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Pre-IVM treatment with C-type natriuretic peptide in the presence of cysteamine enhances bovine oocytes antioxidant defense ability and developmental competence *in vitro*

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Abstract

Background: The use of C-type natriuretic peptide (CNP) combined with cysteamine during pre-*in vitro* maturation (IVM) can help establish an effective pre-IVM system. **Aims:** This study was designed to investigate the effects of CNP combined with cysteamine on meiotic maturation and developmental competence of bovine oocytes *in vitro*. **Methods:** Oocytes were cultured in pre-IVM medium supplemented with 100 nM CNP for 6 h in the absence or presence of 100 µM cysteamine, followed by standard IVM for 24 h. Oocytes matured by standard IVM for 24 h (no pre-IVM) were used as the control. The percentage of oocytes arrested at germinal vesicle (GV) stage in each treatment group was examined at 0 h of IVM, and the nuclear maturation rate was assessed by evaluating the percentages of oocytes that reached metaphase II (M II) stage at 24 h of IVM. After 24 h of IVM, oocytes were subjected to *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC). Cleavage rates were assessed 48 h post-insemination. Blastocyst production rates were recorded on day 8 after IVF. In addition, intra-oocyte glutathione (GSH) and reactive oxygen species (ROS) content for each treatment were tested at 0 h and 24 h of IVM. **Results:** No differences were observed between the CNP pre-IVM treatment and control groups in the rate of bovine oocytes maturing to the M II stage, cleavage and blastocysts production rates after IVF, and intra-oocyte GSH levels. Notably, the presence of cysteamine during pre-IVM culture with CNP significantly improved the rate of embryos developed to the blastocyst stage after *in vitro* maturation and fertilization, moreover, it increased the levels of GSH and reduced the levels of ROS in bovine oocytes. **Conclusion:** The improvement to IVM bovine oocyte developmental competence through pre-IVM with CNP combined with cysteamine may be associated with an increased antioxidant defense. Therefore, such an approach may be a good option for establishing a pre-IVM system.

Key words: Bovine, CNP, Cysteamine, Developmental competence, Oocyte

Introduction

It is commonly accepted that the developmental competence of *in vitro* matured bovine oocytes is compromised in comparison to that of their *in vivo* counterparts, which may be in part due to insufficient cytoplasmic maturity (Rizos *et al*., 2002; Sutton *et al*., 2003). To improve the developmental competence of *in vitro* matured bovine oocytes, extensive studies have focused on the development of oocyte *in vitro* maturation (IVM) culture systems using various pharmacological cyclic adenosine monophosphate (cAMP) modulators (Sato *et al*., 1990; Guixue *et al*., 2001; Mayes *et al*., 2002; Albuz *et al*., 2010; Zeng *et al*., 2014; Farghaly *et al*., 2015). Notably, recent studies have shown that C-type natriuretic peptide (CNP; also known as NPPC), could temporarily sustain the meiotic arrest of bovine oocytes cultured *in vitro* for 6-8 h through sustaining sufficient levels of cAMP (Franciosi *et al*., 2014; Soto-Heras *et al*., 2019). Moreover, pre-IVM with 100 nM CNP for 6 h increased the number of cells per blastocyst compared with standard IVM protocol (no pre-IVM), suggesting that CNP as a cAMP modulator during pre-IVM culture exerts positive effects on oocyte developmental competence (Franciosi e*t al*., 2014). A recent study has also showed that pre-IVM using cAMP modulator [forskolin (FSK) + non-specific phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX)] delays meiotic resumption, and facilitates cumulus cell (CCs) transfer and accumulation of glutathione (GSH) within the oocyte during pre-IVM and IVM (Li *et al*., 2016). However, whether pre-IVM treatment with CNP leads to increased GSH accumulation in the bovine oocyte remains unclear.

Previous studies have shown that GSH synthesis can be stimulated by the addition of low-molecular-weight thiol compounds during the IVM of oocytes (Takahashi *et al*., 1993; de Matos *et al*., 2000; Zhou *et al*., 2008). Cysteamine is a low-molecular-weight thiol that increases the intra-oocyte GSH levels and enhances oocyte developmental competence in different species when present during IVM (Grupen *et al*., 1995; de Matos *et al*., 2002; Izumi *et al*., 2013). Additionally, previous studies with adult bovine oocytes have shown that the addition of 100 µM cysteamine to the culture medium during IVM significantly improves the percentage of the embryos' development to the blastocyst stage compared to the control group (de Matos *et al*., 2002; Merton *et al*., 2013). However, it is unknown whether the presence of cysteamine can accelerate GSH accumulation in bovine

oocytes during pre-IVM with CNP and thus enhance the developmental competence of oocyte.

The purpose of the present study was to investigate whether pre-IVM with 100 nM CNP alone or in combination with 100 µM cysteamine can promote the accumulation of GSH in bovine oocytes and exert a positive effect on oocyte developmental competence.

Materials and Methods

The protocols for the animal studies were approved by the Laboratory Animal Resource Center of Inner Mongolia University for the Nationalities and the study was conducted in accordance with the Animal Care and Use Statute of China.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cumulus-oocyte complex (COC) collection

Ovaries belonged to a random pool of Holstein pluriparous dairy cows slaughtered in a local abattoir at the end of their economic life. These abattoir-obtained ovaries were transported to the laboratory within 2 h in sterilized saline at 26-28°C. Cumulus-oocyte complexes (COCs) were aspirated with a 10 ml syringe from the antral follicles 3-6 mm in diameter. After examination under a stereomicroscope (Olympus SZ40, Tokyo, Japan), only COCs with a homogeneous cytoplasm and compact multi-layered CCs were selected, and washed three times in N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)-buffered TCM199 medium (Gibco 11150059, USA) supplemented with 0.1% (w/v) polyvinyl alcohol (PVA).

COC pre-IVM and IVM

The basic pre-IVM medium was TCM199 (Gibco 12340-030, USA) supplemented with Earle's salts and 10% fetal bovine serum (FBS). The millimolar levels of cysteamine and µmole for CNP were stored at -20°C, dissolved in sterile distilled water and diluted freshly for each experiment. Depending on the experiment, CNP (100 nM) or cysteamine (100 µM) + CNP (100 nM) were added to the basic pre-IVM medium. Groups of ~50 COCs were transferred into a 500-µL pre-IVM medium in 4-well culture plates (Nunclon; VWR, Bridgeport, NJ, USA). The COCs were cultured in pre-IVM medium for 6 h at 38.5°C under 5% CO_2 in humidified air as described previously (Franciosi *et al*., 2014).

Following the pre-IVM treatment, the COCs were washed three times in *in vitro* maturation medium, before being transferred to IVM culture wells. Groups of ~50 COCs were matured *in vitro* using TCM199 with Earle's salts supplemented with 10% FBS, 10 µg/ml follicle stimulating hormone (FSH, Follitropin-V; Bioniche, Belleville, ON, Canada), 1 µg/ml luteinizing hormone (LH) (Lutrophin-V; Bioniche, Belleville, ON, Canada) and 1 µg/ml estradiol in 4-well culture plates. The COCs were incubated for 24 h at 38.5°C under 5% CO_2 in humidified air. A standard IVM treatment in which COCs were directly *in vitro* matured for 24 h and no pre-IVM period served as the control for this set of experiments.

Evaluation of oocyte meiotic arrest and maturation

To evaluate the nuclear stage after pre-IVM and IVM, oocytes were mechanically separated from surrounding CCs by repeated pipetting at 0 (end of pre-IVM) and 24 h of IVM culture, respectively. Denuded oocytes were placed on slides, mounted with a coverslip with paraffin and fixed with ethanol:acetic acid (3:1 v/v) over-night. Fixed oocytes were stained with 1% orcein. The oocytes were examined under an inverted microscope (Olympus IX70, Tokyo, Japan). We measured oocyte meiotic arrest by the percentage of oocytes at germinal vesicle (GV) stage after pre-IVM, and ocyte maturation by the percentage of oocytes at metaphase II (M II) stage after IVM.

In vitro fertilization and culture

In vitro matured COCs were washed three times and transferred to 50 µL Brackett-Oliphant medium (B-O medium) for fertilization (Nedambale *et al*., 2006). Frozen-thawed semen from a bull of proven fertility was used to fertilize oocytes. Motile spermatozoa (50 µL), obtained by the swim-up procedure in B-O medium for 30 min at 38.5°C in a humidified atmosphere of 5% CO_2 in air, were added to each 50 µL fertilization droplet containing oocytes at a final concentration of 1×10^6. Fertilization was carried out for 6 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

After fertilization, CCs were stripped from presumptive zygotes and washed in Charles Rosenkran's 1 amino acid (CR1) medium (Rosenkrans *et al*., 1993). The presumptive zygotes were transferred to 100 µL CR1 culture medium containing 6 mg/ml BSA at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 and incubated for 2 days before they were transferred into the CR1 culture medium with 10% (v/v) FBS and cultured for an additional 6 days. The culture medium was changed every 2 days during the culture period.

Measurement of intra-oocyte GSH content

Intra-oocyte GSH content was measured as previously described (Ozawa *et al*., 2002). Oocytes were carefully denuded by repeated pipetting and washed 3 times in calcium-and magnesium-free phosphate buffer solution (PBS) containing 1 mg/ml polyvinylpyrrolidone (PVP). A group of 20-30 oocytes in 5 µL of PBS was transferred to a microtube, to which 5 µL of 1.25 M phosphoric acid was then added. The microtubes were kept frozen at -80°C and thawed at room temperature. The oocyte GSH content was determined by 5,5´-dithio-(bis 2-nitrobenzoic acid) (DTNB)-GSH reductase recycling micro-GSH assay. Briefly, 350 µL of 0.33 mg/ml nicotinamide adenine dinucleotide phosphate (NADPH) and 50 µL of 6 mM DTNB were added to the microtubes, then 90 µL of distilled water was added and mixed in a microfuge tube. Five µL of 250 U/ml GSH reductase was added to start the reaction. Absorbance

was monitored at 412 nm using a spectrophotometer for 3 min, with readings recorded every 30 s as described previously (Ozawa *et al*., 2002). Total GSH content in oocytes was calculated from a constructed standard curve.

Detection of intra-oocyte reactive oxygen species (ROS) levels

Intra-oocyte ROS levels were measured as previously described (Zavareh *et al*., 2016). Briefly, oocytes were completely denuded by vortex agitation in physiological saline solution for 1 min and then washed to eliminate CCs. A group of 15-20 denuded oocytes were incubated in 40 mM Tris-HCl buffer pH = 7 at 37°C for 30 min in the presence of 5 µM l2´,7´-dichlorodihydrofluorescein diacetate (DCHFDA). They were then washed, sonicated at 50 W for 1 min and centrifuged at 4°C and 10,000 g for 20 min. Fluorescence was monitored in the supernatant using a spectrofluorometer at 488 nm excitation and at 525 nm emission. Standards curves were conducted by using known amounts of H_2O_2 as described previously (Zavareh *et al*., 2016). Data for ROS production were expressed as µM H_2O_2/denuded oocyte.

Experimental design

Experiment 1

The effect of the addition of CNP to pre-IVM medium in the absence or presence of cysteamine on bovine oocytes meiotic arrest and maturation was investigated. Oocytes were cultured in pre-IVM medium supplemented with 100 nM CNP (Franciosi *et al*., 2014) with or without 100 µM cysteamine (Zhou *et al*., 2008) for 6 h, followed by standard IVM for 24 h. Oocytes matured by standard IVM for 24 h (no pre-IVM) were used as the control. At 0 h of IVM (end of pre-IVM treatment), oocytes from each treatment group were fixed and examined for the percentage of oocytes at the GV stage. After 24 h of IVM, oocytes from each treatment group were fixed and examined for the percentage of oocytes at the M II stage.

Experiment 2

The effect of the addition of CNP to pre-IVM medium in the absence or presence of cysteamine on bovine oocytes developmental competence was evaluated. Oocytes were treated in pre-IVM medium supplemented with 100 nM CNP with or without 100 µM cysteamine for 6 h, followed by standard IVM for 24 h. Oocytes matured by standard IVM for 24 h (no pre-IVM) were used as the control. After IVM, oocytes from each treatment group were subjected to *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC). Cleavage rates were assessed at 48 h post-insemination. Blastocyst production rates were recorded on day 8 after IVF.

Experiment 3

The effect of the addition of CNP to pre-IVM medium in the absence or presence of cysteamine on the level of bovine intra-oocyte GSH content was assessed. Oocytes were treated in pre-IVM medium supplemented with 100 nM CNP with or without 100 µM cysteamine for 6 h, followed by standard IVM for 24 h. Oocytes matured by standard IVM for 24 h (no pre-IVM) were used as the control. Intra-oocyte GSH content for each treatment was tested at 0 h and 24 h of IVM.

Experiment 4

The effect of the addition of CNP to pre-IVM medium in the absence or presence of cysteamine on the level of bovine intra-oocyte ROS content was examined. Oocytes were treated in pre-IVM medium supplemented with 100 nM CNP with or without 100 µM cysteamine for 6 h, followed by standard IVM for 24 h. Oocytes matured by standard IVM for 24 h (no pre-IVM) were used as the control. Intra-oocyte ROS content for each treatment was measured at 0 h and 24 h of IVM.

Statistical analysis

Statistical analysis was performed with the SPSS 18 for Windows software package. All percentage values were subjected to arcsine transformation before statistical analysis. All data among the treatment groups were analyzed by one-way ANOVA. A post hoc multiple comparison test was performed using the least significant difference (LSD) test to analyze differences among treatment groups. Experiments were repeated four times. $P<0.05$ was considered to be statistically significant.

Results

Experiment 1

As shown in Figs. 1A-B, before IVM, no differences were observed between the groups on the proportion of oocyte sustained at the GV stage, pre-IVM with either CNP or CNP + cysteamine maintained a great proportion of oocytes arrested at the GV stage (88.1 ± 2.0 and 87.7 ± 2.6% for CNP and CNP + cysteamine, respectively), indicating that CNP exerted an inhibitory effect on meiotic spontaneous maturation of bovine oocytes *in vitro*. Furthermore, after IVM, there was also no significant difference between the groups in the proportion of oocyte that proceeded to the M II stage (86.6 ± 3.8 and 87.9 ± 4.3% for CNP and CNP + cysteamine, respectively vs 83.8 ± 4.4% for control; $P>0.05$).

Experiment 2

As shown in Table 1, no differences were observed between the groups on cleavage rates. However, pre-IVM with CNP + cysteamine significantly increased blastocyst rates after IVM and IVF in comparison with the control (no pre-IVM) group ($P<0.05$), even though pre-IVM with CNP alone did not significantly improve blastocyst rates ($P>0.05$). No differences were observed between CNP and CNP + cysteamine groups on cleavage and blastocyst rates (Table 1).

Experiment 3

As shown in Table 2, pre-IVM with CNP + cysteamine significantly affected intra-oocyte GSH

content, with levels at the end of pre-IVM (0 h IVM) significantly higher, in comparison with the control (no pre-IVM) and pre-IVM with CNP group (P<0.05). This pattern persisted after 24 h of IVM, where intra-oocyte GSH was significantly higher in pre-IVM with the CNP + cysteamine group compared with the control (no pre-IVM) and pre-IVM with CNP group (P<0.05). However, pre-IVM with CNP alone did not significantly affect intra-oocyte GSH content.

Experiment 4

As shown in Table 3, pre-IVM with CNP + cysteamine significantly affected intra-oocyte ROS levels, with levels at the end of pre-IVM (0 h IVM) significantly lower, in comparison with the control (no pre-IVM) and pre-IVM with CNP group (P<0.05). This

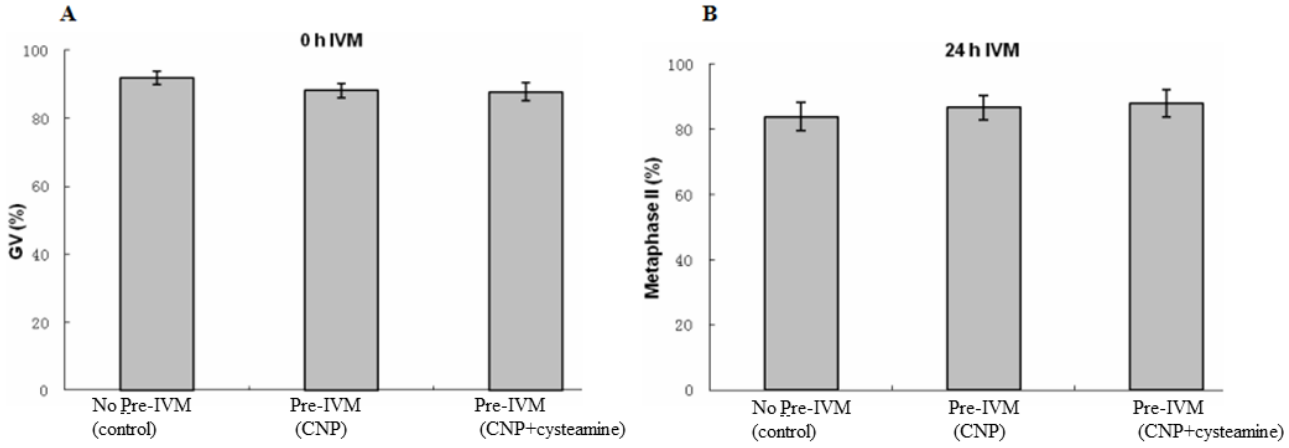

Fig. 1: Effect of pre-IVM with CNP in the absence or presence of cysteamine on meiotic arrest and maturation in bovine oocyte. Bovine immature COCs were subjected to 6 h pre-IVM culture with CNP in absence or presence of cysteamine, followed by standard IVM for 24 h. Oocytes matured by standard IVM for 24 h were used as the control. (**A**) After pre-IVM treatment (0 h IVM), the proportion of oocytes with GV was evaluated (n ≥ 146 COCs per group of four replicate experiments). (**B**) After 24 h of IVM, the proportion of oocytes with M II was evaluated (n ≥ 149 COCs per group of four replicate experiments). Data are expressed as mean±SD values. No significant difference was observed among groups. IVM: *In vitro* maturation, CNP: C-type natriuretic peptide, COCs: Cumulus-oocyte complexes, GV: Germinal vesicle, and M II: Metaphase II

Table 1: Effects of pre-IVM with CNP in the absence or presence of cysteamine followed by standard IVM on developmental competence of bovine oocyte

Treatment	Oocytes (n)	% Cleavage	% Blastocyst from cleaved
No pre-IVM (control)	197	79.7 ± 4.8 (n=157)	28.6 ± 5.3^{b} (n=45)
Pre-IVM (CNP)	225	82.2 ± 6.0 (n=185)	33.5 ± 4.9^{ab} (n=62)
Pre-IVM (CNP+cysteamine)	234	83.5± 5.1 (n=196)	40.3 ± 4.3^{a} (n=79)

Note: Data are expressed as mean±SD of four replicates. Different superscripted letters in the same column indicate a statistically significant difference (P<0.05). IVM: *In vitro* maturation, CNP: C-type natriuretic peptide, and n: Number

Table 2: Effects of pre-IVM with CNP in the absence or presence of cysteamine on bovine intra-oocyte glutathione (GSH) content at 0 h and 24 h of IVM

Treatment	IVM (0 h)		IVM (24 h)	
	Oocytes (n)	GSH content (pmol/oocyte)	Oocyte (n)	GSH content (pmol/oocyte)
No pre-IVM (control)	132	2.4 ± 0.6^{b}	129	4.6 ± 0.9^{b}
Pre-IVM (CNP)	146	3.1 ± 0.3^{b}	136	5.2 ± 0.7^{b}
Pre-IVM (CNP+cysteamine)	138	5.2 ± 0.8^{a}	142	7.9 ± 0.8^{a}

Note: Data are expressed as mean±SD of four replicates. Different superscripted letters in the same column indicate a statistically significant difference (P<0.05). IVM: *In vitro* maturation, CNP: C-type natriuretic peptide, GSH: Glutathione, and n: Number

Table 3: Effects of pre-IVM with CNP in the absence or presence of cysteamine on bovine intra-oocyte ROS level at 0 h and 24 h of IVM

Treatment	IVM (0 h)		IVM (24 h)	
	Oocytes (n)	ROS level (μM H_2O_2/oocyte)	Oocyte (n)	ROS level (μM H_2O_2/oocyte)
No pre-IVM (control)	89	2.9 ± 0.3^{a}	95	4.6 ± 0.5^{a}
Pre-IVM (CNP)	96	2.4 ± 0.3^{a}	87	4.2 ± 0.7^{a}
Pre-IVM (CNP+cysteamine)	92	1.5 ± 0.5^{b}	90	2.9 ± 0.5^{b}

Note: Data are expressed as mean±SD of four replicates. Different superscripted letters in the same column indicate a statistically significant difference (P<0.05). IVM: *In vitro* maturation, CNP: C-type natriuretic peptide, ROS: Reactive oxygen species and n: Number

pattern persisted after 24 h of IVM, where intra-oocyte ROS was significantly lower in pre-IVM with CNP + cysteamine group compared with the control and pre-IVM with CNP group ($P<0.05$). However, pre-IVM with CNP alone did not significantly affect intra-oocyte ROS levels.

Discussion

The present study showed that pre-IVM treatment with CNP, in combination with cysteamine, improved bovine oocyte developmental competence, increased intra-oocyte GSH and lowered intra-oocyte H_2O_2. Previous studies demonstrated that increased GSH levels were highly correlated with oocyte developmental competence (de Matos *et al*., 1995). These findings suggest that the enhancement of an oocyte's antioxidant defense may partly contribute to the improved oocyte developmental competence observed following pre-IVM in the presence of CNP + cysteamine.

It is widely acknowledged that bovine oocytes matured *in vitro* are developmentally compromised (Tesfaye *et al*., 2009; Gilchrist *et al*., 2011). The low efficiency of oocyte IVM technology limits the exploration and utilization of oocyte sources from cattle with desirable traits. Therefore, extensive studies using cAMP modulators have been conducted to develop a two-step culture or pre-IVM systems (Albuz *et al*., 2010; Luciano *et al*., 2011; Lodde *et al*., 2013; Zeng *et al*., 2014; Farghaly *et al*., 2015; Li *et al*., 2016). Among the systems that have been developed, a novel approach is simulated physiological oocyte maturation (SPOM) (Albuz *et al*., 2010; Li *et al*., 2016; Razza *et al*., 2019). This system involves a short period of pre-IVM in the presence of FSK and IBMX, and an IVM period in the presence or absence of PDE inhibitors such as cilostamide (Albuz *et al*., 2010; Li *et al*., 2016). However, whether the novel system developed using these pharmacological agents can enhance bovine oocyte developmental competence *in vitro* remains controversial (Albuz *et al*., 2010; Guimarães *et al*., 2015; Li *et al*., 2016). Recently, given the physiologically inhibitory role of CNP in the modulation of oocyte meiotic maturation, it is proposed that CNP may have potential value as an alternative to develop a promising bovine oocyte IVM system. Notably, a recent study showed that pre-IVM with CNP for 6 h improved bovine oocyte developmental competence, as measured by the increased cell number of blastocysts when compared to standard IVM protocol (control group) (Franciosi *et al*., 2014). More importantly, our studies showed that the presence of cysteamine during pre-IVM with CNP further improved blastocyst rates compared with standard IVM protocol (control group), revealing that the presence of cysteamine during pre-IVM with CNP exerts a beneficial effect on the acquisition of bovine oocyte developmental competence.

It is widely accepted that GSH plays an important role in oocyte maturation, fertilization and early embryonic development, and the increased accumulation of intracellular GSH is one of the markers of oocyte cytoplasmic maturation (Ferreira *et al*., 2009). A recent study suggested that cAMP-modulated pre-IVM treatment using the IBMX, in coordination with adenylate cyclase activator FSK, leads to a significantly increased accumulation of intra-oocyte GSH by prolonging CCs-oocyte gap junctions (GJ) communication (Li *et al*., 2016). However, the current study showed that the use of CNP as a cAMP-modulator during pre-IVM, did not significantly improve intra-oocyte GSH levels, albeit with an increasing trend. The between-study discrepancies may be explained by the differences between mechanisms in the regulation of cAMP levels in COCs during pre-IVM with either FSK+ IBMX or CNP. Several studies have already shown that pre-IVM with FSK will increase the concentration of cAMP in CCs that are transferred to oocytes through GJ communication, and will generate better stability to high levels of cAMP in oocytes in coordination with PDE inhibitor, IBMX, preventing cAMP degradation, (Albuz *et al*., 2010; Zeng *et al*., 2014; Li *et al*., 2016). Presently, it has been considered that CNP can not increase the levels of cAMP in CCs, but can induce the production of cyclic guanosine monophosphate (cGMP), which is then transferred through GJ into the oocyte where it inhibits phosphodiesterase type 3 (PDE3), thus sustaining high levels of cAMP in the oocyte (Zhang *et al*., 2010; Xi *et al*., 2018). Moreover, it is well established that GSH is synthesized largely by CCs because denuded oocytes have a limited capability to synthesize GSH (Curnow *et al*., 2010; Ozawa *et al*., 2010).

Previous studies have demonstrated that the addition of cysteamine during IVM increases GSH content in bovine oocytes, resulting in an improvement of blastocysts rates (de Matos *et al*., 1995). Cysteamine reduces cystine to cysteine, promoting cysteine uptake in mammalian cells (Bannai *et al*., 1984) and therefore enhancing GSH synthesis. In this study, although pre-IVM treatment with CNP alone did not effect intra-oocyte GSH levels, cysteamine supplementation in pre-IVM medium with CNP significantly increased intra-oocyte GSH levels compared to the control (no pre-IVM) and pre-IVM treatment with CNP. These results suggest that cysteamine may act individually on GSH synthesis in bovine CCs, after which it is transferred to the oocyte via GJ with an extended communication functionality by CNP, which primarily contributes to intra-oocyte GSH accumulation, thereby promoting cytoplasmic maturation in bovine oocytes. The present study also showed that an increase in intra-oocyte GSH level mediated by the use of cysteamine during pre-IVM was associated with a decrease in ROS levels within oocytes, suggesting that the beneficial effects of cysteamine could also be attributed to its roles as an antioxidant and a free radical scavenger responsible for the improved developmental competence of bovine oocytes.

To summarize, our results suggest that cAMP-modulated pre-IVM treatment using CNP has a positive effect on bovine oocyte developmental competence. Furthermore, cysteamine supplementation in a pre-IVM medium with CNP further increased blastocyst rates and

GSH content in bovine oocytes, suggesting that the use of CNP combined with cysteamine during pre-IVM would be beneficial to establishing an effective pre-IVM system aimed to enhance the developmental potential of bovine oocytes maturated *in vitro*.

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Conflict of interest

The authors declare no conflict of interest.

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