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Effect of pre-maturation with C-type natriuretic peptide and 3-isobutyl-1-methylxanthine on cumulus-oocyte communication and oocyte developmental competence in cattle

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ABSTRACT

In vitro embryo production depends on oocyte competence, which is acquired during folliculogenesis, involving cytoplasmic and nuclear processes. *In vitro* maturation (IVM) induces spontaneous resumption of meiosis, preventing full competence acquisition. The incorporation of a pre-IVM phase with supplementation with C-type natriuretic peptide (CNP) and 3-Isobutyl-1-methylxanthine (IBMX) was used with the aim of improving developmental competence of cattle oocytes. In a preliminary experiment, COCs were cultured with increasing CNP concentrations and nuclear stage assessment was performed. Supplementation with both 100 and 200 nM CNP resulted in more germinal vesicle (GV) arrest at 6 h of culture than those in the control group (79.3%, 76.4% and 59.2%, respectively). In a second experiment, use of 100 nM CNP plus 500 μM IBMX resulted in retention of more oocytes in the GV stage (92.0%) at 6 h of culture compared to supplementation with either CNP or IBMX alone (74.8% and 86.7%, respectively). A subsequent assessment of the effect of the pre-IVM system (6-h of culture with CNP plus IBMX), followed by 20-h of IVM, with comparison to the control at 24-h of IVM was performed. Blastocyst development rate was greater after the pre-IVM phase (45.1% compared with 34.5%). The inclusion of the pre-IVM phase also resulted in an enhanced mitochondrial activity in matured oocytes and sustained integrity of transzonal projections for longer after IVM. In conclusion, CNP and IBMX function synergistically to arrest meiosis in cattle oocytes during a pre-IVM phase, which improves cumulus-oocyte communication and embryo development.

1. Introduction

In vitro embryo production is an important artificial reproductive technology for cattle breeding programs because when coupled to genetic selection, there is an increase in the rate of genetic gain relative to natural mating and artificial insemination (Granleese et al., 2015). Nevertheless, both production of blastocysts and subsequent pregnancy rate post-transfer are less than for *in vivo*-

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produced embryos, limiting widespread adoption (Rizos et al., 2002).

During folliculogenesis, oocytes go through changes in the nucleus and cytoplasm that are essential for acquiring developmental competence (Gilchrist and Thompson, 2007), which is defined as the capacity to develop to the blastocyst stage, induce pregnancy and result in normally developed offspring to the time of parturition (Sirard et al., 2006). Oocytes are arrested at the germinal vesicle (GV) developmental stage within the follicle, but a spontaneous resumption of meiotic maturation occurs when removed and cultured *in vitro* (Edwards, 1965). This results in the asynchrony of meiotic and cytoplasmic maturation processes. To counter this disconnection, meiotic inhibitors have been used so that there is a time period to complete cytoplasmic maturation before IVM (Gilchrist et al., 2016). Variable results, however, have been observed following oocyte meiosis arrest *in vitro* with meiotic inhibitors of various classes. The most efficacious of these approaches to improve oocyte quality has been when meiosis is inhibited by an intra-oocyte cAMP-regulator during the pre-maturation (pre-IVM) phase of development (Gilchrist et al., 2016).

In vivo, meiotic arrest is controlled by the relatively greater intra-oocyte cAMP concentrations (Cho et al., 2018). The maintenance of these concentrations of cAMP also prolongs the gap junction communication (GJC) between cumulus cells (CC) and oocytes (Albuz et al., 2010; Li et al., 2016), which is essential for acquiring oocyte competence (Gilchrist, 2010). Furthermore, when there is cAMP-mediated IVM, there is an effect on oocyte metabolism. For example, there is a stimulation of CC glycolysis (Zeng et al., 2014), and an increase in oocyte glutathione (GSH) concentrations, reflecting improved oocyte antioxidant defence (Li et al., 2016; Zeng et al., 2014), and enhances mitochondrial and oxidative metabolism (Xi et al., 2018; Zeng et al., 2014).

One such cAMP modulator is 3-isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase (PDE) inhibitor. Oocyte cAMP is hydrolysed by PDE3A (Zhang et al., 2010), which is inhibited by IBMX thereby preventing the degradation of cAMP. In cattle oocytes during the pre-IVM phase, supplementation with IBMX + forskolin (activates adenylyl cyclase activity) delays meiotic resumption and increases blastocyst rate and quality (Albuz et al., 2010; Li et al., 2016). The C-type natriuretic peptide (CNP) is secreted by granulosa cells and stimulates the production of cGMP by CC which inhibits the PDE3/4 (Zhang et al., 2010). In mice, during the pre-IVM phase, supplementation with CNP and oestradiol results in maintenance of meiotic arrest for 48 h, and there is an increased blastocyst development rate to that comparable of oocytes matured *in vivo* (Romero et al., 2016). In cattle, during the pre-IVM phase, supplementation with CNP can result in an improved blastocyst yield and quality (Franciosi et al., 2014; Xi et al., 2018; Zhang et al., 2017a), as with other livestock species (Zhang et al., 2018, 2015; Zhang et al., 2017b). Meiotic arrest, however, can only be sustained for about 6 h and blastocyst development is only slightly improved as a result of this sustained period of meiotic arrest (Franciosi et al., 2014; Xi et al., 2018; Zhang et al., 2017a).

In the present study, it was hypothesised that supplementation with a combination of IBMX and CNP in the pre-IVM phase will prolong meiotic arrest, enhance cumulus-oocyte communication and improve oocyte quality.

2. Materials and methods

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

2.1. COC collection and culture

Cattle ovaries were obtained from adult cows of various ages at an abattoir and transported to the laboratory in warm saline (30–35 ∘C) within 2 h after recovery. The COCs were aspirated from 3 to 8-mm follicles with an 18-gauge needle and a 10-mL syringe. The COCs were maintained in follicular fluid until transferred to IVM or pre-IVM medium. The COCs were incubated at 38.5 ∘C with 6% CO_2 in air in a humidified atmosphere for different time periods. Control culture medium for Pre-IVM consisted of VitroMat (IVF Vet Solutions, Adelaide, Australia) supplemented with 4 mg/mL fatty acid-free bovine serum albumin (BSA; ICPbio Ltd, Auckland, NZ) and 100 nM 17β-Estradiol. Pre-IVM medium was supplemented with CNP and IBMX depending on the experimental design. The IBMX was previously diluted in DMSO (0.1% final DMSO concentration). The IVM culture medium that was used was VitroMat supplemented with 4 mg/mL BSA and 100 mIU/mL recombinant human follicle stimulating hormone (FSH; Puregon, Organon).

2.2. Assessment of meiotic arrest

At 6 and 24 h after the pre-IVM phase was completed, oocytes were mechanically denuded by pipetting and fixed in 4% (v/v) paraformaldehyde for 30 min. Fixed oocytes were incubated with 1 μL/mL 4′,6-diamidino-2-phenylindole (DAPI) solution in phosphate buffer saline (PBS) with 4 mg/mL BSA for 15 min at room temperature (RT). Oocytes were washed in PBS with 1 mg/mL BSA and mounted on a slide with glycerol. The nuclear maturation stage was analysed using an epifluorescence microscope (Olympus BX51; excitation: 340–380 nm; emission: 440–480 nm). Nuclear stage was classified as: germinal vesicle (GV) and germinal vesicle break down (GVBD) at 6 h; and GV, GVBD, Metaphase I (MI) and Metaphase II (MII) at 24 h of the culture period.

2.3. Assessment of oocyte glutathione concentrations and mitochondrial activity

Monochlorobimane (MCB) and MitoTracker™ deep red FM (Molecular Probes; Eugene, OR) were used together to quantitatively assess cytoplasmic maturation, using the procedures described by Sutton-McDowall et al. (2015). The MCB binds to thiol compounds and has a high affinity for GSH (99% of intracellular fluorescence) (Keelan et al., 2001). The MitoTracker™ deep red stain emits more fluorescence with increasing mitochondria membrane potential, thus was used to assess mitochondrial activity. Oocytes were denuded by mechanical pipetting and cultured first with 12.5 μM MCB for 15 min, and second with 200 nM MitoTracker™ deep red FM

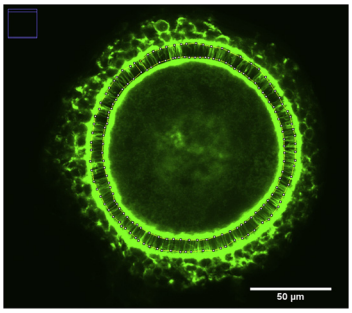

Fig. 1. Assessment of the transzonal projections density in a cumulus-oocyte complex with ImageJ software version 1.51 h. The zona pellucida area between the oocyte and cumulus cells was delimited with the polygon selection tool. Mean average pixel intensity of the delimited region was calculated.

for 15 min, in PBS with 4 mg/mL BSA at 38.5 °C. Oocytes were washed in PBS with 1 mg/mL BSA and transferred in 5-µL drops to a glass-bottom confocal dish. Oocytes were analysed with a Fluoview FV10i confocal microscope (Olympus) using the following filters: MCB 358 nM excitation and 461 nM emission; MitoTracker™ Deep Red FM 644 nM excitation and 665 nm emission. The magnification, laser intensity and image capturing parameters were set and maintained for all replicates. Mean fluorescence intensity in each oocyte was determined using ImageJ software (Version 1.51 h; National Institute of Health, Bethesda, MD, USA). Additionally, three patterns of mitochondrial distribution were observed: peripheral (mitochondria beneath the plasma membrane); homogenous (disperse mitochondria throughout the cytoplasm); and semi-peripheral (disperse mitochondria throughout the cytoplasm with less intensity in the center).

2.4. Assessment of transzonal projections

Transzonal projections (TZPs) were assessed by fluorescein isothiocyanate (FITC) conjugated phalloidin, which stains actin filaments (F-actin) using an adapted protocol from Liu et al. (2010). Briefly, COCs were partially denuded, then fixed in cold 4% (v/v) paraformaldehyde for 20 min. At RT, COCs were permeabilized in 0.25% Triton X-100 in PBS with 4 mg/mL BSA for 30 min and stained with 5 µg/mL phalloidin-FITC solution in PBS with 4 mg/mL BSA for 60 min. Three washes with PBS-BSA were performed between each step. The COCs were mounted with fluorescence mounting medium (Agilent, Santa Clara, CA, USA) on coverslips with a reinforcement ring and kept at -20 °C until analysis. TZPs fluorescence signals were examined with a Fluoview FV10i confocal microscope with 495 nm excitation and 513 nm emission. Images were processed with ImageJ software. As described by Romero et al. (2016), TZPs were observed as continuous filaments between the oocyte and cumulus cells. The TZP density was determined by measuring the mean pixel intensity within the zona pellucida, delimited by the polygon selection tool (Fig. 1).

2.5. In vitro embryo production

Procedures for *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC) were adapted using the procedures previously reported by Hussein et al. (2006). Briefly, after IVM, COCs were washed in Wash medium (IVF Vet Solutions) and co-cultured with 1×10^6 sperm/mL in 500 µL VitroFert (IVF Vet Solutions) supplemented with 4 mg/mL BSA, 10 IU/mL Heparin (DBL, Hospira, Australia), 12.5 µM hypotaurine, 25 µM penicillamine and 1.25 µM epinephrine at 38.5 °C in 6% CO_2 and a humidified air. Frozen sperm from a single bull of proven fertility was thawed at 30 to 35 °C and selected with Bovipure density gradient (NidaCon International AB, Mölndal, Sweden) with a 25-min centrifugation at 300 × *g* at RT. At 24 h post-IVF, presumptive zygotes were washed and denuded by gently pipetting in wash medium. The zygotes were cultured *in vitro* in Cleave medium (IVF Vet Solutions) supplemented with 4 mg/mL BSA, in 20-µl drops (5 zygotes/drop) overlaid with paraffin oil at 38.5 °C with humidified 7% O_2, 6% CO_2, balance N_2. At 5 days post-IVF, embryos were transferred into 20-µL drops of VitroBlast (IVF Vet Solutions) supplemented with 4 mg/mL BSA and under the same culture conditions. Cleavage and blastocyst rate were recorded at 8 days post-IVF. Blastocysts were directly fixed in ethanol with 25 µg/mL Hoechst 33342 (Molecular Probes, Eugene, OR, USA) and kept at 4 °C overnight. Stained blastocysts were mounted on a slide with a drop of glycerol and observed using an epifluorescence microscope (Olympus BX51; excitation: 340–380 nm; emission = 440–480 nm) to count the blastocyst cell number.

2.6. Experimental design

2.6.1. Experiment 1: effects of CNP on oocyte meiotic arrest

To test the effect of CNP on the maintenance of the oocyte meiotic arrest, recovered COCs were cultured for 24 h in a pre-IVM medium supplemented with CNP at different concentrations. The experimental groups consisted of supplementations with: 0 (Control), 50, 100 and 200 nM CNP. Oocyte meiotic stage was assessed at 6 and 24 h of culture. Between 34 and 39 oocytes were evaluated per treatment and time point with there being four replicates.

2.6.2. Experiment 2: effects of CNP combined with IBMX on oocyte meiotic arrest

To evaluate if the supplementation with the combination of CNP and IBMX resulted in an enhanced delay of oocyte meiotic arrest, aspirated COCs were cultured for 24 h in a pre-IVM medium supplemented with 100 nM CNP, 500 μM IBMX, or a combination of both. There were four experimental groups: Control, CNP, IBMX and CNP + IBMX. Oocyte meiotic stage was assessed at 6 and 24 h. Between 36 and 41 oocytes were evaluated per treatment and time point with four replicates being conducted.

2.6.3. Experiment 3: effects of Pre-IVM with CNP and IBMX on oocyte embryo developmental competence

To evaluate if during the pre-IVM phase the supplementation with a combination of CNP and IBMX resulted in a greater yield of more developmentally competent oocytes, COCs were *in vitro* matured, fertilized and embryos were subsequently cultured. Pre-IVM medium contained either 100 nM CNP, 500 μM IBMX or a combination of the two compounds. Between the pre-IVM and IVM phases, COCs were washed five times in IVM medium to remove any residual CNP or IBMX. The use of pre-IVM system approach (6 h of pre-IVM followed by 20 h of IVM) was compared to use of a conventional IVM approach of 24 h. The four experimental groups comprised of Control (24 h IVM), CNP pre-IVM, IBMX pre-IVM, and CNP + IBMX pre-IVM groups. Blastocyst yield was recorded at 8 days of the post-IVF phase and blastocysts were stained with Hoechst 33343. Between 183 and 192 COCs were cultured per treatment with five replicates being conducted and 60–68 blastocysts were stained per treatment group.

2.6.4. Experiment 4: effect of supplementation during the Pre-IVM phase with CNP plus IBMX on glutathione concentration and mitochondrial activity

To determine if the pre-IVM treatments altered the oocyte antioxidant defence and energy metabolism, COCs were denuded and stained with MCB and MitoTracker™ deep red FM. The COCs from two treatment groups were assessed: Pre-IVM (6-h pre-IVM with 100 nM CNP + 500 μM IBMX, followed by 20 h IVM) and Control (20-h IVM). A total of 45 oocytes per treatment were evaluated with three replicates being conducted.

2.6.5. Experiment 5: effect during Pre-IVM phase of supplementation with CNP plus IBMX on the cumulus-oocyte connections

To determine if the pre-IVM maintained TZPs between cumulus cells and oocytes after IVM, COCs were stained with phalloidin-FITC at different time points after pre-IVM and IVM and the TZPs integrity was observed using confocal microscopy. A total of five groups of COCs were analysed: 0 h control (immature COCs after aspiration), 6 h Pre-IVM, 6 h IVM, 20 h IVM, 6 h Pre-IVM + 20 h IVM. There were 45 COCs that were evaluated per group with four replicates being conducted.

2.7. Statistical analysis

Data were analysed by two-way ANOVA followed by Tukey's multiple-comparison *post-hoc* test. Treatment was specified as the fixed factor and replicate as the random variable. Prior to use of the ANOVA, data that were not normally distributed (blastocyst rate and mitochondrial distribution) were square root arcsine transformed, and normality and homogeneity of variance were reassessed (and confirmed). The statistical analyses were performed with SAS/STAT® software v 9.4 (SAS institute Inc., Cary, NC, USA). Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Supplementation with CNP maintains meiotic arrest for up to 6 h and combination with IBMX increases the efficiency of meiotic arrest (Experiments 1 and 2)

In Experiment 1, the effect was evaluated of 6 and 24 h of pre-IVM treatment with different CNP concentrations (50, 100 and 200 nM) on oocyte meiotic progression (Fig. 2). Supplementation with both 100 and 200 nM of CNP, resulted in maintenance the oocyte in the GV developmental stage at 6 h compared to what occurred in the control group without CNP ($P < 0.05$), but there were no differences at 24 h. In Experiment 2, the supplementation with a combination of CNP (100 nM) and IBMX (500 μM) occurred to assess if supplementation with IBMX enhanced the effect of CNP on the oocyte nuclear stage (Fig. 3). The supplementation with a combination of IBMX and CNP resulted in the maintenance of meiotic arrest for as long as 6 h with a greater rate of meiotic arrest occurring than with supplementation with CNP alone ($P < 0.01$), but there were no differences among treatment groups at 24 h of culture.

3.2. Pre-IVM with CNP and IBMX during 6 h followed by a conventional IVM improves embryo development (Experiment 3)

Embryo development was assessed at 8 days post-fertilization after 6 h in the pre-IVM phase followed by 20 h in the IVM phase, compared to the control at 24 h in the IVM phase (Fig. 4). During the Pre-IVM phase, supplementation with CNP plus IBMX resulted in a reduction in rate of blastocyst development compared to what occurred with the control group ($P < 0.05$). During the Pre-IVM phase, supplementation with IBMX resulted in an increased cleavage rate compared with the control group ($P < 0.05$) but there was no effect of further supplementation on blastocyst development rate. During the Pre-IVM phase, supplementation with CNP did not affect cleavage and embryo development rates. There were no differences in blastocyst total cell number when there was comparison among experimental groups.

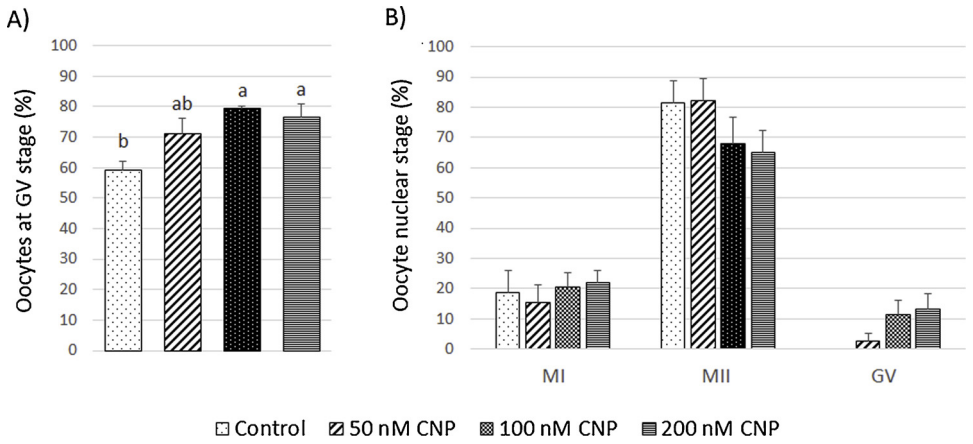

Fig. 2. Effect of CNP on the progression of nuclear maturation in cattle oocytes matured *in vitro* for 6 (A) and 24 (B) h with supplementation of 0 (Control), 50, 100 or 200 nM CNP. Oocyte nuclear maturation was assessed with DAPI and classified as: Germinal vesicle (GV), Metaphase (MI) and Metaphase II (MII). Each bar represents mean + s.e.m. Four replicates were performed with at least 33 oocytes assessed per treatment and time point. Different superscript letters (a–c) in each column represent differences ($P < 0.05$).

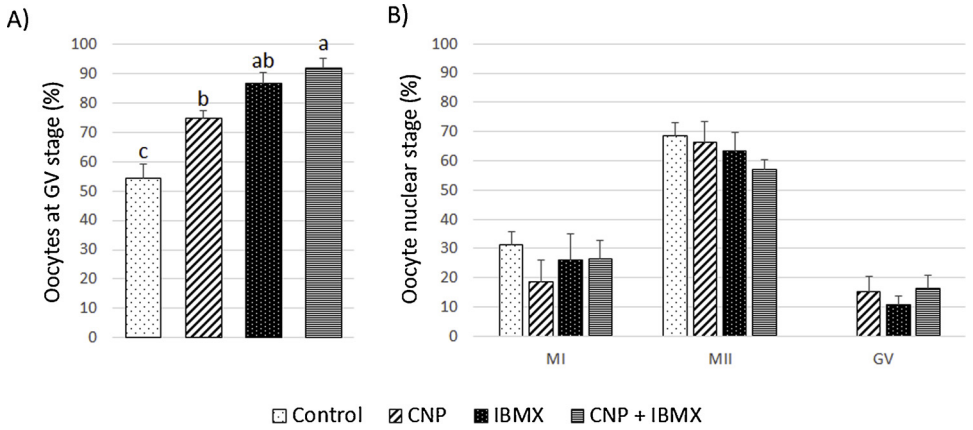

Fig. 3. Effect of CNP and IBMX on the progression of nuclear maturation in cattle oocytes *in vitro* cultured for 6 (A) and 24 (B) h with 0 (Control), 100 nM CNP, 500 μM IBMX or 100 nM CNP + 500 μM IBMX. Oocyte nuclear maturation was assessed with DAPI and classified as: Germinal Vesicle (GV), Metaphase (MI) and Metaphase II (MII). Each bar represents mean + s.e.m. Four replicates were performed with at least 33 oocytes assessed per treatment and time point. Different superscript letters (a–c) in each column represent differences ($P < 0.05$).

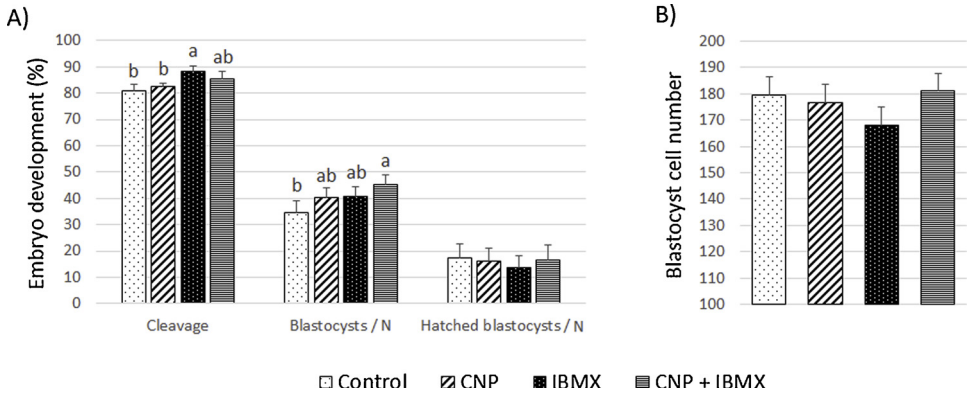

Fig. 4. Effect of 6 h pre-IVM with CNP and IBMX followed by standard IVM on cattle embryo yield (A) and total cell number (B) at 8 days post-fertilization. COCs were cultured in a Pre-IVM medium for 6 h supplemented with 100 nM CNP, 500 μM IBMX or 100 nM CNP + 500 μM IBMX, followed by 20 h IVM. A group of COCs were IVM for 24 h without previous pre-IVM (Control). Five replicates were performed with at least 183 oocytes cultured and 60 blastocysts assessed for cell number per treatment. n = n◦ of immature oocytes. Each bar represents mean + s.e.m. Different superscript letters (a–c) in each column represent differences ($P < 0.05$).

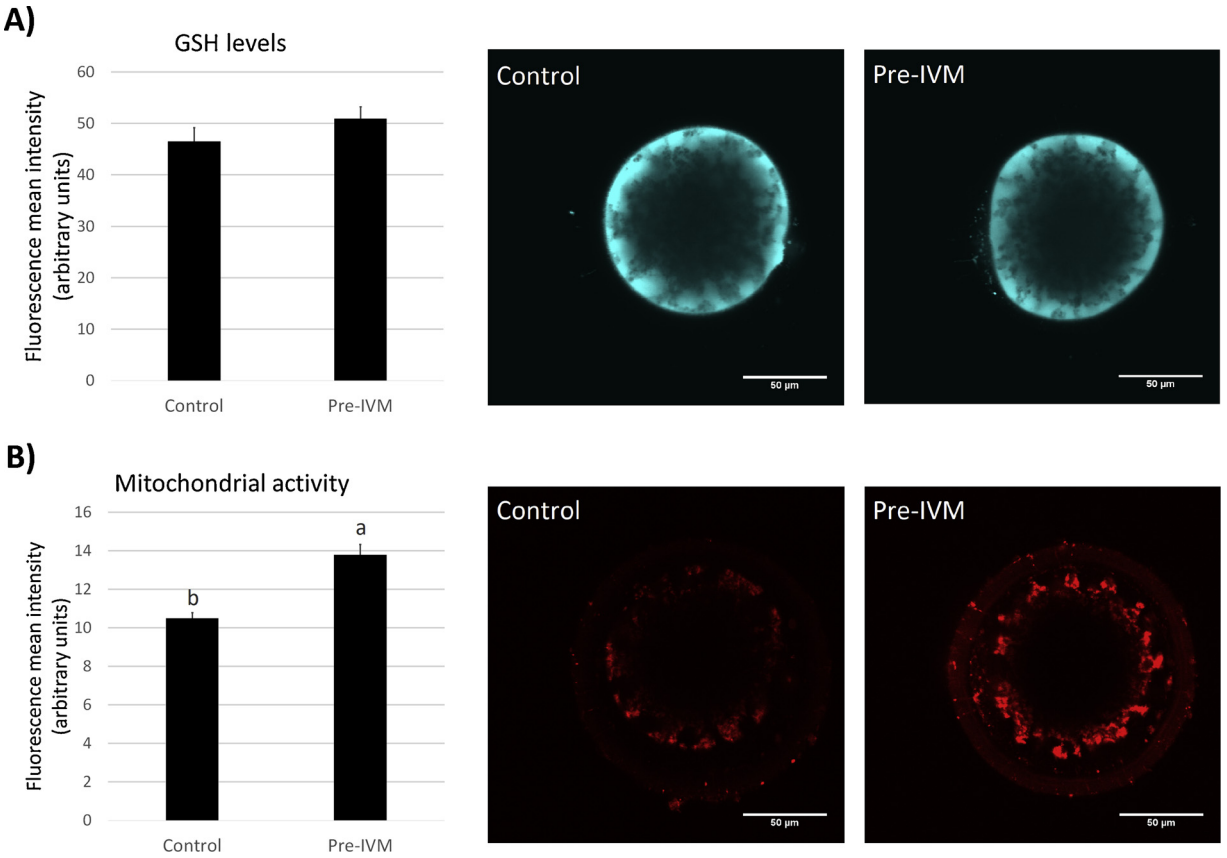

Fig. 5. Effect of 6 h pre−IVM with CNP plus IBMX on GSH levels (A) and mitochondrial activity (B) of cattle oocytes. COCs were either cultured for 6 h in a pre−IVM with 100 nM CNP plus 500 µM IBMX followed by 20 h IVM (Pre−IVM group), or culture for 20 h (Control). 45 oocytes per group were stained with MCB and MitoTracker™ deep red FM in three replicates and the oocyte average pixel intensity was quantified with Image J. Each bar represents mean + s.e.m. Different superscript letters (a–b) in each column represent differences ($P < 0.0001$).

3.3. Pre-IVM with CNP and IBMX improves mitochondrial activity and does not affect GSH concentrations of matured oocytes (Experiment 4)

Oocyte GSH concentrations (MCB staining) and mitochondrial activity (MitoTracker™ deep red FM staining) were assessed after the IVM phase (Fig. 5). During the Pre-IVM phase, supplementation with CNP plus IBMX for 6 h followed by 20 h of IVM culture resulted in greater mitochondrial activity compared to what occurred with 20 h of IVM culture in the control group ($P < 0.001$), but no effect of treatment on the GSH content. There were no differences in the mitochondrial distribution patterns: control group - 9.4% peripheral, 59.3% semi-peripheral and 31.3% homogenous distribution; pre-IVM group - 5.9% peripheral, 47.7% semi-peripheral and 46.4% homogenous distribution.

3.4. Supplementation during Pre-IVM phase with CNP and IBMX maintains transzonal projections of matured oocytes (Experiment 5)

The TZP density of COCs was evaluated with Phalloidin-FITC staining after follicular recovery (Control 0 h), 6 h of IVM, 6 h of pre-IVM, 20 h of IVM, and 6 h of pre-IVM + 20 h of IVM (Fig. 6). There was an increase in the density of TZPs after 6 h of the pre-IVM and 6 h IVM phases, compared to what occurred in the control 0 h group ($P < 0.05$). There was also a decrease in TZP density after 20 h of IVM and 6 h of pre-IVM + 20 h of IVM, compared to what occurred with the 6 h of pre-IVM and 6 h of IVM cultures ($P < 0.05$). The inclusion of the pre-IVM phase resulted in maintenance of the density of TZPs after 20 h of IVM compared to control 20-h IVM ($P < 0.05$).

4. Discussion

In the present study, the effect of supplementation of CNP with and without IBMX on the oocyte meiotic maturation was investigated. It was hypothesized that a combination of both cAMP modulators in a pre-IVM phase would result in maintenance of the germinal vesicle stage for a longer time than when there was supplementation with these compounds alone. The aim was to improve oocyte developmental competence in cattle by applying this biphasic IVM protocol to *in vitro* embryo production.

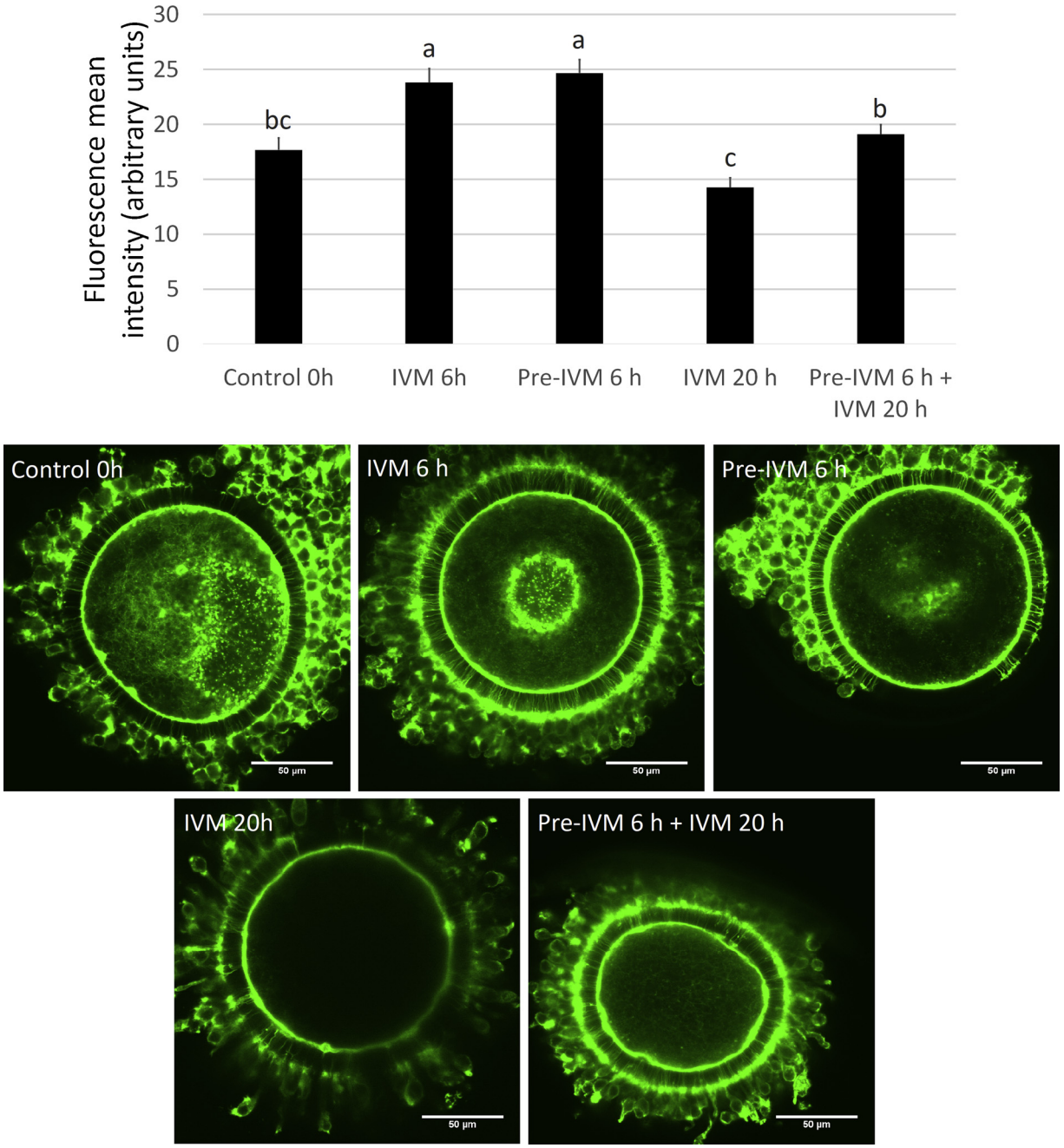

Fig. 6. Effect of 6 h pre−IVM with CNP plus IBMX on transzonal projection population of cattle COCs. COCs were stained with Phalloidin−FITC after recovery (Control 0 h), 6 h of conventional IVM, 6 h of pre−IVM with 100 nM CNP + 500 µM IBMX, 20 h of IVM, and 6 h of pre−IVM followed by 20 h IVM. Average pixel intensity in the region between the oocyte and cumulus cells was quantified with ImageJ. 45 COCs per group were assessed in four replicates. Each bar represents mean + s.e.m. Different superscript letters (a–c) in each column represent differences ($P < 0.05$).

Supplementation with a combination of CNP and IBMX resulted in maintenance of meiotic arrest for 6 h in more than 90% of the oocytes, a greater rate than when either of the meiotic inhibitors were tested individually. In previous studies with cattle COCs, supplementation with CNP (Xi et al., 2018; Zhang et al., 2017a) and its precursor (NPPC) (Franciosi et al., 2014) resulted in maintenance of the GV developmental stage for 6–8 h, and supplementation with IBMX, when used in combination with forskolin (an adenylate cyclase activator), resulted in GV stage maintenance for 9 h (Albuz et al., 2010). Oocyte meiotic arrest is sustained when there are relatively greater intra-oocyte cAMP concentrations (Cho et al., 2018). While IBMX is a broad spectrum PDE inhibitor which prevents cAMP hydrolysis, CNP stimulates the synthesis of cGMP which antagonises PDE activity (Gilchrist et al., 2016). Results of

the present study suggest a synergy between both meiotic inhibitors. However, there was not a prolongation of these effects for 24 h, whereas in other species, CNP supplementation resulted in an arrest of meiosis for at least 24 h in mouse (Romero et al., 2016) and human (Sánchez et al., 2017) COCs. These differences between species could be explained by the greater PDE8 activity (60%) in cattle CC compared to a predominant PDE4 activity in mouse CCs where IBMX does not inhibit PDE8 (Sasseville et al., 2009). Furthermore, PDE8 has 100-fold greater affinity for cAMP than PDE4 (Bender, 2006), hence the greater cGMP concentrations induced by CNP could be more efficient at inhibiting PDE4 than PDE8.

Based on these previously reported results, the use of pre-IVM system (6 h of pre-IVM followed by 20 h of IVM) was compared to use of a conventional IVM procedure of 24 h. The shorter IVM period, compared to the conventional 24 h, was chosen to prevent oocyte aging due to the increase of the total time of culture, and to design a more practical protocol that could be adapted to laboratory working hours and be translated for use in commercial breeding programs. The supplementation during the pre-IVM phase with CNP and IBMX improved oocyte developmental competence as indicated by a greater blastocyst development rate, although there was not an effect on blastocyst cell number. These results are consistent with those from previous studies in which the meiotic arrest induced by supplementation during the pre-IVM phase when there was supplementation with CNP or PDE inhibitors improved blastocyst development in cattle (Albuz et al., 2010; Li et al., 2016; Sugimura et al., 2018; Xi et al., 2018; Zhang et al., 2017a). In the present study, however, supplementation with CNP alone did not improve blastocyst rate development compared to what occurred during IVM in the control group. The inconsistencies in results among studies could be related to different maturation periods. For example, Xi et al. (2018) reported that supplementation during the pre-IVM phase with CNP only enhanced blastocyst development rate when it was followed by a longer IVM (26 h).

Cumulus-oocyte communication is essential for oocyte maturation (Russell et al., 2016). With some studies, there have been reports that supplementation with cAMP modulators such as CNP and IBMX resulted in a prolonged cumulus-oocyte GJC, which otherwise rapidly decreases during meiotic maturation (Albuz et al., 2010; Franciosi et al., 2014; Li et al., 2016; Luciano et al., 2011). Open GJs allow the bidirectional transfer of important maturation-related molecules between cumulus cells and the oocyte, which has a positive effect on oocyte GSH concentrations (Li et al., 2016), oocyte chromatin re-modelling and transcription (Franciosi et al., 2014; Luciano et al., 2011), and oocyte metabolism (Zeng et al., 2014). In the present study there was no change in oocyte GSH concentrations after the IVM phase. Mitochondrial activity, however, was enhanced, which in some studies has been associated with greater embryo developmental competence (Ge et al., 2012). The assessment of other biphasic IVM protocols when culturing cattle oocytes has provided evidence that there is a greater mitochondrial activity (Huang et al., 2016) or an increase in mtDNA copy number with use of these culture systems (Xi et al., 2018; Zhang et al., 2017a). As reviewed by Van Blerkom (2011), mitochondrial ATP synthesis is essential for oocyte maturation and early embryo development. Nevertheless, conclusions about benefit of differences in mitochondrial activity should be made cautiously, because mitochondria are also responsible for ROS production and induction of apoptosis (reviewed by Dumollard et al., 2007). Simultaneous quantification of intra-oocyte GSH, ROS concentrations and mitochondrial activity provide for a more complete assessment (Sutton-McDowall et al., 2015). Nevertheless, in the present study mitochondrial activity was considered a marker of improved oocyte quality, as it was associated with improved embryo development in the pre-IVM group.

Communication between cumulus cells and the oocyte is partly mediated by TZPs; actin filaments that connect CC cells to the oolemma by traversing the zona pellucida (Macaulay et al., 2014). Prior to maturation, the TZPs connect with oocyte GJs (Hyttel et al., 1997). Macaulay et al. (2014) reported that in cattle COCs, TZPs were already withdrawing from the oolemma at 9 h of IVM and were completely separated by 22 h. In the present study, however, there was an increase in TZPs after 6 h of culture in either pre-IVM or IVM medium and some still remained, although at a lesser density, after 20 h of conventional IVM. It has been reported that supplementation during pre-IVM phase with CNP can result in maintenance of TZPs in mouse (Romero et al., 2016) and human COCs (Sánchez et al., 2017). In the present experiment COCs which had undergone a 6 h pre-IVM had a greater density of TZPs after 20 h of IVM compared to COCs after 20 h of IVM alone. This could have relevance to oocyte competence, as TZPs allow the transfer of mRNA and metabolic molecules essential for oocyte maturation (Macaulay et al., 2016, 2014).

5. Conclusion

In conclusion, supplementation with a combination of CNP and IBMX maintained meiotic arrest for 6 h in cattle oocytes. A biphasic IVM protocol consisting of 6 h of pre-IVM with supplementation of CNP plus IBMX followed by 20 h of IVM was developed. The use of this two-step maturation system resulted in improvement in the cumulus-oocyte communication by TZPs which led to enhanced oocyte developmental competence. The results provide additional evidence about the benefits of a pre-maturation IVM protocol on *in vitro* embryo production in livestock species, when compared to use of the conventional IVM system. The efficacy of the biphasic IVM approach in cattle also has relevance for human assisted reproductive technology, because improving human IVM efficiency would enable a reduction in use of hormonal ovarian stimulation and its side effects.

Conflicts of interest

J. G. Thompson is the Founder of a company, ART Lab Solutions Pty Ltd, which manufactures bovine IVF media. All other authors declare they have no conflicts of interest whatsoever.

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