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Extending prematuration with cAMP-modulators enhances the cumulus contribution to oocyte antioxidant defence and oocyte quality via gap-junctions

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Running title: cAMP treatment *in vitro* improves oocyte antioxidant defence

ABSTRACT

STUDY QUESTION: Can bovine oocyte antioxidant defence and oocyte quality be improved by extending the duration of pre-*in vitro* maturation (IVM) with cyclic adenosine mono-phosphate (cAMP) modulators?

SUMMARY ANSWER: Lengthening the duration of cAMP-modulated pre-IVM elevates intra-oocyte reduced glutathione (GSH) content and reduces hydrogen peroxide (H_2O_2) via increased cumulus cell-oocyte gap-junctional communication (GJC), associated with an improvement in subsequent embryo development and quality.

WHAT IS KNOWN ALREADY: Oocytes are susceptible to oxidative stress and the oocyte's most important antioxidant glutathione is supplied, at least in part, by cumulus cells. A temporary inhibition of spontaneous meiotic resumption in oocytes can be achieved by preventing a fall in cAMP, and cyclic AMP-modulated pre-IVM maintains cumulus-oocyte GJC and improves subsequent embryo development.

STUDY DESIGN, SIZE, DURATION: This study consisted of a series of ten experiments using bovine oocytes *in vitro*, each with multiple replicates. A range of pre-IVM durations were examined as the key study treatments which were compared to a control. The study was designed to examine if one of the oocyte's major antioxidant defences can be enhanced by pre-IVM with cAMP modulators, and to examine the contribution of cumulus-oocyte GJC on these processes.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Immature bovine cumulus-oocyte complexes were treated *in vitro* without (control) or with the cAMP modulators; 100 μM forskolin (FSK) and 500 μM 3-isobutyl-1-methyxanthine (IBMX), for 0, 2, 4 or 6 h (pre-IVM phase) prior to IVM. Oocyte developmental competence was assessed by embryo development and quality post-IVM/IVF. Cumulus-oocyte GJC, intra-oocyte GSH and H_2O_2 were quantified at various time points during pre-IVM and IVM, in the presence and absence of functional inhibitors: carbenoxolone to block GJC and buthionine sulfoximide (BSO) to inhibit glutathione synthesis.

MAIN RESULTS AND THE ROLE OF CHANCE: Pre-IVM with FSK+IBMX increased subsequent blastocyst formation rate and quality compared to standard IVM ($P < 0.05$),

regardless of pre-IVM duration. The final blastocyst yields (proportion of blastocysts/immature oocyte) were 26.3% for the control, compared to 39.2%, 35.2% and 34.2%, for the 2 h, 4 h and 6 h pre-IVM FSK+IBMX treatments, respectively. In contrast to standard IVM (control), pre-IVM with cAMP-modulators maintained open gap-junctions between cumulus cells and oocytes for the duration (6 h) of pre-IVM examined, and persisted for a further 8 h in the IVM phase. Cyclic AMP-modulated pre-IVM increased intra-oocyte GSH levels at the completion of both pre-IVM and IVM, in a pre-IVM duration-dependent manner ($P < 0.05$), which was ablated when GJC was blocked using carbenoxolone ($P < 0.05$). By 4 h of pre-IVM treatment with cAMP-modulators, oocyte H_2O_2 levels were reduced compared the control ($P < 0.05$), although this beneficial effect was lost when oocytes were co-treated with BSO. Inhibiting glutathione synthesis with BSO during pre-IVM ablated any positive benefits of cAMP-mediated pre-IVM on oocyte developmental competence ($P < 0.01$).

LIMITATIONS, REASONS FOR CAUTION: It is unclear if the improvement in oocyte antioxidant defence and developmental competence reported here is due to direct transfer of total and/or reduced glutathione from cumulus cells to the oocyte via gap-junctions, or whether a GSH synthesis signal and/or amino acid substrates are supplied to the oocyte via gap-junctions. Embryo transfer experiments are required to determine if the cAMP-mediated improvement in blastocyst rates leads to improved live birth rates.

WIDER IMPLICATIONS OF THE FINDINGS: IVM offers significant benefits to infertile and cancer patients and has the potential to significantly alter ART practice, if IVM efficiency in embryo production could be improved closer to that of conventional IVF (using ovarian hyperstimulation). Pre-IVM with cAMP-modulators is a simple and reliable means to improve IVM outcomes.

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Key words: oocyte *in vitro* maturation / cyclic AMP / gap junctional communication / glutathione / oocyte quality

Introduction

Conventional IVF, or the generation of embryos and pregnancies following ovarian hyperstimulation, has proven a great medical advance. Yet the technology remains inefficient, with only an 18% success rate (live birth/initiated IVF cycle; (Macaldowie *et al.,* 2014)), it is expensive and ovarian hyperstimulation is not without significant health risks (Market-Velker *et al.,* 2010; Seggers *et al.,* 2014). Oocyte *in vitro* maturation (IVM) is a related useful technique to generate mature oocytes and embryos for clinical infertility treatment but uses minimal or no ovarian hyperstimulation (Edwards 1965). Clinical application of IVM has remained limited due to lower embryo yield and subsequent pregnancy rates compared to conventional IVF (Nagai 2001; Gremeau *et al.,* 2012), although recent improvements in pregnancy rates are providing significant promise (Ortega-Hrepich *et al.,* 2013; Walls *et al.,* 2015). IVM is particularly attractive to patients who suffer from polycystic ovary syndrome as these patients have a high antral follicle count and are at risk of developing ovarian hyperstimulation syndrome during conventional IVF. Furthermore, with ever increasing cancer survival rates, IVM has an important place in modern fertility preservation approaches, using oocytes collected either from in vivo or ex vivo ovaries (Smitz *et al.,* 2011). In recent years, improvement has been made in the efficacy of IVM in animals and translating these advances to human IVM stands to bring significant benefits to health care providers and to patients (Gilchrist *et al.,* 2011).

It is widely accepted that the low efficiencies of IVM are in part due to precocious oocyte meiotic resumption following artificial removal of cumulus-oocyte complexes (COCs) from antral follicles and subsequent culture (Gilchrist and Thompson 2007). We and others have demonstrated that a temporary inhibition of spontaneous meiotic resumption, achieved by preventing a fall in intra-oocyte cyclic adenosine monophosphate (cAMP) level through the use of cAMP modulators prior to maturation (pre-IVM), better recapitulates some of the oocyte maturation processes that occur in vivo, leading to greater oocyte developmental competence (Luciano *et al.,* 1999; Guixue *et al.,* 2001; Shu *et al.,* 2008; Albuz *et al.,* 2010; Rose *et al.,* 2013; Zeng *et al.,* 2013; Franciosi *et al.,* 2014; Richani *et al.,* 2014; Zeng *et al.,* 2014). There

are several options to prevent the reduction in cAMP during this pre-IVM period, including the use of specific and non-specific phosphodiesterase (PDE) inhibitors or dibutyryl-cAMP (Funahashi *et al.,* 1997; Gilchrist 2011). We have previously shown that using the non-specific PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX), in conjunction with increasing cAMP synthesis in COCs with the adenylate cyclase activator, FSK, significantly improves oocyte developmental competence as measured by blastocyst development rates and post-transfer fetal yield (Albuz *et al.,* 2010; Zeng *et al.,* 2013; Richani *et al.,* 2014; Zeng *et al.,* 2014). The application of cAMP-modulated IVM is now at the pre-clinical stage in human assisted reproductive technology (ART) (Spits *et al.,* 2015). Moreover, in the mouse, blastocyst yield and quality was greater when the pre-IVM period was lengthened beyond 1 hour (Richani *et al.,* 2014) and 20 hours of dibutyryl-cAMP treatment is routinely used in porcine IVM (Funahashi *et al.,* 1997), suggesting that the duration of cAMP-modulated pre-IVM has important effects on oocyte developmental competence.

Treatment of COCs with cAMP modulators prolongs cumulus-oocyte gap junctional communication (GJC) during IVM (Thomas *et al.,* 2004; Shu *et al.,* 2008; Luciano *et al.,* 2011; Franciosi *et al.,* 2014). Bidirectional crosstalk between the oocyte and cumulus cells (CCs) are essential for oocyte growth, for enabling nutrients and other small molecules to transfer between them, together ensuring that the oocyte acquires the molecular machinery required to support early embryo development (Zuccotti *et al.,* 1998; Albertini *et al.,* 2001; Gilchrist *et al.,* 2004). One such factor exchanged between CCs and the oocyte is reduced glutathione (GSH). CC-oocyte GJC has been reported to regulate intra-oocyte GSH synthesis and accumulation (de Matos *et al.,* 1997; Nagai 2001; de Matos *et al.,* 2002). GSH plays important roles as a reducing agent (and therefore an antioxidant) in oocyte maturation, fertilization and embryonic development (Curnow *et al.,* 2008; Takeo *et al.,* 2015). We hypothesize that one mechanism by which pre-IVM with cAMP modulators improves oocyte competence is to facilitate CC transfer and accumulation of GSH within the oocyte during the pre-IVM period. Furthermore, we assessed if intra-oocyte GSH levels are increased by extending the duration of cAMP-mediated pre-IVM (2, 4, 6 h). We undertook these experiments using bovine oocytes as it has proven a valuable experimental model to understand the complexities of human

oocyte developmental competence and the technological approaches needed to improve human IVM.

Materials and Methods

COC collection

Bovine COCs were obtained from abattoir-derived ovaries of primarily cycling animals (but of different breeds and fecundity) and transported to the laboratory at 35-37°C within 5 h of collection. COCs were aspirated using an 18-gauge needle and a 10-ml syringe from 3-8 mm diameter follicles of mixed growth and atresia status (Sirois and Fortune 1988), and retained in follicular fluid until transferred into pre-IVM medium (Albuz *et al.,* 2010).

COC *in vitro* maturation

Oocytes were matured in vitro using the simulated physiological oocyte maturation (SPOM) version 2 system (Zeng *et al.,* 2014; Gilchrist *et al.,* 2015). Differing duration of pre-IVM (0, 2, 4, 6 h) was a central variable examined in most experiments. Accordingly, the ensuing IVM interval was either, 1) varied in some experiments to give a constant total time in vitro, or 2) kept constant such that total time in vitro was extended.

pre-IVM

Depending on the experimental design, immature COCs were removed from aspirated follicular fluid, washed three times and cultured in Vitromat (IVF Vet Solutions, Adelaide, Australia) containing 4 mg/ml fatty acid-free bovine serum albumin (BSA; ICPbio Ltd, Auckland, NZ), with or without 100 μM FSK (Sigma) and 500 μM IBMX (Sigma), during pre-IVM periods (0, 2, 4 or 6 h). Millimolar stock concentrations of the cAMP modulators were stored at —20°C dissolved in anhydrous dimethylsulphoxide (Sigma) solutions and were diluted fresh for each experiment.

IVM

Following the pre-IVM treatments, bovine COCs were washed three times before maturation in Vitromat + 4 mg/ml BSA + 50 μg/ml gentamycin (Sigma) + 100 mIU/ml recombinant human FSH (Puregon, Organon) for 14 – 24 h, depending on experimental design. Each replicate

included thirty to forty COCs per treatment and were cultured in pre-equilibrated 500 µl drops overlaid with paraffin oil (Sigma) and incubated at 38.5°C with 6% CO_2 in humidified air for the time intervals indicated.

Assessment of oocytes

Oocytes were stained using the orcein staining methods, as described and validated previously (Prentice-Biensch *et al.,* 2012), for the assessment of oocyte nuclear maturation. Briefly, oocytes were stripped of CCs, mounted onto a glass slide and overlaid with a wax supported coverslip. Denuded oocytes were fixed in ethanol: acetic acid (3:1, v/v) by using capillary action to run the solution between the slide and coverslip, then stored at 4°C for 24 h. Oocytes were stained with 1% orcein (w/v) in 45% acetic acid (v/v) for 20 min by the same process and then cleared with a mix of 20% glycerol and 20% acetic acid in water. Chromosomal configurations were evaluated using phase-contrast microscopy (Olympus; Tokyo, Japan) and the stages of meiosis recorded.

Intra-oocyte GSH content was measured as described previously (Keelan *et al.,* 2001) using monochlorobimane (MCB, Sigma), a probe which reacts with reduced thiol groups (-SH) to form fluorescence adducts. As MCB has the highest affinity for GSH, 99% of positive fluorescence is attributed to binding with GSH (Keelan *et al.,* 2001). COCs were cultured intact for variable intervals depending on the treatment, oocytes were denuded of CCs and then incubated in wash medium (Vitrowash, IVF Vet Solutions) containing 12.5 µM MCB at 39°C in darkness for 30 mins. Oocytes were washed twice in wash medium and transferred to 5 µl of wash medium overlaid with paraffin oil in a glass bottom confocal dish (Cell E&G; Houston, TX, USA). Oocytes were examined by using an Olympus Fluoview FV10i confocal microscope (Olympus; Tokyo, Japan) with an excitation wavelength of 358 nm and emission 461 nm. Relative differences in GSH content, represented by the MCB-GSH fluorescence, were determined using ImageJ software 1.33u (National Institutes of Health, Bethesda, MD, USA) and normalized to the mean value of the background (excluding oocytes) in each image. Microscope laser settings were standardized using Inspeck green fluorescence beads (Molecular Probes, Eugene, OR, USA).

Intra-oocyte H_2O_2 levels were measured by peroxyfluor-1 (PF-1), a specific fluorophore for H_2O_2 (with some cross-reactivity with $ONOO^-$, as previously described (Purdey *et al.,* 2015). In brief, following denuding, oocytes were transferred to wash medium containing 100 µM PF-1 at 39°C in darkness. After 30 min incubation, oocytes were washed once in VitroWash, and oocytes transferred into 5 µl of wash medium overlaid with paraffin oil in a glass bottom confocal dish. Fluorescence intensity was determined by confocal microscopy (excitation: 488 nm, emission: 520 nm) using the same procedure as described above.

Assessment of CC-oocyte GJC

Assessment of GJC between CCs and the oocyte during pre-IVM or IVM was determined by lucifer yellow (LY; Sigma) dye transfer following microinjection of dye into the ooplasm of intact COCs, as previously described (Luciano *et al.,* 2004). Briefly, a 3% w/v LY solution in 5 mM lithium chloride was injected into the oocyte. If CC-oocyte gap junctions are open then the dye is free to diffuse into the CCs which can be observed and scored, whereas no dye transfer occurs if GJC has ceased. The transfer of dye to surrounding CCs was assessed by confocal microscope within 15 min of injection. Using a qualitative scoring system a semi-quantitative GJC index can be calculated (Sugimura *et al.,* 2014). In brief, CC LY levels were scored as +2 when the dye was transferred to the entire cumulus layer; +1 when the dye was transferred to limited number of CC layers (primarily the corona radiate); and 0 when there was minimal to no dye transfer into any CCs.

***In vitro* embryo production and differential staining**

In vitro embryo production followed methods previously described (Hussein *et al.,* 2006). In brief, following IVM, COCs were washed twice in wash medium and co-cultured with motile sperm from a single bull of proven fertility (prepared using a discontinuous Percoll gradient, GE Healthcare) in 500 µl of pre-equilibrated IVF medium (VitroFert, IVF Vet Solutions, + 4 mg/ml BSA + 10 IU/ml heparin), at a final concentration of 1×10^6/ml frozen-thawed sperm and cultured at 38.5°C in 6% CO_2 in humidified air. After 22 h from the addition of sperm (day 1), presumptive zygotes were denuded of CCs by gentle pipetting and cultured in groups of

5-10 in 20 µl drops of VitroCleave (IVF Vet Solutions) + 4 mg/ml BSA, overlaid with paraffin oil at 38.5°C in 7% O_2, 6% CO_2, N_2 balance. On day 5, 5-10 embryos were transferred into 20 µl of VitroBlast (IVF Vet Solutions) + 4 mg/ml BSA, overlaid with paraffin and cultured at 38.5°C in 7% O_2, 6% CO_2, N_2 balance. The rates of early and final cleavage embryo development were assessed at 22 h on day 1 and day 5, respectively. Blinded embryo assessments, including yield of blastocysts and hatched blastocysts, were performed on day 8. On day 8 post IVF, blastocysts in each group were processed using a differential staining protocol to determine inner cell mass (ICM) and trophectoderm (TE) cell numbers, as previously described (Hussein *et al.,* 2006). Differential staining was performed as ICM cell numbers provide a robust measure of blastocyst quality, implantation and fetal developmental potential (Lane and Gardner 1997; Sudiman *et al.,* 2014). The zona pellucida was removed by incubation in 0.5% pronase in Dulbecco's phosphate-buffered saline (PBS) at 39°C. Embryos were washed in protein-free PBS and incubated in 10% 2,4,6-trinitrobenzene sulfonic acid in PBS for 10 min at 4°C in the dark; 10 mins in anti-2,4-dinitrophenol (1:10 w/v) at 39°C, followed by 10 mins in complement (2 µg/ml solution of propidium iodide: guinea pig serum, 1:1). Embryos were transferred to (97%) ethanol containing 25 µg/ml of Hoechst 33342 (bisbenzimide) at 4°C for overnight staining. Embryos were mounted on microscope slides in 100% glycerol and the number of ICM and TE cells was determined using an epifluoresence microscope (excitation = 340-380 nm and emission = 440-480 nm), whereby nuclei appeared blue and pink, respectively.

Statistical analysis

Number of COCs per treatment and data set replicate numbers are provided in the figure legends for each experiment. Proportional data for embryo development were arcsine transformed before statistical analyses. Treatment effects were assessed by one-way or two-way ANOVA followed by Turkey's multiple-comparison post-hoc test to identify individual differences between means, using Prism 5.00 GraphPad for Windows (GraphPad Software, San Diego, CA, USA). Two sample *t*-tests were used where only two sample means were compared. Statistical significance was taken as $P < 0.05$.

Results

Effect of pre-IVM duration on bovine oocyte meiotic and developmental competence

The relationship between the duration of cAMP-modulated pre-IVM and the subsequent temporal kinetics of bovine oocyte meiotic maturation remains unclear. Here we examined the effect of 0 h, 2 h, 4 h, and 6 h pre-IVM treatment with forskolin and IBMX, followed by FSH-stimulated IVM, on oocyte meiotic maturation (Figure 1). Decreased pre-IVM duration, with a combined total 20 h of culture (pre-IVM + IVM), led to an incremental increase in metaphase II (MII) rates (Figure 1). However, only the 6 h pre-IVM treatment (6 h pre-IVM + 14 h IVM) yielded significantly fewer MII oocytes compared with no pre-IVM (0 h pre-IVM + 20 h IVM; $P < 0.05$). In contrast, when the pre-IVM time varied (0 h, 2 h, 4 h, 6 h) followed by 20 h IVM, there was no difference in the yield of MII oocytes. These data demonstrate that extended pre-IVM (e.g. 6h) delays total time to MII and hence a total (pre-IVM + IVM) culture period of 26 h was chosen for oocyte developmental competence experiments.

This led us to examine subsequent embryo development following pre-IVM periods of 0 h, 2 h, 4 h, 6 h, combined with IVM for 24 h, 24 h, 22 h, 20 h, respectively (Figure 2A). An increase in the yield of early cleaved embryos at 22h post-IVF was observed with 4 h and 6 h pre-IVM periods compared with no pre-IVM ($P<0.05$; Figure 2B), demonstrating that prolonging pre-IVM duration accelerates 2-cell embryo development, which is a strong predictor of pregnancy success (Sugimura *et al.,* 2012). Although final cleavage rates (as assessed on day 5) were not significantly different between groups (Figure 2C), all pre-IVM treatments increased the proportion of day 8 blastocysts/total embryos cleaved compared to no pre-IVM ($P<0.05$; Figure 2D). Hence, the final blastocyst yields (proportion of blastocysts/immature oocyte) were 26.3% for the control, compared to 39.2%, 35.2% and 34.2%, for the 2 h, 4 h and 6 h pre-IVM treatments, respectively. Only the 6 h pre-IVM period treatment yielded significantly more hatched blastocysts than no pre-IVM treatment ($P<0.05$; Figure 2E). Importantly, all the pre-IVM treatments improved blastocyst quality, reflected by an increase in total number of cells compared with no pre-IVM, made up largely by an increased ICM cell number (Figure 2F; $P<0.05$), and notably, the 6 h pre-IVM treatment yielded the highest ratio

of ICM to total cells among all treatments, and was significantly different to that of no pre-IVM ($P<0.05$; Figure 2F inset).

Effect of pre-IVM duration on bovine CC-oocyte GJC during pre-IVM and IVM stages

GJC integrity was measured by assessing LY dye transfer from the oocyte to the surrounding cumulus vestment (Figure 3A) following 0 h, 2 h, 4 h, and 6 h of pre-IVM with or without forskolin and IBMX (Figure 3B), or after 0 h, 4 h, 8 h and 12 h of IVM in the presence of FSH (Figure 3C). As shown in Figure 3B, during pre-IVM, GJC in the control group (no cAMP modulators) fell sharply and progressively until 6 h of culture. On the contrary, the GJC remained high for up to 6 h of culture in all pre-IVM groups in the presence of FSK and IBMX. With regards to the combination of pre-IVM and IVM, during the first 8 hours of the IVM phase, GJC remained significantly ($P<0.05$) higher than control (no pre-IVM) when COCs had been previously exposed to a cAMP-mediated pre-IVM treatment of at least 2 hours (Figure 3C). By 12 h of culture no differences were observed among any groups, indicating that under these conditions, the limit to CC-oocyte GJC is 12 h of IVM.

Effect of pre-IVM on the level of bovine intra-oocyte GSH content during IVM

The representative images of intra-oocyte MCB fluorescence at 0 h and 24 h of IVM, indicative of GSH levels, are presented in Figure 4A. The duration of pre-IVM exposure to FSK+IBMX significantly affected intra-oocyte GSH content, with levels at the end of pre-IVM (0h IVM) significantly higher after 4 h or 6 h of pre-IVM, in comparison with 2 h and the control (no pre-IVM) group (Figure 4B; $P<0.05$). This pattern persisted after 24 h of IVM, where intra-oocyte GSH was higher with increasing length of the pre-IVM period, with a significantly ($P<0.05$) higher content observed in the 6 h pre-IVM group compared with the control. This indicates that longer pre-IVM periods influence intra-oocyte GSH accumulation, not only during the pre-IVM stages but also possibly throughout IVM. Based on this, we hypothesized that GSH accumulation in the oocyte from pre-IVM treatment was due to the enhanced GJC.

Effect of inhibiting GJC on GSH accumulation

To assess the contribution of CC-oocyte GJC to intra-oocyte GSH levels following pre-IVM

treatment with FSK and IBMX, we treated COCs during pre-IVM with carbenoxolone (CBX), a known gap junction inhibitor which we previously validated in bovine COCs (Thomas *et al.,* 2004), and assessed LY dye transfer from the oocyte to the cumulus vestment and intra-oocyte MCB fluorescence. CBX was effective at blocking CC-oocyte GJC (Figure 5A; $P<0.01$) at 4 h of culture in the presence of FSK and IBMX. The intra-oocyte GSH content was significantly decreased (Figure 5B; $P<0.05$) by CBX in both control and FSK+IBMX treated COCs. These results provide further evidence that CCs contribute to the accumulation of GSH in the oocyte via a GJC-mediated mechanism and that intra-oocyte GSH levels are enhanced by FSK and IBMX during the pre-IVM period.

Effect of inhibition of GSH synthesis on H_2O_2 production and embryonic development

As intra-oocyte GSH levels are regarded as important in managing reducing reactive oxygen species (ROS) in the oocyte (Guerin *et al.,* 2001), we assessed the effect of depleted oocyte GSH levels on H_2O_2 content of oocytes and their subsequent developmental capacity. We depleted oocyte GSH levels by treating COCs with 10 mM buthionine sulfoximide (BSO) a validated inhibitor of glutathione synthase (Sutovsky and Schatten 1997). BSO treatment ablated the pre-IVM (FSK+IBMX)-induced increase in intra-oocyte GSH (Figure 6C; $P<0.05$). H_2O_2 production in the oocyte was evaluated with PF-1 staining (Figure 6A; (Purdey *et al.,* 2015)). Pre-IVM with FSK+IBMX significantly decreased intra-oocyte H_2O_2 levels (Figures 6A(b) and 6B), but this response was eliminated with BSO treatment, suggesting that reduction of GSH levels coincides with an increase in H_2O_2 production in oocytes.

The effect of inhibiting GSH synthesis during the 4 hours of pre-IVM on subsequent embryonic development was examined after IVF and embryo culture (Figure 6D). Although no significant difference in cleavage rates was observed, 4 h of BSO-treatment of oocytes significantly ($P<0.01$) reduced day 8 blastocyst and hatched blastocyst yields, indicating that the synthesis of GSH in the pre-IVM period has a marked effect on subsequent embryo developmental potential.

Discussion

Here we report that increasing the length of pre-IVM treatment with FSK and IBMX positively impacts GJC in bovine COCs, which in turn increases intra-oocyte GSH and lowers intra-oocyte H_2O_2, establishing a mechanism contributing to the improved oocyte developmental competence observed following pre-IVM in the presence of cAMP modulators. The use of cAMP modulators during pre-IVM and/or IVM, to provide a high intracellular cAMP levels within CCs and the oocyte, is associated with prolonged CC-oocyte GJC (Luciano *et al.,* 2004; Thomas *et al.,* 2004; Thomas *et al.,* 2004; Shu *et al.,* 2008; Albuz *et al.,* 2010; Franciosi *et al.,* 2014), in addition to the inhibition, or at least a delay, in the timing of germinal vesicle breakdown (GVBD). The resumption and completion of meiosis is particularly delayed when cAMP modulating agents (FSK + IBMX) are present during pre-IVM stage and combined with cilostamide, a PDE3 inhibitor, during the IVM phase, as per the 'simulated physiological oocyte maturation' (SPOM) version 1 system (Albuz *et al.,* 2010). Further work showed that the beneficial impact of the SPOM system may lie specifically in the pre-IVM period (Zeng *et al.,* 2013; Richani *et al.,* 2014; Zeng *et al.,* 2014), which led to the concept of SPOM version 2 (SPOMv2), wherein there is an absence of cAMP modulators during IVM (Gilchrist *et al.,* 2015), and is the system used in the current study. Numerous mechanisms have been identified as contributing to enhanced developmental competence following SPOMv2, such as enhanced COC oxygen consumption and oocyte oxidative metabolism (Zeng *et al.,* 2013; Richani *et al.,* 2014; Zeng *et al.,* 2014) and accentuating epidermal growth factor (EGF) signaling in CCs (Zeng *et al.,* 2013; Richani *et al.,* 2014; Zeng *et al.,* 2014). Here we add to these concepts by revealing that extending the pre-IVM period for up to 6 hours further enhances oocyte developmental competence, most likely by increasing the oocyte's anti-oxidant defence and decreasing ROS levels.

The pre-ovulatory gonadotrophin surge leads to a transient increase of cAMP in the somatic compartment of the follicle, including in CCs (Tsafriri *et al.,* 1972; Yoshimura *et al.,* 1992; Mattioli *et al.,* 1994; Albuz *et al.,* 2010), but a simultaneous fall in intra-oocyte cGMP and cAMP (Norris *et al.,* 2009). This cAMP surge in the COC is typically impaired or absent using standard clinical IVM protocols, with likely deleterious consequences for oocyte

developmental competence, which is the impetus for developing cAMP-modulated IVM systems (Gilchrist 2011). Such IVM systems typically use a pre-IVM phase containing cAMP elevating agents; COCs are commonly exposed to cAMP modulators for 0.25 - 2 hours prior to IVM (Luciano *et al.,* 1999; Guixue *et al.,* 2001; Albuz *et al.,* 2010), but this can be up to 20 hours, for example using porcine oocytes (Funahashi *et al.,* 1997) coinciding with GVBD in that species.

While the beneficial effects of cAMP modulated pre-IVM on oocyte competence are well accepted, the optimal duration of pre-IVM is largely unknown. A recent study shows extending pre-IVM exposure to cAMP modulators beyond 1-2h appears beneficial to mouse oocyte quality (Richani *et al.,* 2014). In this study we investigated the effect of 0, 2, 4 and 6 hours of pre-IVM with FSK + IBMX followed by standard IVM on subsequent embryo development. Consistent with previous studies using a 2 hour pre-IVM system, all pre-IVM durations examined here led to notable improvements in subsequent embryo development and quality, compared to standard IVM. The 6 h pre-IVM treatment had an optimal effect on oocyte development competence, as reflected in the fastest rate of 2-cell embryo formation, the highest yield of hatched blastocysts and notably the highest ratio of ICM to total cells among all treatments. It has previously been shown that rapid cleave of bovine embryos is a strong predictor of embryo quality and pregnancy success, as assessed by embryo karyotyping, expression of interferon tau and pregnancy rates (Sugimura *et al.,* 2012). In addition, in an IVM context, it is common to see the effects of treatments that improve oocyte quality having little or no effect on MII and total cleavage rates, but manifesting in increased blastocyst rates and ICM cell numbers, both of which are associated with improved implantation and fetal survival rates (Albuz *et al.,* 2010; Sudiman *et al.,* 2014).

One complication of using a cAMP modulated IVM system and altering the pre-IVM interval is the effect on oocyte meiotic kinetics (Gilchrist *et al.,* 2015), which is compounded by the fact that FSK acts initially as an inhibitor and then an inducer of meiosis (Dekel *et al.,* 1988; Yoshimura *et al.,* 1992). Hence, we examined the time required to reach MII using the

different pre-IVM durations with the consequence that, with a 6 hour pre-IVM internal, the IVM phase was shortened to 20 hours (total of 26 hours).

In the current study, pre-IVM with FSK+IBMX fully sustained GJC functionality in COCs throughout the 6 hours examined, in notable contrast to the loss of GJC that occurred in untreated COCs. Furthermore, the effect of pre-IVM on GJC persisted a further 8 hours into the IVM phase. Hence, in the pre-IVM 6h group, GJC was higher than in control COCs for a total of 14 hours. Stimulation of CC cAMP synthesis by FSK or gonadotrophins enhances Cx43 (connexin 43) expression and stimulates phosphorylation of Cx43 at specific residues, possibly via a protein kinase A-dependent mechanism, collectively sustaining GJC functionality (Granot and Dekel 1994; Thomas *et al.,* 2004; Yogo *et al.,* 2006; Sasseville *et al.,* 2009). The use of FSH in conjunction with PDE inhibitors or natriuretic peptide precursor (NPPC), which leads to oocyte PDE inhibition, also sustains CC-oocyte GJC (Thomas *et al.,* 2004; Franciosi *et al.,* 2014). In the current study, the sustained somatic cell support to the oocyte through at least half of oocyte maturation is likely to be a key factor in the enhanced quality of subsequent oocytes (Gilchrist and Richani 2013). One proposed benefit of CC support is the ordered cessation of oocyte transcription and appropriate remodeling of chromatin in preparation for meiosis (Luciano *et al.,* 2011; Franciosi *et al.,* 2014). Another benefit of extended CC-oocyte GJC is increased accumulation of beneficial CC nutrients and metabolites in the oocyte, such as glutathione (Gilchrist and Thompson 2007).

Glutathione is the most abundant non-protein thiol in mammalian cells and the reduced form (GSH) plays a key role in cellular defence against oxidative injury as an important substrate for antioxidant enzymatic reactions that neutralize H_2O_2 and potentially toxic electrophiles (Franco and Cidlowski 2009). It is particularly important in mammalian oocytes where multiple actions have been described, including in oxidative stress defence (Guerin *et al.,* 2001), sperm decondensation and male pronuclear formation (Sutovsky and Schatten 1997) as well as oocyte developmental competence (de Matos *et al.,* 1995; de Matos *et al.,* 1996; Curnow *et al.,* 2010)**.** The central role of glutathione in oocyte developmental competence is now so widely accepted that glutathione precursors, e.g. cysteamine and cysteine, are common

449 additives in oocyte IVM media (Thompson *et al.,* 2007). Early cleavage stage embryos have a
450 poor capacity to synthesize glutathione (Gardiner and Reed 1995), hence accumulation in
451 oocytes during maturation is important for fertilization and subsequent developmental
452 competence (de Matos and Furnus 2000)**.**
453
454 The current study demonstrates that pre-IVM with cAMP modulators increases intra-oocyte
455 GSH, consistent with a recent study (Zeng *et al.,* 2014). Moreover, the current results show
456 that extending pre-IVM beyond 2 hours leads to increased GSH accumulation in the oocyte in
457 the pre-IVM phase, with these higher levels of GSH then persisting throughout the IVM phase.
458 Blocking functional CC-oocyte coupling using CBX greatly decreased intra-oocyte GSH
459 content during pre-IVM, suggesting that CCs supply the additional GSH to the oocyte via gap
460 junctions, as previously reported (Mori *et al.,* 2000; Ozawa *et al.,* 2010). This is consistent with
461 the observation that GSH levels are lower in oocytes denuded of CCs and that CCs can
462 increase intra-oocyte GSH levels, but only when they are physically coupled to oocytes (de
463 Matos *et al.,* 1997; Curnow *et al.,* 2010). It is noteworthy that glutathione is predominantly
464 synthesized in the COC in the GV to GVBD period. Upregulation of glutathione synthesis
465 occurs rapidly in response to the pre-ovulatory gonadotrophin surge and an increase in
466 intra-oocyte glutathione, from basal levels in GV oocytes to maximal levels in MII oocytes, is
467 needed in preparation for fertilization and sperm head decondensation (Perreault *et al.,* 1988;
468 Zuelke *et al.,* 2003). This acute phase of glutathione synthesis during the GV to GVBD period
469 coincides exactly with the period in which oocytes and CCs are coupled via GJC (Thomas *et*
470 *al.,* 2004). Hence, our current findings suggest that an extended cAMP-modulated pre-IVM
471 treatment leads to beneficial accumulation of GSH in the oocyte by prolonging CC-oocyte
472 GJC. This is further supported by the observation that GSH accumulation in the oocyte is only
473 prevented when GJC is blocked in the first half (GV-MI), but not when blocked in the second
474 half (MI-MII), of oocyte maturation (Ozawa *et al.,* 2010). Whilst it seems likely that this would
475 be mediated by direct gap-junctional transfer of glutathione itself from CCs to the oocyte, with
476 the current results, we cannot exclude the possibility that CCs are providing a critical signal
477 and/or metabolite via GJC, which leads the oocyte itself to synthesize additional glutathione.
478

The oocyte and zygote are particularly sensitive to damage by ROS and one of their principal anti-oxidant defences is GSH, allowing the conversion of H_2O_2 to water. Accordingly, increasing intra-oocyte GSH by cAMP-mediated pre-IVM suppressed oocyte H_2O_2, which in turn was negated by inhibition of glutathione synthesis during this period. This provides strong evidence that cAMP-mediated pre-IVM provides an important mechanism for oocyte defence against ROS. It would be expected that removing this defence mechanism would adversely affect oocyte quality, and indeed that is what we observed, in the form of reduced pre-implantation embryo development when oocyte GSH accumulation was prevented in just the 4 hour pre-IVM window.

This study shows that extending the duration of cAMP-mediated pre-IVM, to approximate a large part of the GV-GVBD interval, leads to improvements in subsequent oocyte quality. This adds to the growing body of evidence that cAMP-mediated pre-IVM/IVM has great benefit to the oocyte in terms of its capacity to support preimplantation embryo development (Gilchrist 2011). Such IVM approaches are intended to better simulate in vitro, as close as possible, the natural process of oocyte maturation in vivo. A fundamental mode of action of cAMP-mediated pre-IVM systems appears to be the retention of CC-oocyte gap-junctional coupling during the critical first half of oocyte meiosis in vitro. This enhanced functional CC-oocyte coupling affects oocyte chromatin remodeling and transcription (Luciano *et al.,* 2011; Franciosi *et al.,* 2014), oocyte metabolism (Zeng *et al.,* 2013; Zeng *et al.,* 2014), and accumulation of intra-oocyte GSH (current study). As a result of prolonging GJC in COCs, GSH accumulates in the oocyte providing essential protection against oxidative stress. This provides further support to the notion that the appropriate functioning of CCs is required to provide critical metabolites to the oocyte that are required for healthy preimplantation embryo development. Given that the current lower success rate of clinical IVM is the primary impediment to its uptake, application of this simple cAMP-mediated pre-IVM technique stands to bring important benefit if applied to human ART.

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Authors' roles

The study was jointly designed and funded by RBG and JGT. HJL performed all experiments with assistance from MLSM (GSH and H_2O_2 assays), XQW (oocyte maturation, embryology and differential staining experiments), and SS (GJC assay). HJL, JGT and RBG wrote the manuscript which was edited and approved by all authors.

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Conflict of Interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1: Effect of pre-*in vitro* maturation (IVM) duration on bovine oocyte meiotic maturation. Immature cumulus-oocyte complexes (COCs) were cultured in standard IVM with FSH (no pre-IVM + 20 h IVM) or pre-IVM medium containing forskolin (FSK) and 3-isobutyl-1-methyxanthine (IBMX) for 2, 4 or 6 h, then either 18, 16 or 14 h, respectively of standard IVM, or 20 h of IVM for each pre-IVM treatment. Metaphase II rates were assessed (n≥89 COCs per group over 4 replicate experiments). Data are mean ± SEM. Asterisk indicates bars are significantly different (P<0.05; 1-way ANOVA).

Figure 2: Effect of pre-IVM duration followed by standard IVM on bovine embryo development. COCs were subjected to 0, 2, 4, 6 h pre-IVM culture in the presence of FSK and IBMX, followed by standard IVM in the presence of FSH for 24, 24, 22 or 20 h, respectively (**A**). Oocyte developmental capacity was assessed (n≥146 COCs pre group of 5 replicate experiments) after IVF by early cleavage rate (**B,** cleaved embryos/immature oocytes at 22 h on day 1), final cleavage rate (**C**, cleaved embryos/immature oocytes assessed on day 5), blastocyst rate (**D**, total blastocysts/total cleaved embryos assessed on day 8), and hatched blastocyst rate (**E**, hatched blastocysts/total cleaved embryos assessed on day 8). Blastocyst quality (**F**, Day 8) was assessed by quantification of the number of trophectoderm (TE), inner cell mass (ICM) and total cells, and the ratio of ICM:total cells (n = 25-29 per group). Data are mean ± SEM. Asterisk within each graph indicates significantly different (P<0.05; 1-way ANOVA) to the control (0h + 24h).

Figure 3: Effect of pre-IVM duration on bovine cumulus-oocyte gap-junctional communication (GJC) during pre-IVM and IVM stages. The functional coupling between oocyte and cumulus cells was assessed after lucifer yellow dye injection. As shown in the representative images (**A**), gap junctions were classified as open (a), partial (b), or closed (c), and scored as 2, 1, 0, respectively, and the gap junction index calculated. A total of 690 oocytes were used in these experiments (3 replicates per group). **B:** COCs were treated with or without FSK + IBMX in pre-IVM and GJC was assessed at 0, 2, 4 or 6 h. "*a*" represents the

lack of difference between time points within the FSK+IBMX treatment. Means with non-common letters (*x, y, z*) are significantly different (*P*<0.05; 1-way ANOVA) between time points within the control treatment. Asterisks indicate means that are significantly different (*P*<0.05; *t*-test) between pre-IVM treatments at that time point. **C:** COCs were treated with or without FSK + IBMX in pre-IVM for 2, 4 or 6 h, and then cultured in IVM with FSH and then GJC was assessed at 0, 4, 8 or 12 h of IVM. Means with no common superscript letters "*a-b, x-y, p-q*" within a time point are significantly different (*P*<0.05; 2-way ANOVA).

Figure 4: Effect of pre-IVM duration on bovine intra-oocyte reduced glutathione (GSH) levels during IVM. Intact immature COCs were subjected to 0, 2, 4 or 6 h pre-IVM culture with FSK and IBMX, followed by standard IVM for 0 h (end of pre-IVM treatment) or 24 h. After that, COCs were denuded of cumulus cells and the naked oocytes were incubated with monochlorobimane (MCB) to form MCB-GSH adducts, and observed under a laser scanning confocal microscope. **A:** images of oocytes from the four pre-IVM treatments at the end of pre-IVM (0 h IVM; upper panel) and at 24 h of IVM (lower panel). **B:** MCB-GSH relative fluorescent values from each pre-IVM group (n=10 COCs per group over 3 replicate experiments) at 0 h or 24 h of IVM. Means within a time point with no common superscripts *"a-b, x-y"* are significantly different (*P*<0.05; 1-way ANOVA).

Figure 5: Effect of the blocked GJC on bovine intra-oocyte GSH. Immature COCs were incubated for 4 h with or without FSK + IBMX and with or without carbenoxolone (CBX), a known blocker of gap junctions. **A:** The blocking action of CBX was validated on FSK + IBMX treated COCs (n=10 per group over 3 replicate experiments) by assessment of the gap junctional index after 4 h of CBX treatment (**; *P* < 0.01; *t*-test). **B:** Intra-oocyte GSH content, represented by MCB-GSH adduct relative fluorescence value, was assessed after 4 h of culture (n=10 oocytes per group over 3 replicate experiments). Means with no common superscripts (*a-c*) are significantly different (*P* < 0.05; 1-way ANOVA). Data are mean ± SEM.

Figure 6: Effect of inhibition of GSH synthesis on bovine oocyte hydrogen peroxide **(H_2O_2) production and embryonic development.** Immature COCs were not cultured

(control) or cultured with FSK + IBMX for 4 h, either in the absence (Pre-4h) or presence of buthionine sulfoximide (BSO; Pre-4h+BSO). COCs were then denuded and intra-oocyte H_2O_2 production was detected using peroxyfluor-1 (PF-1) staining. Representative images **(A)** and quantitative levels **(B)** of H_2O_2 relative fluorescence in oocytes from the control, Pre-4h, and Pre-4h+BSO groups. **C:** The effect of inhibition of GSH synthesis by BSO was validated by measuring intra-oocyte MCB-GSH staining. Each data observation was made on 10 oocytes and the experiments were replicated three times. Columns (mean ± SEM) within a graph with no common superscript letters (*a-b*) are significantly different ($P < 0.05$; 1-way ANOVA). **D:** Oocyte developmental capacity was assessed on COCs subjected to 4 h pre-IVM (FSK + IBMX) either with or without BSO, followed by standard IVM in the presence of FSH for 24 h. After IVF, blastocyst and hatched blastocyst rates were assessed on day 8. Data are mean ± SEM; n=315 total COCs from 4 replicate experiments. Asterisk (**) within each group indicates significantly different ($P<0.01$; *t*-test) to the Pre-4h group.

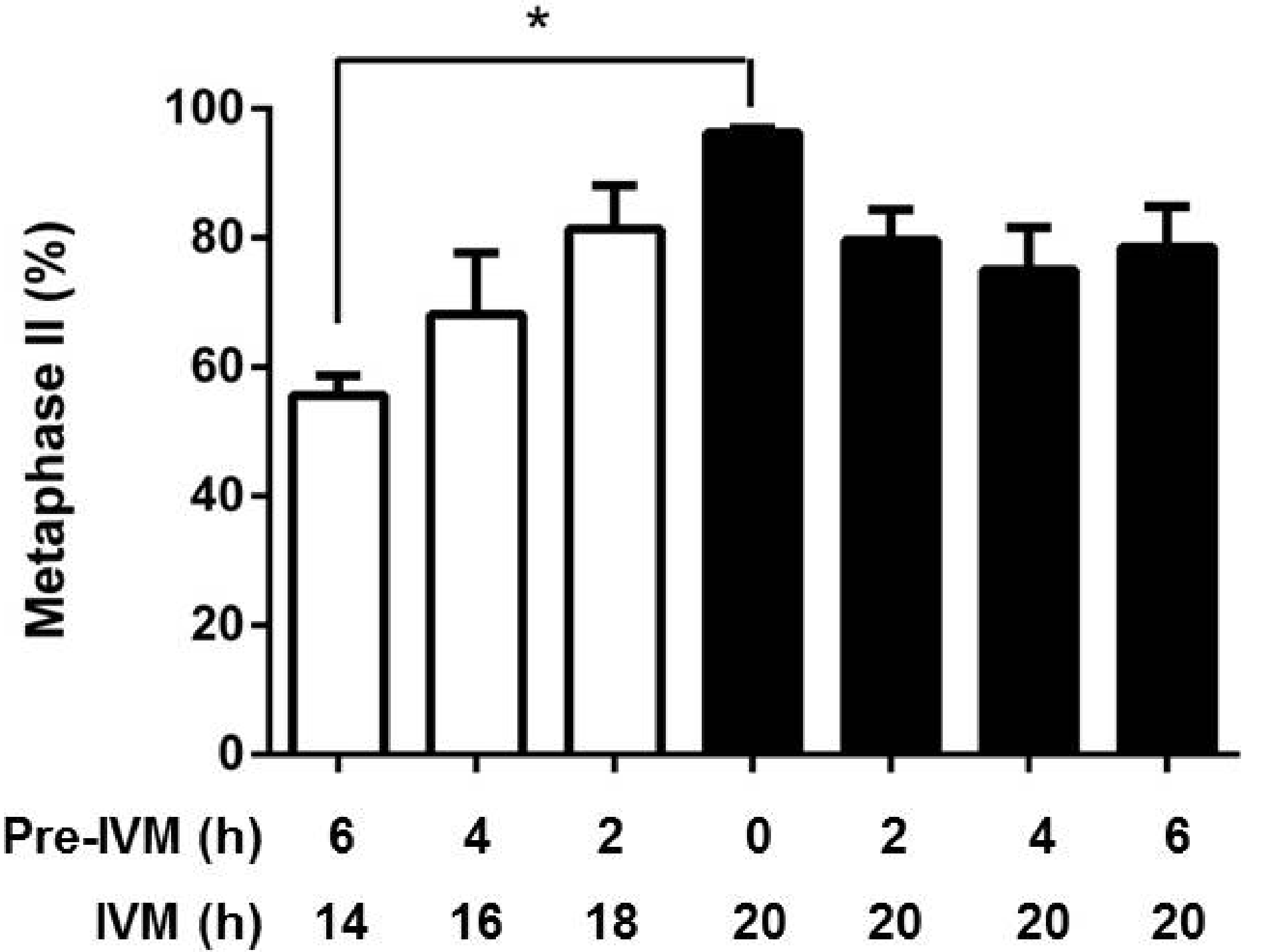
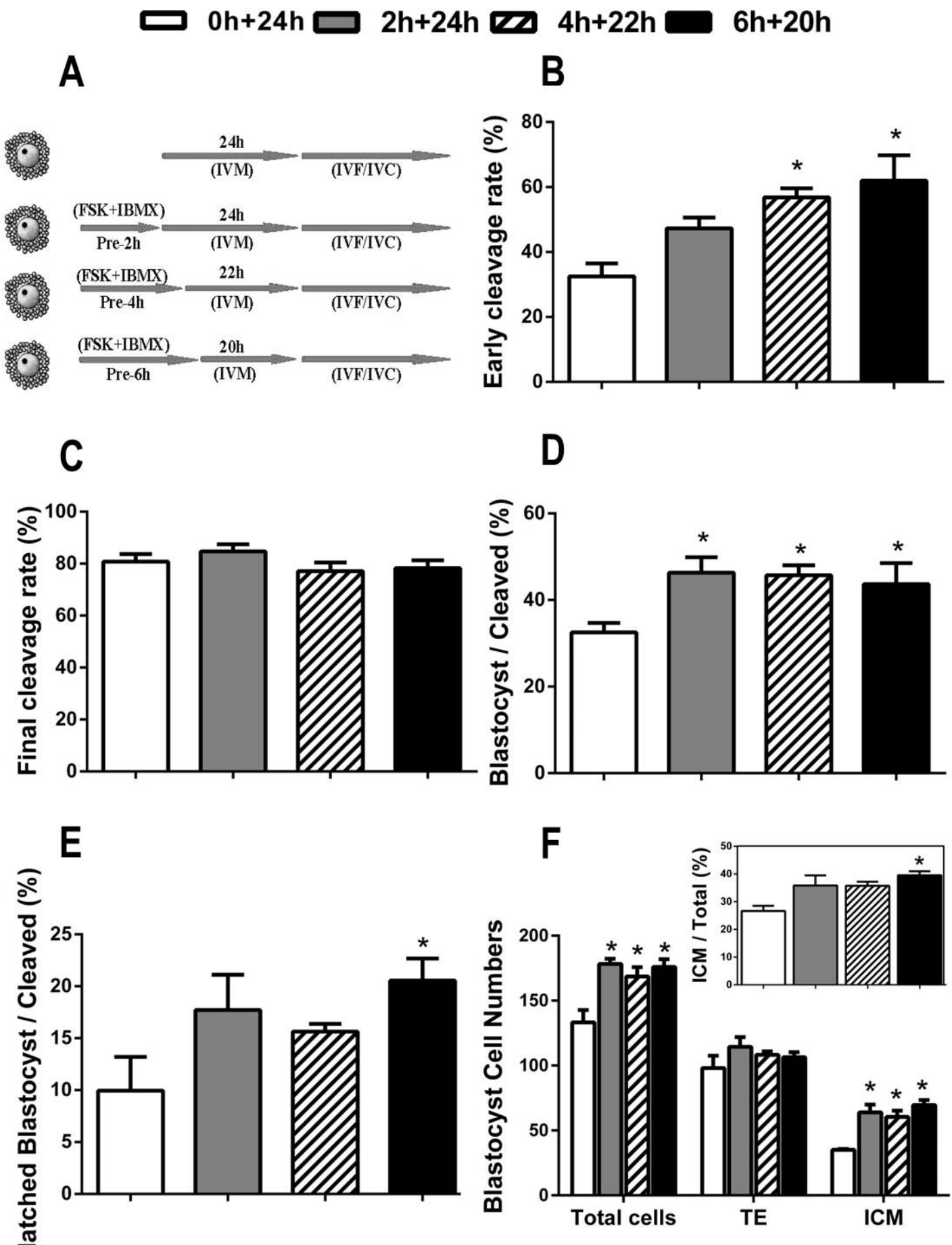
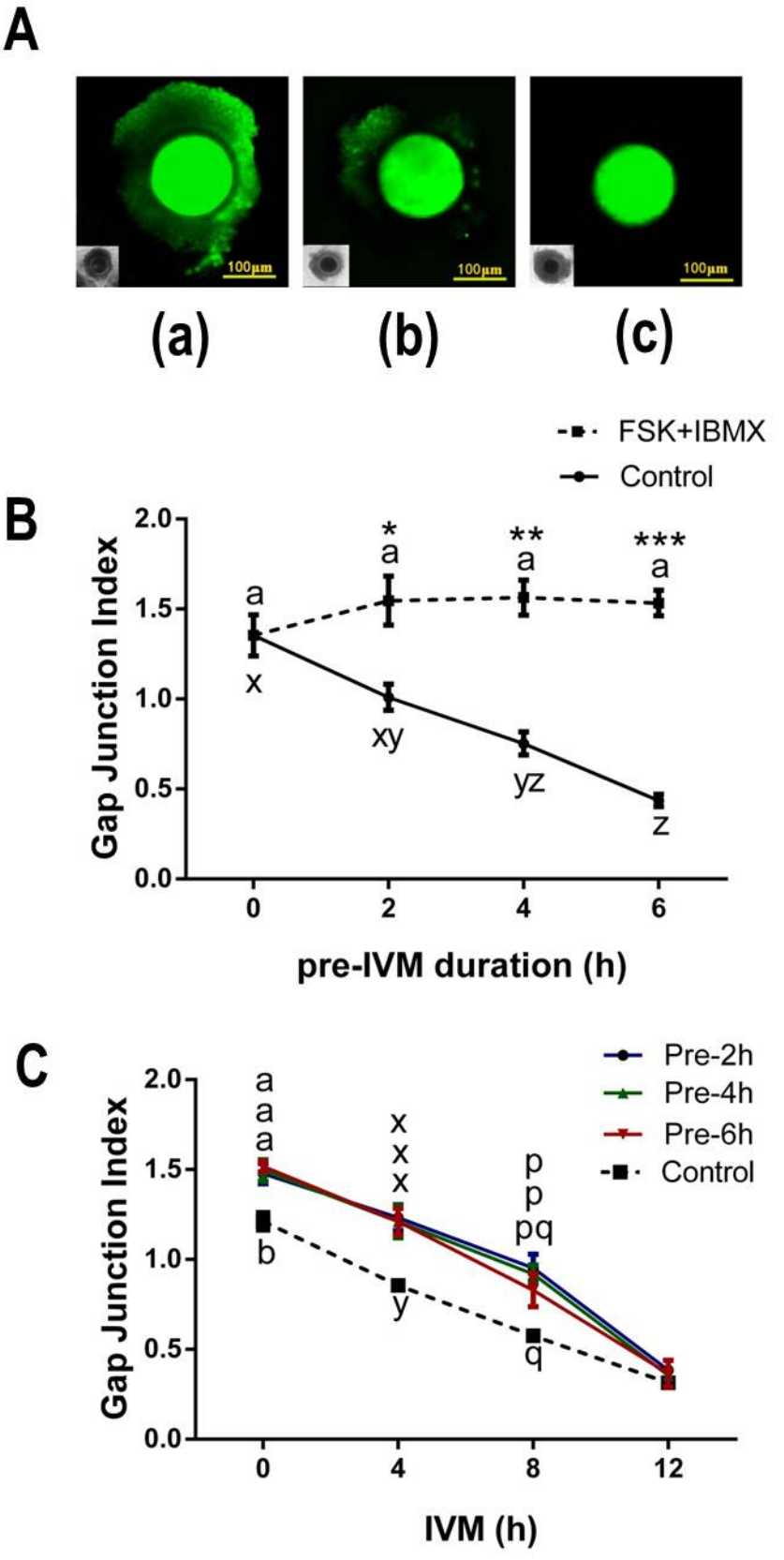
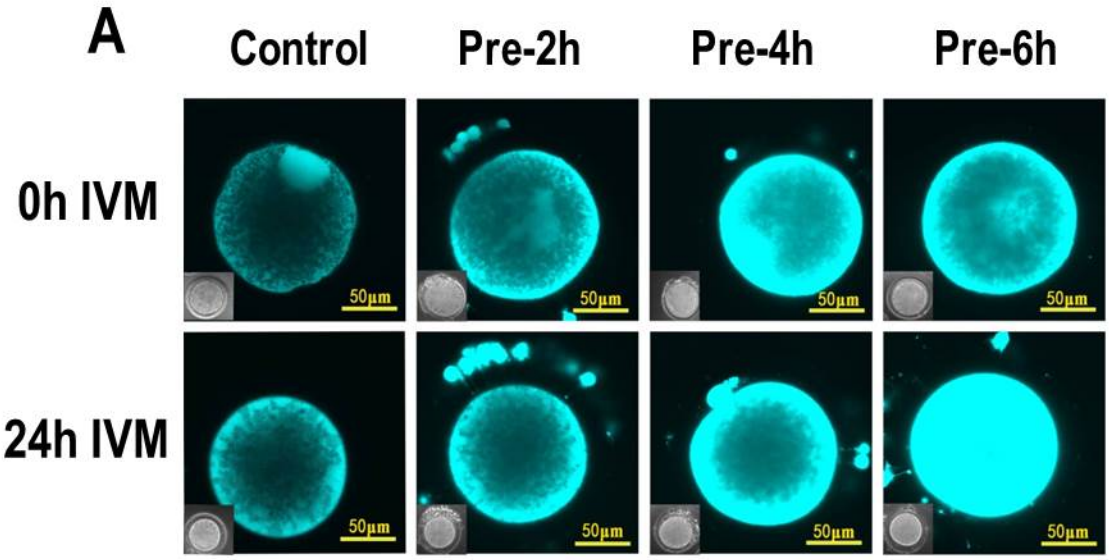
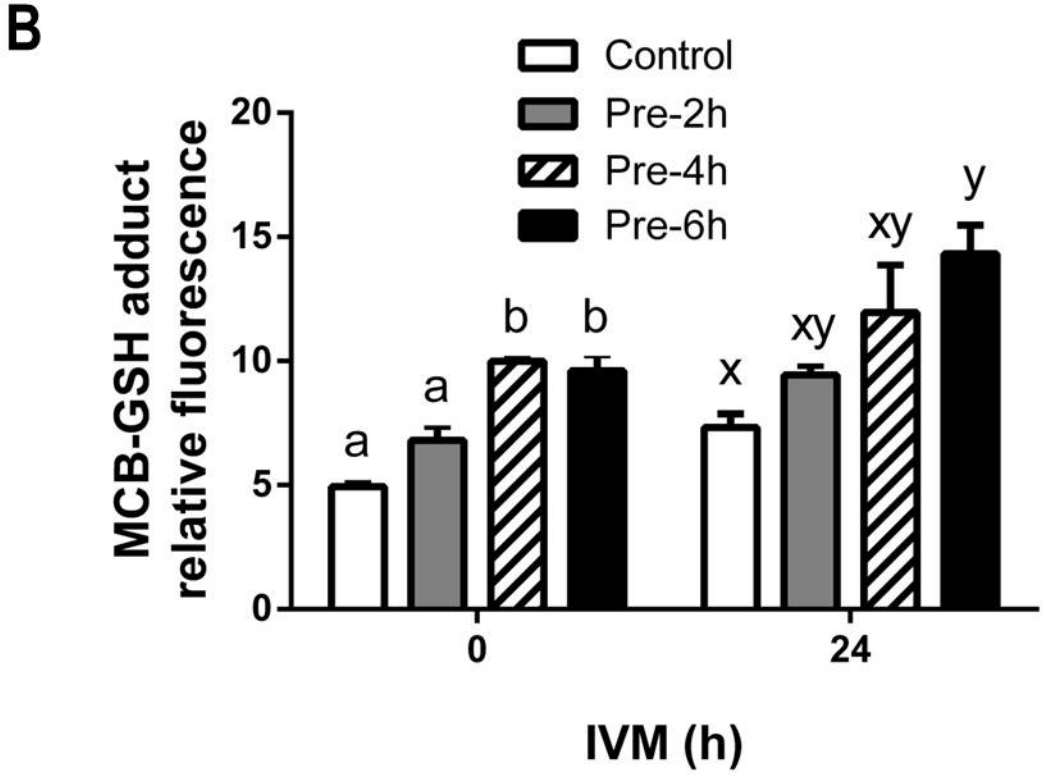

*
Metaphase II (%)
100
80
60
40
20
0
Pre-IVM (h) 6 4 2 0 2 4 6
IVM (h) 14 16 18 20 20 20 20

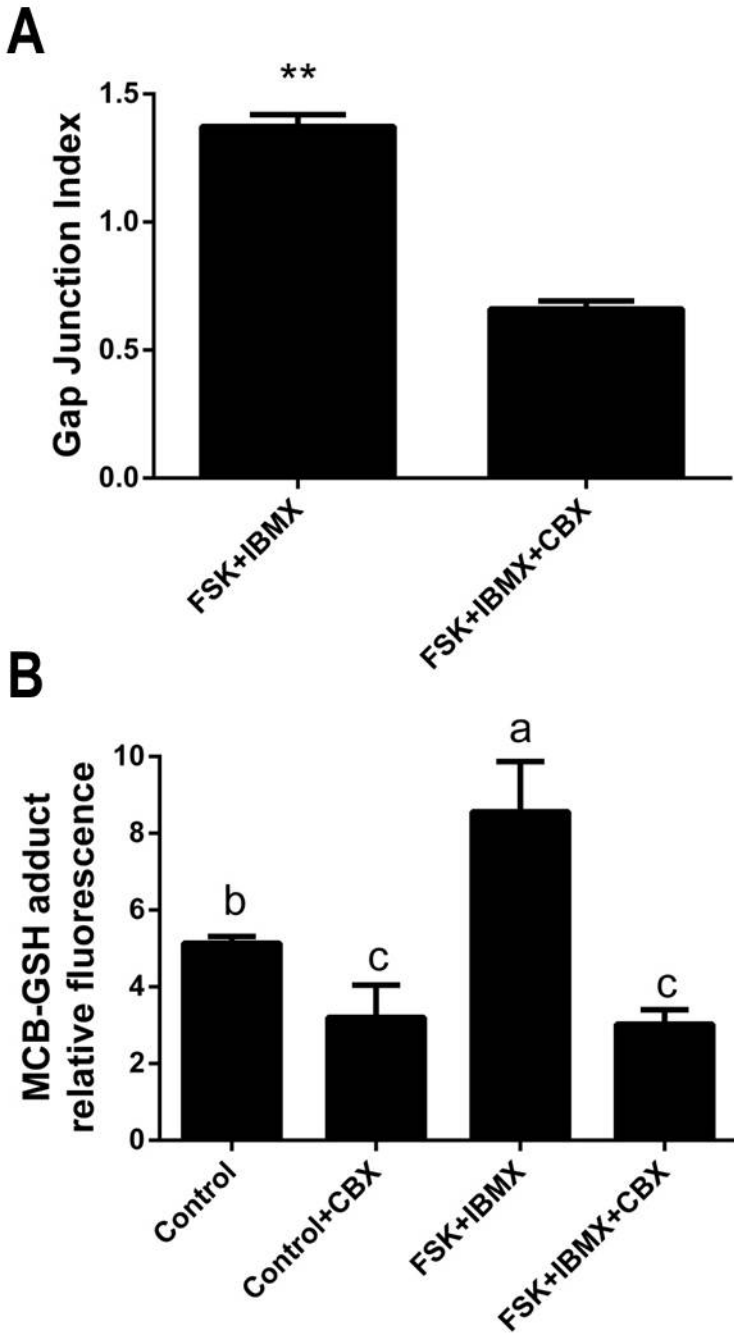
Figure 2

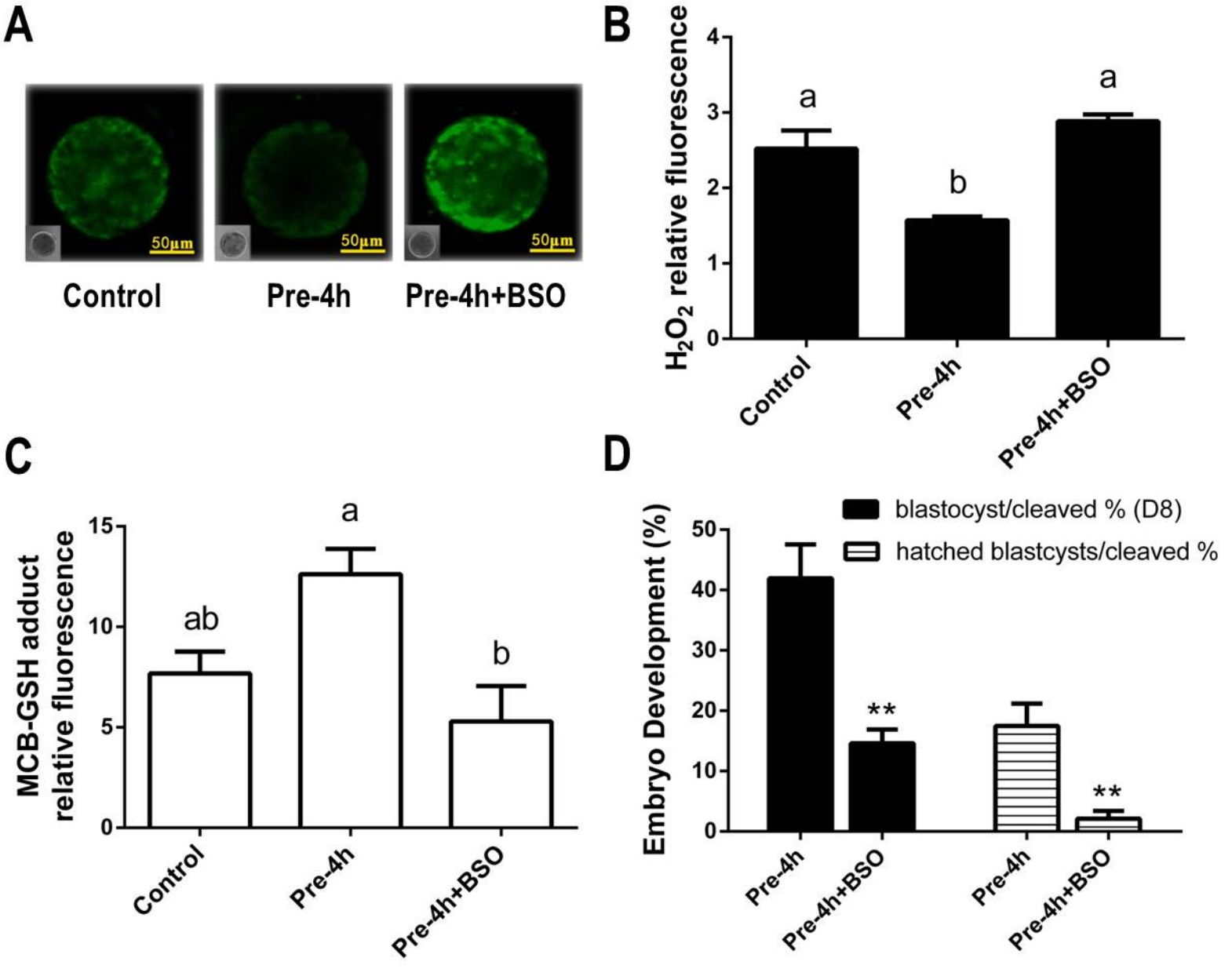

0h+24h
2h+24h
4h+22h
6h+20h
A
24h
(IVM)
(IVF/IVC)
(FSK+IBMX)
Pre-2h
24h
(IVM)
(IVF/IVC)
(FSK+IBMX)
Pre-4h
22h
(IVM)
(IVF/IVC)
(FSK+IBMX)
Pre-6h
20h
(IVM)
(IVF/IVC)
B
Early cleavage rate (%)
C
Final cleavage rate (%)
D
Blastocyst / Cleaved (%)
E
Hatched Blastocyst / Cleaved (%)
F
Blastocyst Cell Numbers
ICM / Total (%)
Total cells
TE
ICM


A
100μm
100μm
100μm
(a)
(b)
(c)
FSK+IBMX
Control
B
Gap Junction Index
2.0
1.5
1.0
0.5
0.0
*
**
***
a
a
a
a
x
xy
yz
z
0
2
4
6
pre-IVM duration (h)
C
Pre-2h
Pre-4h
Pre-6h
Control
Gap Junction Index
a
a
a
x
x
x
p
p
pq
b
y
q
0
4
8
12
IVM (h)


A
Control
Pre-2h
Pre-4h
Pre-6h
0h IVM
24h IVM
50μm


B
Control
Pre-2h
Pre-4h
Pre-6h
MCB-GSH adduct relative fluorescence
20
15
10
5
0
a
a
b
b
x
xy
xy
y
0
24
IVM (h)


A
**
1.5
1.0
0.5
0.0
Gap Junction Index
FSK+IBMX
FSK+IBMX+CBX
B
a
b
c
c
10
8
6
4
2
0
MCB-GSH adduct relative fluorescence
Control
Control+CBX
FSK+IBMX
FSK+IBMX+CBX


A
50μm
50μm
50μm
Control
Pre-4h
Pre-4h+BSO
B
H_2O_2 relative fluorescence
0
1
2
3
4
a
b
a
Control
Pre-4h
Pre-4h+BSO
C
MCB-GSH adduct relative fluorescence
0
5
10
15
ab
a
b
Control
Pre-4h
Pre-4h+BSO
D
Embryo Development (%)
0
10
20
30
40
50
blastocyst/cleaved % (D8)
hatched blastcysts/cleaved %
**
**
Pre-4h
Pre-4h+BSO
Pre-4h
Pre-4h+BSO