a}$
L-arg (mM)			
2.5	$36.50 \pm 4.21^{C,a}$	$90.67 \pm 18.21^{A,a}$	$63.17 \pm 8.14^{B,a}$
5.0	$33.38 \pm 11.97^{B,a}$	$63.69 \pm 9.15^{A,b}$	$64.42 \pm 0.10^{A,B,a}$
10.0	$34.00 \pm 6.21^{B,a}$	$68.17 \pm 8.04^{A,b}$	$59.00 \pm 8.75^{A,a}$

Values represent the means ± standard deviation of six replicates. Different uppercase letters in the same line denote mean difference ($P < 0.05$) between hours, and different lowercase letters in the same column denote mean difference ($P < 0.05$) between treatments. Control +ve: control without follicular HS; control −ve: control with follicular HS.
Abbreviations: COCs, cumulus-oocyte complexes; HSs, hemisections; L-arg, L-arginine.

Table 3
Effects of adding different concentrations of L-arg on the kinetics of 17β-estradiol (ng/mL) production by bovine COCs (n = 20) and eight follicular wall HSs evaluated in the culture medium during IVM.

Treatments	7 h	15 h	22 h
Control +ve	$16.41 \pm 0.45^{a,b}$	$16.78 \pm 0.64^{a,b}$	14.85 ± 0.10^{b}
Control −ve	19.91 ± 0.58^{a}	$18.51 \pm 1.00^{a,b}$	18.85 ± 0.46^{a}
L-arg (mM)			
2.5	$18.37 \pm 0.93^{a,b}$	$16.75 \pm 1.40^{a,b}$	$16.95 \pm 1.48^{a,b}$
5.0	$17.32 \pm 1.29^{a,b}$	$18.62 \pm 1.92^{a,b}$	$18.11 \pm 1.53^{a,b}$
10.0	$17.22 \pm 0.68^{a,b}$	$18.08 \pm 3.71^{a,b}$	$16.55 \pm 0.96^{a,b}$

Values represent the means ± standard deviation of four replicates. Different superscript letters represent mean difference ($P < 0.05$) between treatments in the same column. Control +ve: control without follicular HS; control −ve: control with follicular HS.
Abbreviations: COCs, cumulus-oocyte complexes; HSs, hemisections; L-arg, L-arginine.

therefore, they are necessary elements for cell proliferation and differentiation [45,46]. Thus, there is a possibility that the beneficial effect observed in the supplementation of the medium with L-arg is also a consequence of the activity of polyamines, favoring the proliferation and survival of CC.

The concentrations of NO_3^-/NO_2^- were similar to control −ve at each combination of L-arg concentration and treatment time. The increase in concentration observed in the different treatments and control −ve over time suggests a cumulative effect. However, it is possible that at certain concentrations of this amino acid, there are differences in the patterns of activity of the different isoforms of NOS. A differentiated activity pattern favoring the action of one of the isoforms in particular could be part of the mechanisms that regulate the events of resumption and progression of meiosis [47]. The effects of different concentrations of L-arg on the activity of NOS isoforms were not the object of study in the present study.

The involvement of steroids in the NO signaling pathway as a result of L-arg supplementation was evaluated. Steroids may be involved in the acquisition of nuclear competence of mammalian oocytes [23,24,48–50], and NO may affect the *in vitro* steroidogenesis of follicular cells, depending on its concentration in the culture medium [20,22]. However, we found no difference in the production of P_4 and E_2 in the culture medium after the supplementation with different concentrations of L-arg in this experimental model.

Table 2
Effects of adding different concentrations of L-arg on the kinetics of progesterone (ng/mL) production by bovine COCs (n = 20) and eight follicular wall HSs evaluated in the culture medium during IVM.

Treatments	7 h	15 h	22 h
Control +ve	$5.93 \pm 1.55^{B,b}$	$9.73 \pm 2.34^{A,b}$	$10.87 \pm 1.63^{A,b}$
Control −ve	$85.00 \pm 18.38^{B,a}$	$220.00 \pm 50.91^{A,a}$	$228.00 \pm 8.00^{A,a}$
L-arg (mM)			
2.5	$82.67 \pm 15.01^{C,a}$	$138.67 \pm 15.28^{B,a}$	$222.67 \pm 11.37^{A,a}$
5.0	$84.00 \pm 15.10^{B,a}$	$126.67 \pm 50.29^{B,a}$	$228.67 \pm 62.78^{A,a}$
10.0	$74.00 \pm 16.25^{B,a}$	$200.00 \pm 65.50^{A,a}$	$170.67 \pm 16.29^{A,a}$

Values represent the means ± standard deviation of four replicates. Different uppercase letters in the same line denote mean difference ($P < 0.05$) between hours. Different lowercase letters in the same column denote mean difference ($P < 0.05$) between treatments. control +ve: control without follicular HS; control −ve: control with follicular HS.
Abbreviations: COCs, cumulus-oocyte complexes; HSs, hemisections; L-arg, L-arginine.

The increase in P_4 concentration in control −ve was on average 14.3-fold higher than that in control +ve at 7 hours of cultivation. This sharp increase in P_4 could be involved in the meiosis block observed in this IVM system of COCs in the presence of the follicular wall HSs; however, the addition of L-arg did not affect the P_4 production profile over time. The membrane integrity of CC was not different between the two controls, but the supplementation with L-arg increased integrity, thus ruling out the possibility that the increase in the number of viable cells after addition of HSs may be responsible for the increase in P_4 concentration observed following supplementation with L-arg.

There was difference in the concentration of E_2 between the two controls (1.3 times) ($P < 0.05$) at 22 hours of cultivation. More studies are needed to assess whether this decrease has any physiological significance or whether it was due to a change in the number and/or quality of follicular somatic cells in culture. These results showed that the presence of follicular wall and the supplementation with L-arg did not alter the concentration of E_2 in the culture medium. There may be a negative feedback control to keep E_2 within the concentration range observed in this experiment (14.8 ± 0.1–19.9 ± 0.6 ng/mL), or the oocyte synthesized a factor that stimulates the synthesis of estradiol transported to the follicle wall through gap junctions, which did not occur with the experimental model used in this study.

Given the observed stimulatory effect on the nuclear maturation of oocytes by the addition of 5.0-mM L-arg at 7 hours of cultivation, we evaluated whether L-arg at the same concentration affects the intracellular concentrations of cGMP and cAMP in COCs, as a pathway to stimulate the resumption and progression of meiosis. L-arginine (5.0 mM) increased the concentration of cGMP at 1 hour of cultivation in relation to control −ve. The usual pathway of NO activity is through the cGMP pathway of sGC enzyme activation. Thus, in the presence of the precursor of NO, the L-arg/NO/sGC/cGMP pathway remained active, ensuring higher concentrations of cGMP within the COCs and suggesting its participation in meiosis and progression to MI. Similar results on the profile of the concentration of cGMP in COCs in the first 6 hours of IVM were observed by

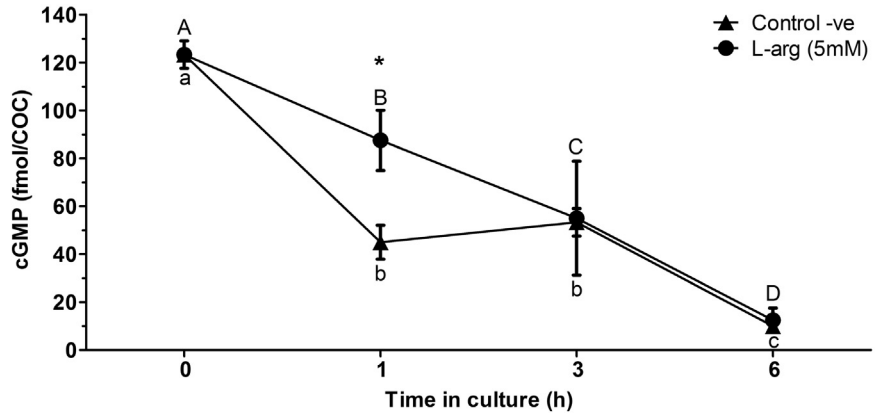

Fig. 4. Effects of the addiction of 5 mM of L-arg on the cGMP concentration (fmol/COC) in bovine COCs (n = 120, from a total of 840 COCs analyzed) cocultured with eight follicular wall HSs during different times of IVM. Values represent the means ± standard deviation of four replicates. The asterisk show significant difference between treatments only at 1 hour of culture ($P < 0.05$). Different lowercase letters denote significant differences between time in culture of control group ($P < 0.05$). Different uppercase letters denote significant differences between time in culture of L-arg group ($P < 0.05$). cGMP, cyclic guanosine monophosphate; COCs, cumulus-oocyte complexes; L-arg, L-arginine.

Schwarz et al. [11]; however, after adding 0.1 μM, SNP occurred a decreased rate of GVBD. Further studies are needed to evaluate the function of maintaining the highest concentration of cGMP compared with control −ve in COCs treated with L-arg or SNP, but triggering opposite results in the meiosis. Most membrane integrity of CC observed in the present study may have contributed to an increase in the concentration of cGMP observed at 1 hour of culture, providing an increased synthesis of cGMP and/or transportation of this nucleotide by gap junctions into the oocyte modulating the resumption of meiosis.

The cAMP concentration of COCs with or without L-arg supplementation exhibited a gradual increase after the first hour of cultivation, reaching a peak at 3 hours. The presence of gonadotropins, particularly FSH, in the maturation medium could have led to this result. Luciano et al. [51] observed that maturation medium supplemented with gonadotropins and/or fetal serum can affect cAMP intracellular concentrations during the first hours of cultivation. Follicle-stimulating hormone activates membrane receptors and G proteins of target cells, which induce the production of cAMP by activating adenylate cyclase [29,52]. The concentration of cAMP in treated COCs with 5-mM L-arg was similar to that of the control −ve under the cultivation conditions studied, demonstrating that the effect of L-arg in the resumption of meiosis does not occur through changes in the concentration of cAMP. Similar profile of the concentration of cGMP and cAMP in bovine COC was also observed by Schwarz et al. [11] after the addition of S-nitroso-*N*-acetylpenicillamine (NO donor) to the IVM medium, where the concentration of cGMP increased only by 1 hour of cultivation. These data suggest that the effect observed in this experiment the concentration of cGMP in COC after supplementation of the medium with 5.0-mM L-arg was caused by the metabolism of L-arg to NO.

It has been proposed that in response to the preovulatory LH peak there is a transient increase in cAMP

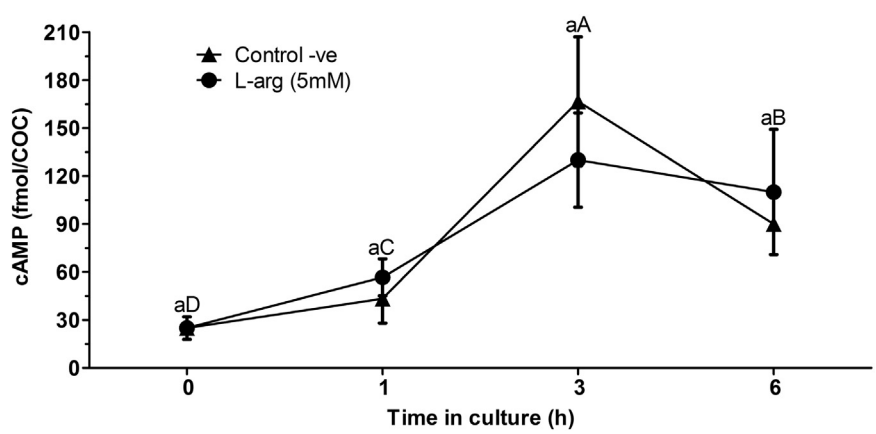

Fig. 5. Effects of the addiction of 5 mM of L-arg on the cAMP concentration (fmol/COC) in bovine COCs (n = 40, from a total of 280 COCs analyzed) cocultured with eight follicular wall HSs during different times of IVM. Values represent the means ± standard deviation of four replicates. Lowercase letters "a" denote no significant differences between groups ($P > 0.05$). Different uppercase letters denote significant differences between time in culture ($P < 0.05$). COCs, cumulus-oocyte complexes; L-arg, L-arginine.

production by granulosa cells and the communication between follicle cells and the oocyte is ended, consequently stopping the transfer of cAMP and cGMP to the oocyte [53]. This decrease in the concentration of cGMP leads to an increase in the hydrolyzing activity of phosphodiesterase 3A, which in turn increases the hydrolysis of cAMP in the oocyte. With this occurs a marked decrease in the intra-oocyte concentration of cAMP, allowing the resumption of meiosis [54]. In this sense, the observed changes in the concentrations of cGMP and cAMP in COCs treated with 5.0-mM L-arg in this experiment were different from those described by Tsafriri and Dekel [54] in rodents, suggesting that L-arg stimulates resumption of meiosis through different pathways in cattle in the presence of follicular wall HSs, not including the decrease in the concentration of cGMP with subsequent decrease of cAMP concentration in bovine COC. More studies are needed to measure the concentration of these nucleotides in the cumulus and oocyte cells to evaluate the profile of the concentration of the same and the role of each compartment on the IVM of oocytes.

There is evidence that arginine activates the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) signaling pathway as an essential mechanism in the immune response of the body's defense cells [55]. The increased activity of MAPK in the somatic cells of the follicle induces nuclear maturation. Mitogen-activated kinase protein activates other substances and phosphorylates proteins (connexin 43) of interconnecting bonds. This block of intercellular communication prevents the entry of inhibitory signals into the oocyte [56]. Under these conditions, L-arg could modulate the signaling pathways that lead to the resumption of meiosis. More studies are needed to evaluate this hypothesis.

In conclusion, our data show that, using this system that partially blocks meiosis, (1) the mechanisms that give the oocyte the ability to restart the meiotic until MI after adding 5.0-mM L-arg do not involve changes in the concentration of P_4 and E_2, nitrate/nitrite in the culture medium and (2) L-arg acts on a pathway that involves changing the cGMP concentration but does not involve changing cAMP concentration. More studies are needed to assess whether the observed effects of L-arg during IVM using this system are via NO or not and what the role is in increasing the viability of CC in the resumption and progression of meiosis until MI.

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Competing interests

None of the authors have any conflicts of interest to declare.

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