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Nitric oxide impacts bovine sperm capacitation in a cGMP-dependent and cGMP-independent manner

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Abstract

We aimed to elucidate whether NO acts in in vitro sperm capacitation in bovine via cGMP/PKG1 pathway. For this, cryopreserved bovine sperm were capacitated in vitro with 20 µg/ml heparin (Control) plus treatments: 1 mM L-arginine (L-arg, NO precursor), 50 µM Rp-8-Bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cGMPS, selective inhibitor of the binding site for cGMP in PKG1), 1 mM 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO, NO scavenger), and the combinations of L-arg + RP-8-Br-cGMPS and L-arg + PTIO. Sperm motility and vigour were determined by phase-contrast microscopy, capacitation status by chlortetracycline staining, and the intracellular concentration of cGMP was measured by ELISA. Data were subjected to analysis of variance and means compared with SNK test at 5% probability. Motility and vigour were lower in sperm treated with PTIO when compared to Control and other treatments ($p < .05$). The L-arg treatment showed the highest percentage of capacitated sperm when compared to the Control and other treatments (Rp-8-Br-cGMPS, L-arg + Rp-8-Br-cGMPS and PTIO) (69.8 ± 3.4%, 51.2 ± 3.0, 51.1 ± 2.1, 51.2 ± 3.0 and 45.5 ± 2.7, respectively) ($p < .05$). The capacitation ratio (%) was lower in treatments with Rp-8-Br-cGMPS, L-arg + Rp-8-Br-cGMPS and PTIO, respectively ($p < .05$). Lastly, cGMP concentration (pmol/ml) was lower in PTIO and L-arg + PTIO (1.3 ± 0.3 and 1.6 ± 0.4) and was higher in Rp-8-Br-cGMPS and L-arg + Rp-8-Br-cGMPS (3.7 ± 0.4 and 4.0 ± 0.5) treatments. We showed that during in vitro capacitation of cattle: (a) NO influences sperm motility and vigour; (b) NO is associated with cGMP synthesis through two independent pathways and (c) the cGMP/PKG1 pathway has a partial role in sperm capacitation and does not involve the L-arg/NO.

KEYWORDS
cattle, cyclic nucleotide, frozen/thawed sperm, L-arginine, signalling pathway, sperm capacitation

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1 | INTRODUCTION

Capacitation is a multifactorial event that primes sperm for fertilization. In vivo, this process is primarily influenced by the female reproductive tract, which affects biochemical and biophysical mechanisms in the sperm. As a result, the sperm is capable of fertilizing the egg (Bailey, 2010). Numerous signalling events and pathways are linked to sperm capacitation (Gangwar & Atreja, 2015), and thus, several substances in in vitro experiments have been reported to mechanistically impact this process. In bovine, heparin is the primary agent that induces sperm capacitation (Parrish, Susko-Parrish, Winer, & First, 1988). In addition, second messengers of cell signalling pathways, such as nitric oxide (NO), play a crucial role in the molecular events that lead to the capacitation (Gangwar & Atreja, 2015).

Previous studies of our and other groups demonstrated that the sperm capacitation requires the action of NO to occur in cattle in vitro (Ferreira-Berbari et al., 2010; Leal, Caldas-Bussiere, Carvalho, Viana, & Quirino, 2009; O'Flaherty, Rodriguez, & Srivastava, 2004). Although many signalling pathways have been extensively investigated during capacitation, the complete mechanism of action of NO still needs to be fully elucidated. In this manner, in vitro studies conducted with agents known to participate in the NO signalling pathway may contribute to a better understanding of NO-mediated sperm capacitation in cattle.

NO is a highly reactive molecule classified as a reactive nitrogen species (RNS) with a very short half-life (3–5 s) (Ignarro, 2000). In mammals, NO is synthesized by nitric oxide synthase (NOS), which converts arginine in citrulline and NO in the presence of oxygen and several cofactors (Schmidt, Pollock, Nakane, Forstermann, & Murad, 1992). During the sperm capacitation of cattle, NO plays a role on plasma membrane integrity, motility and vigour (Leal et al., 2009; Paes de Carvalho et al., 2003; Rodriguez, O'Flaherty, Beconi, & Beorlegui, 2005).

L-arginine (L-arg), a precursor of NO synthesis, has been used to study the role of NO in sperm capacitation and acrosome reaction. In bovine, it was observed that the concentration of 10 mM L-arg improved the parameters of motility, capacitation and acrosome reaction after 45 min (O'Flaherty et al., 2004) and 4 hr of incubation in capacitation medium (Leal et al., 2009), in frozen/thawed and fresh sperm preparations, respectively. An increase in nitrate/nitrite (NO_3^-/ NO_2^-) concentration led to NO metabolite synthesis in addition to an improvement in the percentage of oocytes penetrated by sperm (Leal et al., 2009). In vitro assays with cryopreserved sperm demonstrated that the addition of 1.0 mM L-arg to the capacitation medium increased the percentage of capacitated sperm and decreased the ratios of acrosome reaction (Leal, Caldas-Bussiere, Ohashi, Cordeiro, & Quirino, 2012).

It is known that classical NO signalling occurs through the activation of soluble guanylate cyclase (sGC), which stimulates the synthesis of cyclic guanosine monophosphate (cGMP) and results in the activation of specific kinases, such as cGMP-dependent protein kinases (PKGs) (Martínez-Ruiz, Cadenas, & Lamas, 2011). During capacitation, the PKG and the activation of cyclic nucleotide-gated (CNG) channel pathways are triggered by high cGMP concentrations. In consequence, CNG channels then permit the influx of Ca^{2+} in mouse sperm (Cisneros-Mejorado, Hernández-Soberanis, Islas-Carbajal, & Sánchez, 2014). Moreover, it has been shown that both PKG and CNG channels are part of the cGMP-induced signalling cascade that leads to sperm capacitation in mice (Cisneros-Mejorado et al., 2014; Cisneros-Mejorado & Herrera, 2012). However, this observation has not been proven in cattle.

The Rp-8-Bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cGMPS) was initially described as an antagonist of protein kinase G type I cGMP-dependent (PKG1) (Butt, Pöhler, Genieser, Huggins, & Bucher, 1995). PKG1 belongs to the serine/threonine family of proteins and represents one of the targets of cGMP in many tissues (Kawada, Toyosato, Islam, Yoshida, & Imai, 1997). In contrast, Rp-8-Br-cGMPS is a competitive inhibitor of cGMP due to its competition for the ion channels at the cGMP binding sites in the PKG1 (Wei, Cohen, Yan, Genieser, & Barnstable, 1996). In humans, PKG1 participates in the control of sperm motility (Miraglia et al., 2011). In this same study, the use of Rp-8-Br-cGMPS alone did not alter sperm motility but inhibited the beneficial effects on this parameter after treating sperm with S-nitrosoglutathione (GSNO) and 8-Br-cGMP, a NO donor and an analog of cGMP (Miraglia et al., 2011), respectively.

Therefore, it is possible that the NO synthesis from L-arg in bovine sperm may stimulate the production of cGMP, which in turn triggers the action of the PKGs leading to the capacitation of bovine sperm. Thus, this work aimed to elucidate whether NO impacts in vitro sperm capacitation in bovine via cGMP/PKG1 pathway.

2 | MATERIALS AND METHODS

2.1 | Reagents and experimental groups

All reagents used in this research were obtained from Sigma-Aldrich Brasil Ltda unless otherwise stated. The experiments were conducted with commercial cryopreserved sperm from three Nellore bulls (*Bos taurus indicus*) obtained from Alta Genetics. For this reason, no ethical approval was required.

Briefly, after thawed, we submitted the sperm to a Percoll separation and then we divided the specimen into different treatment groups, followed to an in vitro capacitation. The standard medium used in the capacitation was modified Tyrode's (sp-TALP) supplemented with 6 mg/ml BSA fatty acid-free, 100 IU/ml penicillin, 100 μg/ml streptomycin and 20 μg/ml heparin, used as a capacitation agent (Parrish et al., 1988). To conduct the experiments, we used L-arg as a NO precursor, PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide) as NO scavenger and Rp-8-Br-cGMPS as a competitive inhibitor of cGMP. Then, the described reagents were employed in the media as the following treatments: sperm capacitated with 1 mM L-arg (Leal et al., 2012; Maciel Jr et al., 2018); sperm capacitated with 50 μM Rp-8-Br-cGMPS (Maciel Jr et al., 2014); sperm capacitated with 1 mM PTIO; and the treatments that associate L-arg with Rp-8-Br-cGMPS and L-arg with PTIO. Moreover, a

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positive control of sperm capacitated with the concomitant addition of 5 mM 8-Br-cGMP (Leal et al., 2012) and 1 mM PTIO was used to determine cGMP concentration without L-arg. All evaluations used a negative control without adding any other agent. The adequate concentration of PTIO was found out in pre-tests in relation to capacitation status (Figure S1, Supplementary Material). The six treatments were Control, L-arg, PTIO, L-arg + PTIO, Rp-8-Br-cGMP and L-arg + Rp-8-Br-cGMP.

2.2 | Sperm handling and selection

Briefly, the sperm was thawed at 37°C for 30 s and centrifuged at 600 *g* for 15 min in a Percoll gradient 45/90% (Eid, Lorton, & Parrish, 1994) to select sperm with increased viability. The pellet was washed in 400 µl of wash-TALP medium (Chamberland et al., 2001) and centrifuged at 150 *g* for 5 min. Then, sperm motility, vigour and concentration were visually determined with the aid of a phase-contrast microscope (NIKON—Eclipse E200). Total motility and vigour were also characterized after 3 hr of culture. The sperm concentration was defined by cell counting in a Neubauer chamber.

The selected sperm were then transferred to the capacitation medium. The in vitro sperm capacitation was induced for 3 hr in 200 µl sp-TALP heparin without (Control) or with treatments (described in section 2.1) in an incubator at 38.5°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3 | Evaluation of sperm motility and vigour

The evaluation of sperm motility and vigour (characterized by the flagellar beating force that influences the motility) of the sperm was performed by a subjective method with the aid of phase-contrast microscopy (Nikon Eclipse E200—400 × magnification), according to the Brazilian College of Animal Reproduction (CBRA, 2013). Motility was classified as a percentage, and the vigour was estimated in a scale from zero to five, where zero represented the absence of lateral movement of the head and five with rapid and energetic movement of the tail beat. A total of 72 observations of four replicates from three different bulls were analysed.

After 1 hr in contact with the capacitation medium, the lateral region of the sperm head starts to aggregate and, after hours, it agglutinates in the medium. This process precludes the use of an automated method to determine motility since the software used for this analysis exclusively identifies a predefined sperm size. Thus, despite the sperm agglutination, it is still possible to visually evaluate the sperm features.

2.4 | Evaluation of capacitation pattern by chlortetracycline assay

To assess the percentage of sperm capacitation, we used the chlortetracycline staining technique (CTC), modified by Cormier, Sirard, and Bailey (1997). The stock solution of CTC (0.75 mM CTC, 20 mM Tris-base and 5 mM DL-cysteine) was prepared daily. This CTC solution was then mixed with 4% paraformaldehyde (4%) in 20 mM Tris-base and 0.22 M of 1,4-diazabicyclo octane—DABCO (Invitrogen Molecular Probe). The sperm suspension (25×10^6 sperm/ml) was stained and placed between a slide and a coverslip for epifluorescence microscope observation (Nikon Eclipse TE300—40× magnification, 440 nm excitation and 470 nm emission).

For each evaluation, at least 200 cells were counted and classified into three groups, as described (Fraser, 1995): F (fluorescent), acrosome intact sperm, fluorescing entire surface; C (capacitated), with loss of fluorescence in the post-acrosomal region; and RA (reacted acrosome), with loss of fluorescence in the post-acrosomal and acrosomal region, expressing fluorescence only in the midpiece.

2.5 | cGMP measurement

Previously to the analysis, a standardization of the extraction methodology was conducted, and the ideal sperm concentration was found. Cyclic GMP analysis was conducted according to Miraglia et al. (2011) and Osycka-Salut et al. (2014) with minor modifications.

First, aliquots containing 7×10^6 sperm cells/ml were pre-incubated for 3 min in sp-TALP (0.3% BSA) supplemented with 1 mM IBMX at 38.5°C. IBMX was used before the incubation with treatments as a phosphodiesterase (PDE) inhibitor to prevent the hydrolysis of cGMP, since some PDE isoforms are known to degrade nucleotides (Francis, Sekhar, Ke, & Corbin, 2011). Following, the samples were washed twice in 400 µl of wash-TALP medium and centrifuged at 150 *g* for 5 min to remove IBMX pre-treatment. Then, the sperm were capacitated for 3 hr in an incubator at 38.5°C in a humidified atmosphere of 95% air and 5% CO_2. After this period, the treatment-containing capacitation medium was removed. For this, the samples were centrifuged at 3,000 × *g* for 5 min, the supernatant was removed, and 1 ml of ice-cold absolute ethanol was added over the precipitate, followed by vortexing for 1 min. After complete ethanol evaporation, the pellet was resuspended in 50 mM Tris-HCl, pH 7.4 and 0.1% BSA and kept frozen (−20°C).

The intracellular levels of cGMP were found using the Cyclic GMP EIA Kit (Cayman). For this, three replicates of three bulls were used and samples were submitted to evaluation without the acetylation step, and the methodology of analysis was conducted according to the manufacturer protocol. Finally, the levels of the cGMP were determined using a spectrophotometer (ELISA reader, BioTek) at 405 nm.

To our knowledge, it is the first time that ELISA methodology is employed for cGMP measurement in sperm. To this analysis, the seven treatments were Control, L-arg, PTIO, L-arg + PTIO, Rp-8-Br-cGMP, 8-Br-cGMP + PTIO and L-arg + Rp-8-Br-cGMP.

2.6 | Statistical analysis

The data were tested for the assumptions of the Newman–Keuls test as normality and homogeneity of variance. Motility, vigour, reacted acrosome (%), acrosome intact (%), capacitated (%) and the levels of cGMP were subjected to analysis of variance with repeated

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measures in a mixed model (Proc MIXED) to determine the effects of treatments in all evaluations. LSmeans ± *SEM* were compared by the Tukey's test at 5% probability (SAS, 2011). The data that support the findings of this study are available from the corresponding author upon reasonable request.

3 | RESULTS

When we compared the different capacitation treatments and Control, we found that the sperm motility was reduced in PTIO treatment ($p < .05$), except when compared to the Control and L-arg + PTIO. For vigour, the treatments PTIO and RP-8-Br-cGMPS showed a lower ratio than the treatment with L-arg ($p < .05$) (Table 1).

L-arg showed the highest capacitation ratio and the lowest non-capacitated and acrosome-reacted ratio when compared to the Control and other treatments ($p < .05$). When compared to the Control, L-arg increased the capacitated sperm by 17.4% and decreased the intact acrosome by 52.1% and the reacted acrosome by 17.5% ($p < .05$) (Figure 1).

In contrast to our findings with the treatments with L-arg, the NO scavenger showed the lowest capacitation ratio (45.5 ± 0.9%) and the highest reacted acrosome ratio (32.9 ± 1.0%) ($p < .05$). Moreover, when compared to the Control, PTIO showed an increase ($p < .05$) in the intact acrosome ratio (21.7 ± 0.7% vs. 13.9 ± 0.5%). Nevertheless, L-arg partially reverted the effects of PTIO on the capacitated and intact sperm, but totally reverted the effect on the reacted acrosome ratio ($p < .05$).

Treatment with Rp-8-Br-cGMPS associated or not with L-arg decreased the capacitation ratio (51.2 ± 0.7% and 51.1 ± 0.7%) and increased the intact acrosome ratio (21.2 ± 0.6% and 22.4 ± 0.6%) when compared to the Control ($p < .05$). In this case, L-arg, when associated with the PKG1 inhibitor, could not revert its effect during in vitro capacitation ($p > .05$).

The intracellular measurement of cGMP concentration after in vitro capacitation showed that the ratio of this nucleotide (pmol/ml) was not different between the Control and L-arg treatment (2.2 ± 0.1 and 2.3 ± 0.2, $p > .05$). We also found that cGMP concentration was lower ($p < .05$) in PTIO (1.3 ± 0.3), but when L-arg was added, it could restore cGMP concentration (L-arg + PTIO = 1.6 ± 0.2) when compared to the Control and L-arg. However, cGMP was higher ($p < .05$) in Rp-8-Br-cGMPS and L-arg + Rp-8-Br-cGMPS (3.7 ± 0.2 and 4.0 ± 0.2), when compared to the Control and L-arg treatment. The positive control had the highest cGMP concentration (8-Br-cGMP + PTIO = 4.9 ± 0.4) when compared to all treatments and the Control ($p < .05$), except when compared to L-arg + RP-8-Br-cGMPS (Figure 2).

TABLE 1 LSmeans and standard error of the motility and vigour of frozen/thawed bovine sperm

Treatment	Motility (%)	Vigour (1-5)
Control	47.5 ± 0.2^{ab}	2.3 ± 0.1^{ab}
L-arg	56.3 ± 0.2^{a}	2.5 ± 0.1^{a}
Rp-8-Br-cGMPS	51.7 ± 0.2^{a}	1.9 ± 0.1^{b}
L-arg + Rp−8-Br-cGMPS	51.1 ± 0.2^{a}	2.2 ± 0.1^{ab}
PTIO	35.0 ± 0.4^{b}	1.8 ± 0.2^{b}
L-arg + PTIO	43.3 ± 0.4^{ab}	2.0 ± 0.2^{ab}
8-Br-cGMP + PTIO	55.0 ± 0.4^{a}	2.7 ± 0.2^{ab}

Note: Evaluation results by optical microscopy after 3 hr of in vitro capacitation. LSmeans with different letters in the same column are significantly different with Tukey's test ($p < .05$). Treatments: L-arg (NO precursor), PTIO (NO scavenger), Rp-8-Br-cGMPS (selective inhibitor of the binding site for cGMP in PKG1) and associations.

4 | DISCUSSION

In the present study, we demonstrated that single treatments with PTIO (NO scavenger) decreased the percentage of sperm motility and vigour after 3 hr of in vitro capacitation, and the addition of L-arg to PTIO kept these parameters similar to Control, possibly by the NO synthesis. These results corroborate with the statement that NO action is required for sperm motility (Herrero & Gagnon, 2001; Leal et al., 2009). However, other signalling pathways in addition to NO pathway may influence sperm motility, since we did not observe a total inhibition of sperm motility and vigour with the NO scavenger treatment.

The RNS, such as NO, are produced in the sperm mainly by regular metabolic activity (de Lamirande, Jiang, Zini, Kodama, & Gagnon, 1997). Although excessive synthesis of NO may lead to oxidative stress (De Castro et al., 2016; Morielli & O'Flaherty, 2015), low concentrations may be beneficial to sperm, contributing to sperm motility and signal transduction mechanisms during the sperm capacitation (de Lamirande et al., 1997; O'Flaherty, De Lamirande, & Gagnon, 2006). This dual effect was reported for NO during the sperm capacitation in several studies (Leal et al., 2009; Miraglia et al., 2011; Wang, He, Yan, Cai, & Chen, 2014).

No differences were found in the motility and vigour using Rp-8-Br-cGMPS as a selective inhibitor of cGMP/PKG1 pathway, even in association with L-arg. In humans, Miraglia et al. (2007) also did not observe differences in the sperm motility of the treatment with Rp-8-Br-cGMPS (10 μM) when compared to Control. Moreover, in accordance with our findings, Miraglia et al. (2011) evaluated the kinetics of sperm motility of different treatments after 30, 60 and 90 min, and found that Rp-8-Br-cGMPS alone (10 μM) could not alter motility when compared to controls. However, in the same study, a fivefold lower dose of Rp-8-Br-cGMPS has been shown to be effective in inhibiting the beneficial action of GSNO (NO donor, 5 μM) and 8-Br-cGMP (cGMP analog, 500 μM) on the motility of human sperm (Miraglia et al., 2011). This may occur because the NO donor used by these authors stimulated the NO/sGC/cGMP pathway, while in the present study we used a precursor (L-arg) that can act or not via NO.

According to our findings, the treatment with PTIO decreased the capacitation status of the sperm, but L-arg partially reverted the scavenging effect of PTIO, possibly through the generation of NO.

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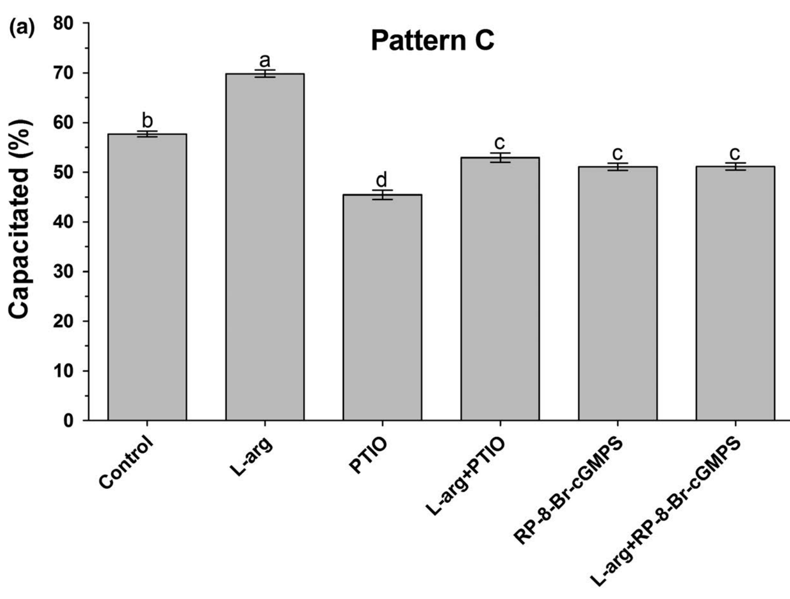

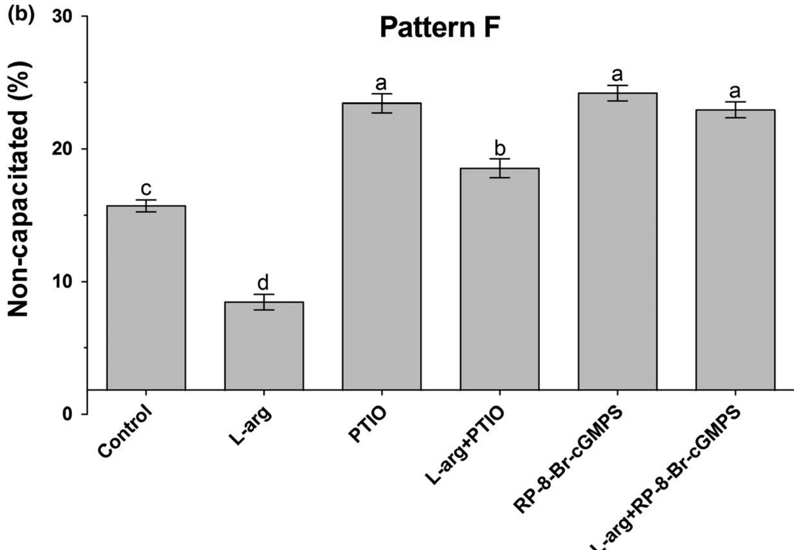

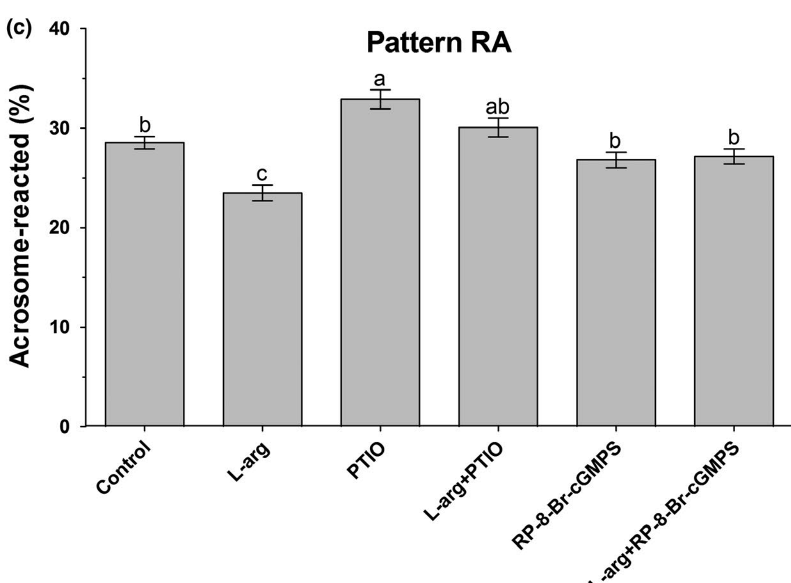

FIGURE 1 Capacitation status assessed by CTC staining between Control and treatments with L-arg, PTIO, Rp-8-Br-cGMPS and their associations during in vitro sperm capacitation in cattle. (a) Pattern C: percentage of capacitated sperm; (b) Pattern F: percentage of acrosome intact sperm; and (c) Pattern RA: percentage of sperm with reacted acrosome. LSmeans with different letters on the same parameter indicate statistical differences according to the Tukey's test ($p < .05$)

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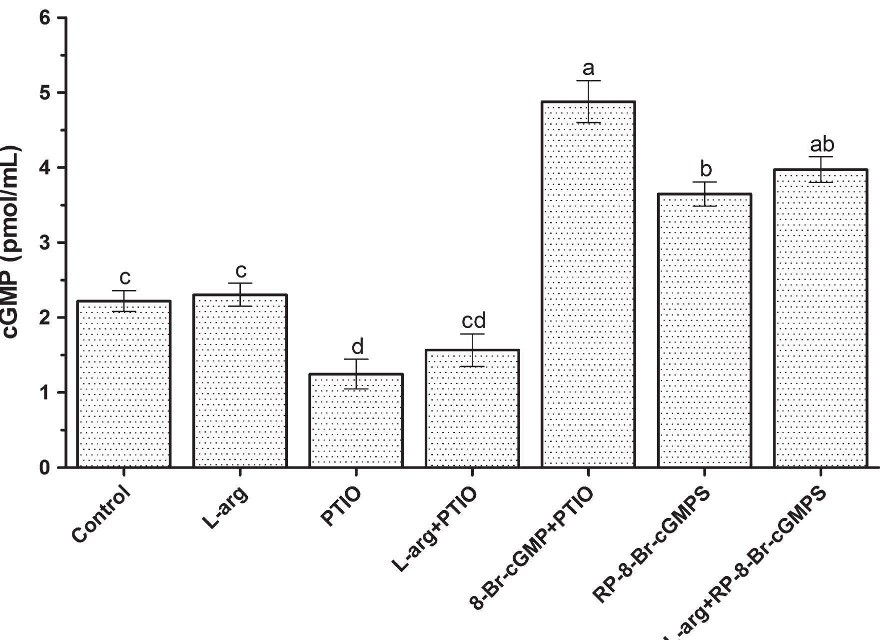

FIGURE 2 Intracellular concentration of cGMP (pmol/ml) after in vitro sperm capacitation in cattle. Different letters indicate statistical differences between treatments according to the Tukey's test ($p < .05$). Treatments: L-arg (NO precursor), PTIO (NO scavenger), Rp-8-Br-cGMPS (selective inhibitor of the binding site for cGMP in PKG1) and associations

L-arg, a natural precursor of NO synthesis, has been used in the in vitro capacitation of cattle (Leal et al., 2009, 2012; O'Flaherty et al., 2004; Rodriguez et al., 2005), buffaloes (Jagan Mohanarao & Atreja, 2012), pigs (Funahashi, 2002) and humans (de Lamirande & Lamothe, 2009). Although its action is attributed to NO, a distinct NO-independent L-arg pathway may be active during capacitation, such as the polyamine synthesis (Racke & Warnken, 2010). Thus, more studies are needed to determine whether L-arg impacts capacitation by regulating other downstream pathways.

Treatments with Rp-8-Br-cGMPS exhibited reduced ratio of capacitated sperm (11.5%) compared to Control. Moreover, when L-arg was added to the Rp-8-Br-cGMPS treatment, we did not observe an increase in the capacitation ratios. Nevertheless, in both cases (*i.e.* when PTIO and Rp-8-Br-cGMPS were used), almost 50% of sperm could achieve capacitation. In this way, one can speculate that although both signalling pathways are active, these pathways promote partial responses and are unlikely unique during sperm capacitation in bovine. Thus, there are other pathways activated by cGMP that were not investigated in this study and may also be active in promoting capacitation (*e.g.* cyclic nucleotide-gated [CNG] channels) (Cisneros-Mejorado & Herrera, 2012).

Post-translational protein modifications are activated by RNS during sperm capacitation to control cellular processes (O'Flaherty & Matsushita-Fournier, 2017). Protein phosphorylation at tyrosine residues occurs during the capacitation process (Roy & Atreja, 2008; Visconti, Krapf, de la Vega-Beltrán, Acevedo, & Darszon, 2011). S-nitrosylation of proteins is influenced by NO independently of cGMP and is temporally and spatially selective, resulting in cell signalling regulation (Lefièvre et al., 2007). It is possible that when L-arg was used in the treatments, it may have led to NO synthesis which in turn led to the phosphorylation (Jagan Mohanarao & Atreja, 2012), tyrosine nitration (Eve Herrero, de Lamirande, & Gagnon, 2001; de Lamirande, Lamothe, & Villemure, 2009) and S-nitrosylation (Lefièvre et al., 2007) of proteins that strongly influence sperm capacitation. Recently, we showed that culture medium supplemented with 1.0 mM L-arg promoted a differential protein abundance pattern that significantly associated with an increased frozen–thawed sperm quality and percentage of capacitated bovine sperm. Thus, proteomic changes may be linked to the molecular mechanisms involved in the action of L-arg on the in vitro sperm capacitation of cattle (Maciel Jr et al., 2018).

We further hypothesized that L-arg leads to NO synthesis, as previously demonstrated by Leal et al. (2009), which in turn could lead to an increase in cGMP production during in vitro sperm capacitation of cattle. It is already established in other cell types that the action of NO on signalling occurs mainly via cGMP (Martínez-Ruiz et al., 2011). For this reason, we standardized the cGMP measurement to elucidate whether after the addition of L-arg to the capacitation medium, an increase in cGMP synthesis would occur. To the best of our knowledge, this is the first time that cGMP was measured in bovine sperm.

Thus, we found that L-arg treatment does not stimulate the cGMP synthesis, as compared to Control. However, when PTIO was used, the cGMP concentration decreased, suggesting that NO is needed to stimulate cGMP synthesis. These data demonstrate that NO has another different source than L-arg to act in the NO/cGMP pathway. A non-enzymatic pathway for NO generation from nitrate/nitrite may be active in sperm, as previously demonstrated in the digestive and cardiovascular systems (Lundberg, Weitzberg, & Gladwin, 2008; McKnight et al., 1997). This alternative pathway might serve as a backup system to ensure NO-like bioactivity when the endogenous L-arg/NO synthase pathway is dysfunctional or when oxygen availability is reduced (Lundberg et al., 2008).

Using the 5 mM of the analog of cGMP with NO scavenger, the concentration of this nucleotide was highest, suggesting that the sperm membranes are permeable to cGMP. We can further

FIGURE 3 Schematic representation of L-arg/NO/cGMP signalling pathway in bovine sperm during in vitro capacitation. The compounds used in this study are named beside their targets. The PTIO is a NO scavenger, and Rp-8-Br-cGMPS is a selective inhibitor of the binding site for cGMP in PKG1

speculate that this concentration was responsible for increasing the intracellular concentration of cGMP by approximately 3.6 pmol/ml when compared to the treatment only with PTIO. This result is essential for future studies that aim to evaluate the action of this nucleotide during bovine sperm capacitation. Finally, using Rp-8-Br-cGMPS, we found that cGMP concentration was higher than Control. The Rp-8-Br-cGMPS is selectively competing for the cGMP binding sites, thus allowing cGMP to remain unbound in the intracellular environment, increasing its concentration in the cytosol. Moreover, Butt et al. (1995) demonstrated that the Rp-8-Br-cGMP does not hydrolyse the cGMP-specific phosphodiesterase (PDE) type V, leading to high cGMP concentrations. Further studies are needed to prove the presence of PDE V in bovine sperm and whether the use of Rp-8-Br-cGMP may also inhibit it, as shown in human platelets.

Cyclic nucleotide-gated channels, composed of two subunits (alpha and beta), are present in the flagellum of mammalian sperm and represent important elements of cGMP and cAMP signalling pathways (Wiesner et al., 1998). It was shown in mouse sperm that CNG channels are more sensitive to cGMP and represent another target of cGMP during sperm capacitation, regulating the Ca^{2+} entry (Cisneros-Mejorado & Herrera, 2012), thus establishing a relationship between cGMP signalling pathway and increased motility.

A scheme for L-arg/NO/cGMP signalling pathway is shown in Figure 3. First, L-arginine is metabolized in L-citrulline and NO by the action of nitric oxide synthase enzymes. NO, in turn, activates soluble guanylate cyclase to generate cGMP, which has as one of its intracellular targets the PKG. Another hypothesis is the NOS-independent NO synthesis, from nitrate/nitrite (NO_3^-/NO_2^-).

In summary, our findings suggest that during in vitro sperm capacitation of cattle: (a) NO acts on sperm motility and vigour; (b) L-arg did not lead to an increased cGMP concentration; however, (c) NO promotes cGMP synthesis through two independent pathways; and (d) cGMP/PKG1 has a partial role in sperm capacitation and does not involve the L-arg/NO.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

V.L. Maciel Jr. conducted the experiment with the collaboration of D.F. Dubeibe, C.S. Paes de Carvalho and A.C.M.S. Leal. C.R. Quirino conducted the statistical analysis, and M.C. Caldas-Bussiere is the senior author. All the authors participated in the experimental design and preparation of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Bailey, J. L. (2010). Factors regulating sperm capacitation. *Systems Biology in Reproductive Medicine*, *56*(5), 334–348. https://doi.org/10.3109/19396368.2010.512377

Butt, E., Pöhler, D., Genieser, H.-G., Huggins, J. P., & Bucher, B. (1995). Inhibition of cyclic GMP-dependent protein kinase-mediated effects by (Rp)-8-bromo-PET-cyclic GMPS. *British Journal of Pharmacology*, *116*(8), 3110–3116. https://doi.org/10.1111/j.1476-5381.1995.tb15112.x

CBRA (2013). *Manual para exame andrológico e avaliação se sêmen animal*, (3rd ed.). Belo Horizonte, Brazil: Colégio Brasileiro de Reprodução Animal.

14390531, 2019, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/rda.13570 by UENF - Universidade do Norte Fluminense Darcy Ribeiro, Wiley Online Library on [09/04/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Chamberland, A., Fournier, V., Tardif, S., Sirard, M. A., Sullivan, R., & Bailey, J. L. (2001). The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. *Theriogenology*, *55*(3), 823–835. https://doi.org/10.1016/S0093-691X(01)00446-0

Cisneros-Mejorado, A., Hernández-Soberanis, L., Islas-Carbajal, M. C., & Sánchez, D. (2014). Capacitation and Ca2+ influx in spermatozoa: Role of CNG channels and protein kinase G. *Andrology*, *2*(1), 145–154. https://doi.org/10.1111/j.2047-2927.2013.00169.x

Cisneros-Mejorado, A., & Herrera, D. P. S. (2012). CGMP and cyclic nucleotide-gated channels participate in mouse sperm capacitation. *FEBS Letters*, *586*(2), 149–153. https://doi.org/10.1016/j.febslet.2011.12.006

Cormier, N., Sirard, M. A., & Bailey, J. L. (1997). Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *Journal of Andrology*, *18*(4), 461–468. https://doi.org/10.1002/j.1939-4640.1997.tb01953.x

de Castro, L. S., de Assis, P. M., Siqueira, A. F. P., Hamilton, T. R. S., Mendes, C. M., Losano, J. D. A., ... Assumpção, M. E. O. A. (2016). Sperm oxidative stress is detrimental to embryo development: A dose-dependent study model and a new and more sensitive oxidative status evaluation. *Oxidative Medicine and Cellular Longevity*, *2016*, 1–12. https://doi.org/10.1155/2016/8213071

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., & Gagnon, C. (1997). Reactive oxygen species and sperm physiology. *Reviews of Reproduction*, *2*, 48–54. https://doi.org/10.1530/ror.0.0020048

de Lamirande, E., & Lamothe, G. (2009). Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. *Free Radical Biology and Medicine*, *46*(4), 502–510. https://doi.org/10.1016/j.freeradbiomed.2008.11.004

de Lamirande, E., Lamothe, G., & Villemure, M. (2009). Control of superoxide and nitric oxide formation during human sperm capacitation. *Free Radical Biology and Medicine*, *46*(10), 1420–1427. https://doi.org/10.1016/j.freeradbiomed.2009.02.022

Eid, L. N., Lorton, S. P., & Parrish, J. J. (1994). Paternal influence on S-phase in the first cell cycle of the bovine embryo. *Biology of Reproduction*, *51*(6), 1232–1237. https://doi.org/10.1095/biolreprod51.6.1232

Ferreira-Berbari, J., Caldas-Bussiere, M. C., Paes de Carvalho, C. S., Viana, K. S., Leal, A., & Quirino, C. R. (2010). Efeito da inibição da óxido nítrico sintase induzível na capacitação in vitro de espermatozoides bovinos. *Arquivo Brasileiro De Medicina Veterinaria E Zootecnia*, *62*(3), 511–520. https://doi.org/10.1590/S0102-09352010000300003

Francis, S. H., Sekhar, K. R., Ke, H., & Corbin, J. D. (2011). Inhibition of cyclic nucleotide phosphodiesterases by methylxanthines and related compounds. In B. B., Fredholm (Ed.), *Handbook of Experimental Pharmacology*, (vol: *200*, pp. 93–103). Berlin, Germany: Springer

Fraser, L. (1995). Ionic control of sperm function. *Reproduction, Fertility and Development*, *7*(4), 905–925. https://doi.org/10.1071/RD9950905

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*, https://doi.org/10.1530/reprod/124.6.857

Gangwar, D. K., & Atreja, S. K. (2015). Signalling events and associated pathways related to the mammalian sperm capacitation. *Reproduction in Domestic Animals*, *50*(5), 705–711. https://doi.org/10.1111/rda.12541

Herrero, M. B., de Lamirande, E., & Gagnon, C. (2001). Tyrosine nitration in human spermatozoa: A physiological function of peroxynitrite, the reaction product of nitric oxide and superoxide. *Molecular Human Reproduction*, *7*(10), 913–921. https://doi.org/10.1093/molehr/7.10.913

Herrero, M. B., & Gagnon, C. (2001). Nitric oxide: A novel mediator of sperm function. *Journal of Andrology*, *22*(3), 349–356. https://doi.org/10.1002/j.1939-4640.2001.tb02188.x

Ignarro, L. J. (2000). *Nitric oxide: biology and pathobiology. Nitric Oxide*. Retrieved from http://books.google.co.jp/books?xml:id=h5FugARr4bgC%5Cnhttp://books.google.com/books?hl=en&lr=&xml:id=h5FugARr4bgC&oi=fnd&pg=PP2&dq=Nitric+Oxide.+Biology+and+Pathobiology&ots=dWCNGZq6Kg&sig=vko-v6mrUju53TL7jtSYxcm4Uwc%5Cnhttp://books.googl

Jagan Mohanarao, G., & Atreja, S. K. (2012). Identification of NO induced and capacitation associated tyrosine phosphoproteins in buffalo (Bubalus bubalis) spermatozoa. *Research in Veterinary Science*, *93*(2), 618–623. https://doi.org/10.1016/j.rvsc.2011.09.017

Kawada, T., Toyosato, A., Islam, M. O., Yoshida, Y., & Imai, S. (1997). cGMP-kinase mediates cGMP- and cAMP-induced Ca2+ desensitization of skinned rat artery. *European Journal of Pharmacology*, *323*(1), 75–82. https://doi.org/10.1016/S0014-2999(97)00028-9

Leal, A. C. M. S., Caldas-Bussiere, M. C., de Carvalho, C. S. P., Viana, K. S., & Quirino, C. R. (2009). Role of nitric oxide on quality of freshly ejaculated bull spermatozoa during heparin-induced in vitro capacitation. *Animal Reproduction Science*, *116*(1–2), 38–49. https://doi.org/10.1016/j.anireprosci.2008.12.020

Leal, A. C. M. S., Caldas-Bussiere, M. C., Ohashi, O. M., Cordeiro, M. S., & Quirino, C. (2012). *Assessment of bovine sperm capacitation pathway l-arginine/no/gmpc through in vitro embryo production. In Proceedings of the 17th International Congress on Animal Reproduction (ICAR)* (Vol. *47*, pp. 586–586). Reprod Domest Anim. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.1439-0531.2012.02119.x/abstract.

Lefièvre, L., Chen, Y., Conner, S. J., Scott, J. L., Publicover, S. J., Ford, W. C. L., & Barratt, C. L. R. (2007). Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics*, *7*(17), 3066–3084. https://doi.org/10.1002/pmic.200700254

Lundberg, J. O., Weitzberg, E., & Gladwin, M. T. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery*, https://doi.org/10.1038/nrd2466

Maciel, V. L. Jr, Caldas-Bussiere, M. C., Paes de Carvalho, C. S., Dubeibe, D. F., Quirino, C. R. (2014). Characterization of the L-arg/PKG1/cGMP signaling pathway in the in vitro capacitation of bovine spermatozoa. In: Proceedings of the 28th Annual Meeting of the Brazilian Embryo Technology Society (SBTE). Natal, RN, p. 308.

Maciel, V. L. Jr, Caldas-Bussiere, M. C., Silveira, V., Reis, R. S., Rios, A. F. L., & Paes de Carvalho, C. S. (2018). L-arginine alters the proteome of frozen-thawed bovine sperm during in vitro capacitation. *Theriogenology*, *119*, 1–9. https://doi.org/10.1016/j.theriogenology.2018.06.018

Martínez-Ruiz, A., Cadenas, S., & Lamas, S. (2011). Nitric oxide signaling: Classical, less classical, and nonclassical mechanisms. *Free Radical Biology and Medicine*, *51*(1), 17–29. https://doi.org/10.1016/j.freeradbiomed.2011.04.010

McKnight, G. M., Smith, L. M., Drummond, R. S., Duncan, C. W., Golden, M., & Benjamin, N. (1997). Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut*, *40*(2), 211–214. https://doi.org/10.1136/gut.40.2.211

Miraglia, E., De Angelis, F., Gazzano, E., Hassanpour, H., Bertagna, A., Aldieri, E., ... Ghigo, D. (2011). Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. *Reproduction*, *141*(1), 47–54. https://doi.org/10.1530/REP-10-0151

Miraglia, E., Rullo, M. L., Bosia, A., Massobrio, M., Revelli, A., & Ghigo, D. (2007). Stimulation of the nitric oxide/cyclic guanosine monophosphate signaling pathway elicits human sperm chemotaxis in vitro. *Fertility and Sterility*, *87*(5), 1059–1063. https://doi.org/10.1016/j.fertnstert.2006.07.1540

14390531, 2019, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/rda.13570 by UENF - Universidade do Norte Fluminense Darcy Ribeiro, Wiley Online Library on [09/04/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Morielli, T., & O'Flaherty, C. (2015). Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. *Reproduction*, *149*(1), 113–123. https://doi.org/10.1530/REP-14-0240

O'Flaherty, C., De Lamirande, E., & Gagnon, C. (2006). Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. *Free Radical Biology and Medicine*, *40*(6), 1045–1055. https://doi.org/10.1016/j.freeradbiomed.2005.10.055

O'Flaherty, C., & Matsushita-Fournier, D. (2017). Reactive oxygen species and protein modifications in spermatozoa. *Biology of Reproduction*, *97*(4), https://doi.org/10.1093/biolre/iox104

O'Flaherty, C., Rodriguez, P., & Srivastava, S. (2004). L-Arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. *Biochimica Et Biophysica Acta-General Subjects*, *1674*(2), 215–221. https://doi.org/10.1016/j.bbagen.2004.06.020

Osycka-Salut, C., Diez, F., Burdet, J., Gervasi, M. G., Franchi, A., Bianciotti, L. G., … Perez-Martinez, S. (2014). Cyclic AMP efflux, via MRPs and A1 adenosine receptors, is critical for bovine sperm capacitation. *Molecular Human Reproduction*, *20*, 89–99. https://10.1093/molehr/gat053

Paes de Carvalho, C. S., Caldas-Bussiere, M. C., Ueno, V. G., Carvalho, F. P., Matta, S. G. C., & Quirino, C. (2003). Efeito do óxido nítrico na capacitação in vitro de espermatozóides de bovinos da raça Nelore. In *Anais do Congresso de Integração em Biologia da Reprodução* (pp. 216–217). Ribeirão Preto, Brazil: Acta Scientiae Veterinariae.

Parrish, J. J., Susko-Parrish, J., Winer, M. A., & First, N. L. (1988). Capacitation of bovine sperm by heparin. *Biology of Reproduction*, *38*(5), 1171–1180. https://doi.org/10.1095/biolreprod38.5.1171

Racke, K., & Warnken, M. (2010). L-Arginine metabolic pathways. *The Open Nitric Oxide Journal*, *49*, 9–19.

Rodriguez, P. C., O'Flaherty, C. M., Beconi, M. T., & Beorlegui, N. B. (2005). Nitric oxide-induced capacitation of cryopreserved bull spermatozoa and assessment of participating regulatory pathways. *Animal Reproduction Science*, *85*(3–4), 231–242. https://doi.org/10.1016/j.anireprosci.2004.05.018

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. *International Journal of Andrology*, *31*(1), 12–24. https://doi.org/10.1111/j.1365-2605.2007.00745.x

SAS (2011). *SAS/STAT 9.3 User's Guide. User's Guide*. Cary, NC; SAS Institute Inc. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Sas/stat+9.3#1

Schmidt, H. H., Pollock, J. S., Nakane, M., Forstermann, U., & Murad, F. (1992). Ca2+/calmodulin-regulated nitric oxide synthases. *Cell Calcium*, *13*(6–7), 427–434. https://doi.org/10.1016/0143-4160(92)90055-W

Visconti, P. E., Krapf, D., de la Vega-Beltrán, J. L., Acevedo, J. J., & Darszon, A. (2011). Ion channels, phosphorylation and mammalian sperm capacitation. *Asian Journal of Andrology*, *13*(3), 395–405. https://doi.org/10.1038/aja.2010.69

Wang, J., He, Q., Yan, X., Cai, Y., & Chen, J. (2014). Effect of exogenous nitric oxide on sperm motility in vitro. *Biological Research*, *47*(1), https://doi.org/10.1186/0717-6287-47-44

Wei, J. Y., Cohen, E. D., Yan, Y. Y., Genieser, H. G., & Barnstable, C. J. (1996). Identification of competitive antagonists of the rod photoreceptor cGMP- gated cation channel: β-phenyl-1, N2-etheno-substituted cGMP analogues as probes of the cGMP-binding site. *Biochemistry*, *35*(51), 16815–16823. https://doi.org/10.1021/bi961763v

Wiesner, B., Weiner, J., Middendorff, R., Hagen, V., Kaupp, U. B., & Weyand, I. (1998). Cyclic nucleotide-gated channels on the flagellum control Ca2+ entry into sperm. *Journal of Cell Biology*, *142*(2), 473–484. https://doi.org/10.1083/jcb.142.2.473

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