Heparin and cAMP modulators interact during pre-*in vitro* maturation to affect mouse and human oocyte meiosis and developmental competence

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Abstract

Study Question: Does heparin ablate the advantageous effects of cAMP modulators during pre-*in vitro* maturation (IVM) and have a deleterious effect in standard oocyte IVM?

Summary Answer: Heparin interrupts energy metabolism and meiotic progression and adversely affects subsequent development of oocytes under conditions of elevated cAMP levels in cumulus-oocyte complexes (COCs) after pre-IVM treatment with forskolin.

What is Known Already: In animal IVM studies, artificial regulation of meiotic resumption by cAMP elevating agents improves subsequent oocyte developmental competence. Heparin has no effect on spontaneous, FSH- or EGF-stimulated meiotic maturation.

Study Design, Size, Duration: An *in vitro* cross-sectional study was conducted using immature mouse and human COCs. Depending on individual experimental design, COCs were treated during pre-IVM with or without heparin, in the presence or absence of forskolin and/or 3-isobutyl-1-methylxanthine (IBMX), and then COC function was assessed by various means.

Participants/Materials, Settings, Methods: Forty two women with polycystic ovaries or polycystic ovarian syndrome donated COCs after oocyte retrieval in a non-hCG-triggered IVM cycle. COCs were collected in pre-IVM treatments and then cultured for 40 hours and meiotic progression was assessed. COCs from 21-24 day old female CBA F1 mice were collected 46 h after stimulation with equine chorionic gonadotrophin. Following treatments, COCs were checked for meiotic progression. Effects on mouse oocyte metabolism were measured by assessing oocyte mitochondrial membrane potential using JC-1 staining and oocyte ATP content. Post-IVM mouse oocyte developmental competence was assessed by *in*

43 *vitro* fertilization (IVF) and embryo production. Blastocyst quality was evaluated by differential staining of
44 inner cell mass and trophectoderm layers.

45 **Main Results and the Role of Chance:** In the absence of heparin in pre-IVM culture, the addition of cAMP
46 modulators did not affect human oocyte MII competence after 40 hours. In standard IVM, heparin
47 supplementation in pre-IVM did not affect MII competence, however when heparin was combined with
48 cAMP modulators, MII competence was significantly reduced from 65% to 15% ($P<0.05$). In mouse
49 experiments, heparin alone in pre-IVM significantly delayed germinal vesicle breakdown (GVBD) so that
50 fewer GVBDs were observed at 0h and 1h of IVM ($P<0.05$), but not by 2h or 3h of IVM. Combined
51 treatment with IBMX and forskolin in the pre-IVM medium produced a large delay in GVBD such that no
52 COCs exhibited GVBD in the first 1h of IVM, and the addition of heparin in pre-IVM further significantly
53 delayed the progression of GVBD ($P<0.05$), in a dose-dependent manner ($P<0.01$). Combined IBMX and
54 forskolin treatment of mouse COCs during pre-IVM significantly increased mitochondrial membrane
55 potential and ATP production in the oocyte at the end of pre-IVM ($P<0.05$), and significantly improved
56 fertilization, embryo development and quality ($P<0.05$). However, heparin abolished the IBMX +
57 forskolin-stimulated increase in mitochondrial membrane potential and ATP production ($P<0.05$), and
58 adversely affected embryonic cleavage, development rates and embryo quality ($P<0.05$). This latter
59 adverse combinational effect was negated when mouse COCs were collected in heparin and IBMX for 15
60 min, washed and then cultured for 45 min in IBMX and forskolin without heparin.

61 **Limitation, Reasons for Caution:** Experiments in mice found that heparin ablation of the advantageous
62 effects of cAMP modulators during pre-IVM was associated with altered oocyte metabolism, but the
63 mechanism by which heparin affects metabolism remains unclear.

Wider Implications of the Findings: This study has revealed a novel and unexpected interaction between heparin and cAMP modulators in pre-IVM in immature mouse and human oocytes, and established a means to collect oocytes using heparin whilst modulating oocyte cAMP to improve developmental potential.

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Introduction

Recovery of immature oocytes followed by *in-vitro* maturation (IVM) is a potentially useful treatment to generate mature oocytes for a range of clinical applications including human infertility treatment and fertility preservation. The major advantages of IVM are avoidance of the risk of ovarian hyperstimulation syndrome (OHSS), reduced cost, simplified treatment, and reduced anxiety that the short-term and long-term side effects of repeated ovarian stimulation may have on maternal and fetal health (Chian *et al*., 2004). However, in comparison with conventional *in vitro* fertilization (IVF), *in vitro* matured oocytes have been shown to be compromised in embryo quality and developmental potential (Trounson *et al*., 2001). In general, current approaches to IVM have much lower pregnancy rates than conventional IVF, with implantation rates rarely exceeding 10 to 15% per embryo transferred and higher early pregnancy losses (Smitz *et al*., 2011).

An innovative approach to IVM for application in infertility treatment and fertility preservation is required to bring this patient-friendly treatment into routine practice. A recent significant clinical innovation is the approach of vitrifying all embryos in an IVM pick-up cycle, which substantially increases implantation rates (De Vos *et al*; 2011, Guzman *et al*; 2012). In terms of laboratory approaches, solid gains in the improvement of oocyte competence have been achieved in animal studies by managing cyclic adenosine mono-phosphate (cAMP) levels during IVM (reviewed; Gilchrist, 2011). Cyclic AMP plays a critical role in maintaining mammalian oocyte meiotic arrest and inducing maturation in mammalian oocytes (Conti *et al.,* 2012). Relatively high levels of cAMP within the oocyte are essential to maintain meiotic arrest, whereas a drop in intra-oocyte concentration of cAMP enables resumption of meiosis and

maturation (Sela-Abramovich *et al*., 2006), and this is the mechanism that allows oocytes to spontaneously mature during IVM. The cornerstone of modern approaches to IVM is to control cumulus-oocyte complex (COC) cAMP levels to allow synchronization of nuclear and cytoplasmic maturation processes within the oocyte (Gilchrist, 2011; Smitz *et al*., 2011). A key outcome of this approach is the maintenance *in vitro* of cumulus-oocyte gap junctions, with the objective to prolong the oocyte maturation period to promote sustained interaction between the immature oocyte with adequately conditioned cumulus cells (Gilchrist, 2011; Luciano *et al*., 2011). Maintenance of an appropriate cAMP concentration seems to be an important requirement to promote chromatin transition and gradual transcriptional silencing, as part of final oocyte differentiation (Luciano *et al*., 2011). A large body of animal and human literature demonstrates that artificial regulation of meiotic resumption by cAMP elevating agents improves subsequent oocyte developmental competence (Funahashi *et al*., 1997; Luciano *et al*., 1999, 2004; Nogueira *et al*., 2003a, b, 2006; Thomas *et al*., 2004; Luciano *et al*., 2011). Our previous study found that combined treatment of COCs with the cAMP modulators cilostamide (type 3-specific phosphodiesterase (PDE) inhibitor) and forskolin, positively influenced human oocyte developmental competence, by exhibiting a synergistic effect on the prevention of loss of gap junctions and the resumption of meiosis, by increasing COC cAMP levels (Shu *et al*., 2008). In another approach termed SPOM (simulated physiological oocyte maturation), we showed using mouse and cow COCs that the first 1–2 h after oocyte collection is critically important and that treatment with forskolin and 3-isobutyl-1-methylxanthine (IBMX, a non-specific PDE inhibitor) during this period has profound long-term consequences on oocyte developmental programming (Albuz *et al*., 2010).

Clinical IVM pick-up aspirates are usually blood laden, which may block the thin IVM aspiration needle, and typically makes the tightly compacted COCs difficult to identify in the aspirate. Heparin acts as an

anticoagulant, preventing the formation of clots and extension of existing clots. It is widely used as an injectable anticoagulant in clinical practice, and can also be used to form an inner anticoagulant surface on various experimental and medical devices. Therefore, during the clinical practice of IVM and IVF, follicular fluid is collected in tubes containing saline or PBS containing heparin. Heparin is sometimes added to collection media in animal IVM research laboratories, although in some species it is often omitted. When the SPOM approach (Albuz *et al*., 2010) was applied in a clinical experiment, we noted an unexpected adverse interaction between heparin and cAMP regulators that prevented human oocyte maturation. Therefore, in this study we hypothesized that heparin ablates the advantageous effects of cAMP modulators during pre-IVM, but has no deleterious effects in standard laboratory IVM practice. Due to the natural shortage of good quality human oocytes for research purposes, the bulk of the mechanistic investigations of this hypothesis were conducted using the mouse model, where the same phenomenon was evident.

MATERIALS AND METHODS

Experimental approvals and reagents

All human oocyte experiments were conducted in Belgium at UZ Brussel, whilst all mouse experiments were conducted at the University of Adelaide, Australia. Human experiments were approved by the local ethics committee and each of the oocyte donors gave written informed consent. All mouse experimental protocols and animal handling procedures were reviewed and approved by the University of Adelaide Animal Ethics Committee. All of the reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Three kinds of heparin purified from porcine intestinal mucosa were used in this study: one high molecular weight (17-19 kDa) heparin (Sigma, H3393-1MU) and two low molecular weight (~6 kDa) liquid heparins from DBL (Hospira Australia Pty Ltd, New Zealand) and Leo (LEO Pharma, Belgium). All mouse experiments were conducted with DBL heparin (DBL) unless otherwise stated.

Clinical and laboratory aspects of human IVM

Forty two consecutive patients and healthy oocyte donors with ultrasound-only polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS) consented to donate oocytes for IVM research at UZ Brussel. Briefly, all women received 150 IU/day highly purified human menopausal gonadotrophin (Menopur; Ferring Pharmaceuticals) for three days, starting on day three after spontaneous menstruation or withdrawal bleeding. An ovarian ultrasound scan was performed on day six to rule out the emergence of a dominant follicle. All patients underwent transvaginal ovarian puncture for immature oocyte retrieval on cycle day seven. No hCG trigger was administered. COCs were retrieved with a 17-gauge single-lumen needle (K-OPS-1230-VUB; Cook Medical, Brisbane, Australia), with an aspiration pressure of 70 mm Hg and collected into pre-IVM media from Cook Medical; either version 1 (without cAMP-modulators) or

version 2 (with forskolin and IBMX), with or without heparin at 10 IU/mL (Heparin Leo®, Leo Pharma, Belgium). Follicular aspirates were filtered (Falcon 1060; 70 mm mesh size) and COCs were collected from the culture dish and incubated in pre-IVM media with treatments for 1 hour. Collected COCs were then washed and cultured individually in 96-well dishes (Costar 3696; Corning. New York, USA), each well containing 75 µL of IVM culture media (version 1; Cook Medical) that had been pre-equilibrated at 37°C and 6% CO_2 and supplemented with 100 mIU/mL FSH and 0.25 µM cilostamide. After 40 hours of IVM culture, COCs were mechanical dissociated using hyaluronidase (Cook Medical) under a stereomicroscope and maturation was assessed under an inverted microscope. These pre-IVM and IVM treatments were designed to test the efficacy of the SPOM IVM approach in humans under clinical conditions (Albuz *et al.,* 2010).

Mouse COC collection and pre-IVM treatments

Immature female CBA F1 mice that were 21-24 days of age were housed in a temperature-controlled and light-controlled room. Immature COCs were collected 46 h after administration of 5 IU of equine chorionic gonadotrophin (eCG; Folligon, Intervet, The Netherlands). Puncture of mouse ovaries and collection of COCs from large antral follicles was performed in HEPES-buffered alpha minimal essential medium (αMEM; Invitrogen, Carlsbad, USA) supplemented with 3 mg/ml bovine serum albumin (BSA) (ICP Biological™, New Zealand) and 1 mg/ml fetuin. All experimental interventions in this study were during the 1 hour pre-IVM phase and hence were conducted in collection medium in HEPES-aMEM medium. Depending on individual experimental design, COCs were exposed during pre-IVM to the adenylate cyclase activator, forskolin (50 µM), with or without the PDE inhibitor IBMX (50 µM) and heparin (50 IU/ml). Millimolar stock concentrations of the forskolin and IBMX were stored at -20°C

188 dissolved in anhydrous dimethylsulphoxide and were diluted fresh for each experiment. COCs were
189 maintained in pre-IVM treatments under atmospheric conditions at 37°C for 1 h. At the end of the
190 pre-IVM phase, COCs were washed twice in their respective IVM treatments, before transfer to IVM
191 drops.
192
193 **Mouse IVM**
194 Mouse COCs were matured in bicarbonate-buffered αMEM medium supplemented with 3 mg/ml BSA.
195 For experiments measuring germinal vesicle breakdown (GVBD) as the primary outcome (figure 2A and
196 figure 3), COCs were cultured in HEPES-buffered αMEM medium supplemented with BSA without FSH for
197 1 h at 37°C under atmospheric conditions, or for 3 h (figure 2B) at 37°C in pre-equilibrated
198 bicarbonate-buffered αMEM medium (with BSA, without FSH) in 6% CO_2 in humidified air. In the
199 experiments where meiosis was assessed (figures 2, 3 and 5), COCs were denuded of cumulus cells at the
200 designated time points and checked for meiotic progression by light microscopy. Where IVM preceded
201 IVF (figure 5), groups of 30-40 COCs were matured in 500 µl drops of pre-equilibrated
202 bicarbonate-buffered αMEM medium supplemented with 50 mIU/ml recombinant human FSH (Puregon,
203 Organon), BSA and 1 mg/ml fetuin, overlaid with mineral oil and incubated at 37°C with 5% CO_2 in
204 humidified air for 18 h prior to IVF.
205
206 **Mouse IVF and embryo culture**
207 The effect of treatments on oocyte developmental competence was assessed by examining the capacity
208 of the oocyte post-IVM to support pre-implantation embryo development. Mouse IVF and embryo
209 culture were performed as previously described (Albuz *et al.*, 2010). All media used were the Vitro

Research Media series generously donated by Cook Medical (Brisbane, Australia). Mouse IVM/embryo experiments were replicated 4 times.

Differential staining of mouse blastocysts

On Day 5 of embryo culture, blastocysts and hatching blastocysts were subjected to a differential staining protocol for identification of cells within the inner cell mass (ICM) and trophectoderm (TE) layers (Dunning *et al*., 2010). The zona was dissolved by incubation in 0.5% pronase at 37°C. Blastocysts were then transferred to 10 mM tri-nitrobenzenesulfonic acid for 10 min at 4°C, followed by incubation in 0.1 mg/ml of anti-dinitrophenyl-BSA for 10 min at 37°C, then 5 min at 37°C in guinea pig serum containing 10 µg/ml of propidium iodide. Blastocysts were then stained overnight in 6 µg/ml of bisbenzimide in ethanol at 4°C. The following day, blastocysts were washed in 100% ethanol and mounted on a siliconized slide in a glycerol drop. The number of pink (TE) and blue (ICM) fluorescent cells was assessed, blinded to treatment group, using a fluorescent microscope and Hg lamp, with an ultraviolet excitation wavelength dichroic mirror at 400× magnification.

Assessment of mouse oocyte metabolism after pre-IVM culture

To determine ATP levels within oocytes, COCs were cultured in their respective pre-IVM treatments for 1 hour and then denuded of cumulus cells. For each sample preparation, 10 oocytes from each treatment were collected in 10 µl of ice cold distilled water and snap-frozen and stored at −80°C for later analysis. The measurement of ATP content in oocytes was performed with an ApoSENSOR^{TM} ATP Assay Kit (BioVision, CA, USA) according to the manufacturer's instructions. The assay utilizes the enzyme luciferase to catalyze the formation of light from ATP and luciferin, and light was measured by using a

TRIAD^{TM} Series Verification Plate (DYNEX, VA, USA). A duplicated 5-point standard curve (0, 0.5, 1, 2 and 3 µM of ATP) was included in the assay. Oocyte ATP content was calculated using the formula derived from the linear regression of the standard curve. Five replicate experiments were conducted using 80-120 oocytes/treatment/replicate.

The mitochondrial membrane potential sensitive fluorescence dye, JC-1 (Molecular Probes, Eugene, OR), was used to measure the activity of oocyte mitochondria. Low-polarized mitochondria ($\delta\Psi m \leq -100$ mV) fluoresce green and high-polarized mitochondria ($\delta\Psi m \geq -140$ mV) fluoresce yellow-red, reflecting the formation of JC-1 multimers or J-aggregates (Reers *et al*., 1995). The stock concentration of the dye was 1 mM in dimethyl sulfoxide. The dye was diluted in culture medium before use. COCs were cultured in their respective pre-IVM treatments for 1 hour, then denuded of cumulus cells and oocytes were cultured for 15 minutes in their respective treatments with 2 µM JC-1. Oocyte fluorescence was observed using a narrow green filter (490-540 nm) and a narrow red filter (570-620nm), using a FluoView FV10i Confocal Microscope (Olympus, Japan). The laser-power and photomultiplier settings were kept constant for all experiments. A single optical scan through the center of the oocyte was used for the analysis. The images were processed and red and green fluorescence intensities of JC-1 in oocytes was measured and analysed using the inbuilt software of the FluoView FV10i Confocal Microscope. Three replicate experiments were conducted using 10-20 oocytes/treatment/replicate.

Statistical analysis

Statistical analyses were conducted using the Statistical Package for Social Sciences 18.0 (SPSS, Chicago, IL). Treatment effects were assessed by Chi-square test (human COC experiment) or one-way analysis of

variance followed by either Dunnett's or Bonferroni's multiple-comparison post-hoc tests to identify individual differences between means. All values are presented as means with their corresponding SEM. Statistical significance was set at $P<0.05$.

Results

Effect of heparin on meiotic maturation of human COCs treated with/without cAMP modulators

In the absence of heparin in pre-IVM, the addition of cAMP modulators (pre-IVM, forskolin + IBMX; IVM, cilostamide) did not affect human oocyte MII competence after 40 hours (Fig 1A). Heparin alone in pre-IVM did not appreciably affect MII competence, however when combined with cAMP modulators, MII competence was significantly ($P<0.05$) reduced from 65% to 15% (Fig 1B), suggesting an adverse interaction between pre-IVM heparin and the pre-IVM cAMP modulators forskolin and IBMX, on human oocyte maturation. Hence, subsequent experiments were conducted using the mouse experimental model to investigate in further detail the nature of such an interaction.

Effect of heparin with/without IBMX and forskolin in pre-IVM on subsequent GVBD of mouse COCs

As expected, treatment with IBMX and/or forskolin during pre-IVM significantly lowered the rates of GVBD at designated time points in IVM, when compared with controls ($p<0.05$). As shown in Fig. 2A and 2B, heparin alone in pre-IVM also significantly ($p<0.05$) decreased GVBD at 0h and 1h of IVM, but not by 2h or 3h of IVM. Furthermore, an additive effect of heparin and IBMX and/or forskolin in pre-IVM was observed, whereby heparin further inhibited GVBD in each IBMX/forskolin treatment combination ($p<0.05$). In the combined treatment of IBMX and forskolin in pre-IVM, oocytes commenced GVBD after 2 h of IVM, however the addition of heparin in pre-IVM further significantly decreased GVBD rates for the

first 3h of the IVM phase ($p<0.05$) (Fig. 2B).

As there are many different heparin variants, each with different vehicles and preservatives, we examined the capacity of different types of heparin to interact with cAMP modulators in pre-IVM, on subsequent meiotic resumption in IVM. When used together with IBMX and forskolin in pre-IVM, both low molecular weight liquid injectable heparin variants containing preservatives (~6kDa; Leo and DBL), and a high molecular weight, ostensibly preservative-free, powdered heparin (17 – 19 kDa; Sigma), inhibited GVBD to comparable extents after 2 hour of IVM ($P<0.05$, Fig. 3A). In the presence of IBMX and forskolin in pre-IVM, there was a significant dose-dependent inhibition of oocyte GVBD during the IVM phase ($P<0.01$, Fig. 3B). This experiment yielded higher GVBD rates than those in Fig 2, which we attribute to a different operator using subtly different denuding techniques and efficiency of assessment which, in the absence of a PDE inhibitor in IVM, have large affects on GVBD rates in mouse oocytes. All other mouse experiments in this study used DBL heparin at 50 IU/ml.

Effect of IBMX, forskolin and heparin on energy metabolism in mouse oocytes

Cyclic AMP is generated from ATP and hence treatment during pre-IVM with IBMX and/or forskolin is likely to impact on COC metabolism. Relative to levels at collection from the follicle, intra-oocyte ATP levels did not change after 1 h of pre-IVM treated with 50 μM IBMX or 50 μM forskolin (Fig. 4A). However, combined IBMX and forskolin treatment of COCs during pre-IVM significantly increased ATP concentration in the oocyte after 1 h ($p<0.05$). Heparin had no affect on oocyte ATP levels in the presence of IBMX, tended to decrease levels in the presence of forskolin ($p=0.102$), and abolished the increase in ATP concentration in the presence of IBMX and forskolin ($p<0.05$), generating less than half

298 the ATP content (Fig. 4A). This latter adverse combinational effect was negated when COCs were
299 collected in heparin and IBMX for 15 mins, washed and then cultured for 45 mins in IBMX and forskolin
300 without heparin, to the extent that ATP levels were restored to levels comparable to IBMX + forskolin
301 ($p>0.05$; Fig 4A).
302
303 We also examined oocyte mitochondrial activity after pre-IVM treatments using the mitochondrial
304 membrane stain JC-1. As shown in figs. 4B and 4C, in the control group of COCs undergoing GVBD, the
305 ratio of red/green fluorescence intensity was more than doubled ($p<0.05$) from 0 h to 1 h of pre-IVM
306 culture. Although the ratio was also significantly increased from collection to 1 h when treating with
307 heparin ($p<0.01$), it was nonetheless lower than the 1 h control ($p<0.01$). With any combination of IBMX
308 +/- forskolin +/- heparin, the majority of oocytes were still at the GV stage after 1 h of pre-IVM (Figs 2,
309 4C), when JC-1 staining was performed. When treated with IBMX, regardless of the presence of heparin,
310 oocyte mitochondrial membrane hyper-polarisation levels were significantly ($p<0.05$) lower than in
311 control oocytes (1 h). In the absence of heparin, when compared with IBMX, forskolin seemed more
312 effective at increasing the ratio of red/green fluorescence intensity in oocytes ($p<0.01$). IBMX notably
313 amplified this stimulating effect of forskolin (Figs 4B, 4C; $p<0.05$), supporting the observed elevated ATP
314 levels in this treatment group (Fig 4A). However, heparin abolished the stimulation of mitochondrial
315 function by forskolin ($p<0.01$), and it was more apparent in treatment with IBMX and forskolin, with less
316 than half the ratio of red/green fluorescence intensity in these oocytes ($p<0.01$).
317
318 **Effect of heparin with/without IBMX and forskolin in pre-IVM on subsequent maturation and**
319 **developmental competence of mouse oocytes**

To determine the developmental consequences of pre-IVM treatment with heparin with/without IBMX and forskolin, following pre-IVM, COCs were subjected to standard IVM (with FSH), IVF and embryo culture to day 5 (Fig. 5). Whilst IBMX and forskolin initially delayed meiotic resumption (Fig. 2), these oocytes were not delayed in reaching MII, but rather the treatment promoted increased maturation of oocytes to the polar body stage compared to the control (Fig. 5A, 87.3%, $P<0.05$). In the absence of IBMX + forskolin, heparin had no effect on the maturation rate when compared to control ($P>0.05$), but significantly ($p<0.05$) reduced maturation in the presence of IBMX + forskolin. Compared with the control, pre-IVM with IBMX and forskolin significantly increased cleavage, blastocyst and hatched blastocyst rates (Fig. 5B-D, $P<0.05$). IBMX and forskolin also improved embryo quality, reflected in an increased ICM cell number and a higher ratio of ICM/total cells compared with the control (Fig. 5E, 5F, $P<0.05$). Heparin alone had no adverse effect on cleavage or embryo development rates or on embryo quality compared to the control ($P<0.05$). However, when combined with IBMX and forskolin, heparin significantly decreased the cleavage rate (Fig 5B; $P<0.05$) compared to the control group, and notably antagonized the beneficial effects of forskolin and IBMX, in terms of cleavage and hatched blastocyst rates, ICM cell numbers and ICM/total cell numbers (Fig 5; $P<0.05$).

As heparin + IBMX + forskolin combined in pre-IVM adversely affected the majority of parameters of COC function assessed in this study, but the combination of heparin + IBMX only weakly inhibited GVBD of oocytes and had no harm on mitochondrial activity (Fig. 1 and 3), the effect of this treatment on oocyte maturation and subsequent developmental competence was assessed. Compared to the control, treatment during pre-IVM with heparin and IBMX for 1 hour had no effect on oocyte maturation, cleavage, blastocyst or hatched blastocyst rates, or on subsequent blastocyst cell numbers (Fig. 5). When

COCs were collected in medium with IBMX and heparin for 15 mins, and then washed and transferred to medium with IBMX and forskolin without heparin for 45 mins (IH-IF; Fig 5), oocyte maturation, cleavage and hatched blastocyst rates were all significantly ($p<0.05$) higher than when COCs underwent pre-IVM with heparin + IBMX + forskolin.

Discussion

Mammalian oocyte maturation *in vitro* and subsequent developmental competence is influenced to a great extent by the culture media and culture conditions employed, including the addition of hormones, growth factors and other specific signal transduction regulators (Gilchrist, 2011). There is now a large body of evidence that management of oocyte and cumulus cell cAMP during IVM is desirable and is an effective means to enhance oocyte developmental competence (Smitz *et al*., 2011). Consistent with previous studies using a cAMP-mediated biphasic-IVM approach (Funahashi *et al.,* 1997; Nogueira *et al*., 2003a; Nogueira *et al*., 2003b; Shu *et al*., 2008; Vanhoutte *et al*., 2009) and with simulated physiological oocyte maturation (Albuz *et al*., 2010), compared to standard IVM, elevating COC cAMP in this study generated higher rates of mouse oocyte maturation and embryo development and improved embryo quality, as reflected in an increased hatched blastocyst rate and ICM size. This was achieved by rapidly increasing cAMP levels in COCs with forskolin and IBMX for just the one hour pre-IVM phase. However, under such elevated cAMP conditions, the inclusion of heparin in pre-IVM media affected human and mouse oocyte maturation, and had a notable detrimental interaction with forskolin on mouse oocyte metabolism and subsequent developmental potential. By contrast, collection medium with IBMX and heparin caused a transient arrest in oocyte meiotic resumption, without compromising subsequent

maturation and fertilization, suggesting that this may be a good clinical option for oocyte collection when using cAMP modulators.

In our study, in the absence of cAMP modulators, heparin had no affect on the capacity of human or mouse oocytes to complete meiosis, but delayed mouse oocyte meiotic resumption. Heparin did not completely prevent GVBD during spontaneous maturation, but delayed it so that after 2 hours oocytes exhibited similar GVBD rates to spontaneously maturating oocytes in heparin-free culture. These results are consistent with previous studies showing generally a benign effect of heparin on standard oocyte meiotic maturation. Heparin has no effect on spontaneous, FSH- or EGF-stimulated murine meiotic maturation (Eppig, 1981; Downs, 1989). In bovine COCs, the addition of heparin under standard IVM conditions may affect the kinetics of GVBD and possibly MII, without notably affecting COC cAMP levels (Fenton *et al*., 1993; Flores-Alonso *et al*., 2008). These minor affects of heparin in standard IVM are consistent with the almost universal use of heparin as an additive to clinical IVM collection media (human and veterinary).

It has however been known for decades that heparin and other glycosaminoglycans affect matrix formation and cumulus expansion of *in vitro* maturing COCs, albeit in a highly species-specific manner. In mice, heparin blocks hyaluronic acid synthesis and cumulus expansion of COCs stimulated by FSH, epidermal growth factor (EGF), prostaglandin E (PGE) or dbcAMP (Eppig, 1981), whereas heparin stimulates cumulus expansion in bovine COCs independent of FSH or cAMP (Fenton *et al*., 1993). Watson *et al.* (2012) recently found that heparin is likely to prevent mouse cumulus expansion by binding to endogenous GDF9 and disrupting its interaction with heparan sulphate proteoglycan coreceptor(s) on cumulus cells, important for GDF9 signaling and cumulus cell function in the periovulatory follicle. Hence,

385 the addition of heparin to IVM COCs is likely to have significant effects on oocyte-secreted GDF9
386 signalling to cumulus cells and thereby on cumulus cell differentiation and function, although this may
387 not account for the more acute temporal effects of heparin observed in this study on oocyte meiotic
388 resumption and maturation.

389 In general, in the current study, there was a notable adverse interaction between heparin and the
390 combined cAMP modulators on most aspects of human and mouse oocyte function assessed. Heparin
391 further inhibited GVBD in mouse oocytes incubated with either IBMX or forskolin, and this was
392 exacerbated when IBMX and forskolin were combined in pre-IVM. Under these pre-IVM conditions,
393 heparin dose-dependently inhibited mouse oocyte GVBD during the IVM phase. In addition, human
394 oocyte maturation was all but prevented when COCs were collected in heparin together with forskolin
395 and IBMX. The mechanism by which heparin additively inhibits GVBD of GV-stage oocytes in the presence
396 of IBMX and forskolin in pre-IVM is currently unclear.
397

398 There is no clear evidence to our knowledge that heparin affects COC cAMP levels, but heparin interrupts
399 the actions of Ca^{2+} in COCs. In the majority of fully grown, immature mouse oocytes, repetitive transient
400 Ca^{2+} oscillations occur every 2–3 minutes and last for 2–4 hours soon after release from the antral follicles
401 (Carroll *et al*., 1994). Ca^{2+} influx occurs in oocytes, mostly via L-type Ca^{2+} channels and gap junctions
402 (Eppig and Downs, 1984), and Ca^{2+} influx decreases as maturation progresses (Tosti *et al*., 2000). The
403 acquisition of meiotic competence is related to the functionality of the IP_3 pathway and, correspondingly,
404 to the oocyte's ability to generate spontaneous cytoplasmic IP_3-dependent calcium oscillations (Lefèvre *et*
405 *al*., 1997). Heparin is a well known inositol 1,4,5-trisphosphate (IP_3) receptor antagonist and

406 microinjection of heparin into bovine oocytes suppresses spontaneous Ca^{2+} oscillations and inhibits GVBD
407 (Homa, 1991).
408
409 In the current study, it was evident that when mouse COCs were stimulated with forskolin, heparin
410 interrupted the metabolism of the oocyte with decreased mitochondrial activity and ATP production. This
411 may be as a result of interrupting the actions of high cAMP in regulating Ca^{2+} transport processes from
412 endoplasmic reticulum (ER) to mitochondria. Increased cAMP can induce Ca^{2+} rises and the accumulation
413 of Ca^{2+} (Dolphin, 1999), which can affect communication between the endoplasmic reticulum (ER) and
414 mitochondria (Vandecasteele *et al*., 2001; Mendes *et al*., 2005; Szabadkai *an*d Duchen, 2008). The ER and
415 mitochondria are closed endomembrane networks which control different aspects of cellular metabolism.
416 Close apposition of IP_3-gated channels (IP_3 receptors) to the mitochondrial surface enables the uptake of
417 Ca^{2+} by mitochondria during cell stimulation (Vandecasteele *et al*., 2001; Mendes *et al*., 2005). Heparin
418 prevents IP_3 interacting with its receptor, and thus inhibits the Ca^{2+} release from internal stores and
419 induces a Ca^{2+} drop. Thus, we suggest that the notably decreased oocyte metabolism may be caused by
420 heparin-mediated interruption of cAMP/calcium intra-cellular signaling and that this in turn adversely
421 affected oocyte meiotic and developmental competence.

422 In this study, treatment of mouse COCs during pre-IVM with IBMX and forskolin had notable positive
423 effects on oocyte meiotic maturation, embryo cleavage and blastocyst rates and embryo quality, as well
424 as impacting COC metabolism, particularly mitochondrial activity and ATP availability. This is generally
425 consistent with our previous mouse and bovine study (Albuz *et al*., 2010) which showed that oocyte
426 developmental competence is improved by treating COCs at collection with either forskolin or IBMX to
427 prevent loss of COC cAMP and to substantially increase COC cAMP levels, as occurs during *in vivo* oocyte

maturation (Schultz *et al*., 1983). Perhaps surprisingly, combined IBMX and forskolin treatment of mouse COCs during pre-IVM did not deplete the oocyte of ATP but rather significantly increased ATP production in the oocyte after 1 hour. In such an *in vitro* scenario, when COCs are treated with forskolin, cAMP is principally generated by cumulus cells from cumulus cell ATP and is then transported to the oocyte via gap junctions (Thomas *et al*., 2002). The increased oocyte ATP levels after forskolin and IBMX treatment correlated to increased oocyte mitochondrial activity. Cyclic AMP can diffuse in the cell where it can set up localized gradients in subcellular structures (Zaccolo *et al*., 2002), and it can also be produced directly in the mitochondrial matrix by the soluble adenylyl-cyclase localized in this compartment (Zippin *et al*., 2003). Cyclic nucleotide (cAMP and cGMP) phosphodiesterases have been found in the inner and outer mitochondrial membranes (Cercek and Houslay, 1982). These adenylyl-cyclases and phosphodiesterases can modulate the mitochondrial level of cAMP (Baillie *et al*., 2005). In humans (Papa *et al*., 2001) and other mammals (Technikova-Dobrova *et al*., 2001), cAMP-dependent phosphorylation of complex I subunits is associated with stimulation of the NADH-ubiquinone oxidoreductase activity of the complex, resulting in enhancement of overall cellular respiration (Papa, 2006). This may be a means by which forskolin and IBMX could stimulate mitochondrial membrane potential and thereby ATP production by the oocyte, to generate high oocyte maturation and blastocyst rates and improved embryo quality.

Heparin is a necessary reagent used in clinical IVM aspirations as it prevents blood clotting and greatly assists COC collection. By contrast, research IVM laboratories using oocytes from animals *post-mortem*, typically use no heparin (mouse) or low/no heparin (domestic species) during the oocyte collection phase. As the use of heparin with forskolin was harmful to oocyte maturation and subsequent competence (current study), we sought to explore this interaction further in order to find an optimal medium combination for clinical COC aspiration and collection, which enhances embryo yield from IVM oocytes.

When mouse COCs were collected with heparin and IBMX, but without forskolin, no adverse effect of heparin on mitochondrial membrane polarity or ATP synthesis was detected, and similar oocyte maturation and developmental competence rates were achieved, compared to the control with heparin. Moreover, a feasible option to achieve high blastocyst formation rates, was to collect COCs in medium with IBMX and heparin, then transfer to pre-IVM medium with forskolin and IBMX (IH-15min + IF-45min; Figure 5).

This study has confirmed that, under standard pre-IVM and IVM conditions, heparin exposure during pre-IVM has benign effects on oocyte maturation and developmental potential, but that under elevated cAMP conditions during pre-IVM, heparin adversely affects oocyte energy metabolism, oocyte meiotic maturation and subsequent embryo development. Collecting oocytes with IBMX and heparin may be a good option for pre-IVM where forskolin is used in clinical applications.

Authors'roles

R.B.G., J.S. and H.T.Z secured funding for the project. R.B.G., J.G.T. and H.T.Z designed the study and interpreted the results. L.G, M.D.V and J.S performed the human experiments, and H.T.Z. performed the majority of mouse experiments with some contributions from Z.R., X.W., M.L.S. and L.J.R. H.T.Z. and R.B.G. wrote the manuscript and all authors contributed to editing. All authors approved the final version.

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Conflict of Interest

RBG and JGT are consultants to Cook Medical, the other authors have no conflicting interests to declare.

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Figure Legends

Figure 1. Interaction of heparin with cAMP modulators (forskolin and 3-isobutyl-1-methylxanthine [IBMX]) on human oocyte meiotic maturation. Immature human cumulus-oocyte complexes (COCs) were aspirated from antral follicles and held in pre-*in vitro* maturation (IVM) treatment medium for 1 h, either A) without heparin or B) with heparin with or without forskolin and IBMX, and then cultured in IVM medium for 40 h. Columns represent the maturation rate after IVM culture. Columns from left to right represent the following number of COCs (A, 71 and 102; B 34 and 240). *Significantly different ($P<0.05$) to –cAMP modulator.

Figure 2. Effect of heparin with and without 3-isobutyl-1-methylxanthine (IBMX) and forskolin during pre-*in vitro* maturation (IVM), on germinal vesicle breakdown (GVBD) rates of mouse oocytes after 1 hour of IVM (A), and on the kinetics of GVBD during pre-IVM and IVM (B). Mouse cumulus-oocyte complexes (COCs) were collected and held in each treatment medium for 1 h (pre-IVM), then washed, cultured in treatment-free IVM medium for 1 h (A) or for defined periods up to 3h (B), then denuded and assessed for GVBD. Columns of (A) from left to right represent the following number of mouse COCs (294, 359, 137, 205, 159, 268, 232 and 226). Lines of (B) from left to right (0h, 1h, 2h, 3h) represent the following number of mouse COCs (control: 131, 119, 137, 148; heparin: 127, 152, 136, 159; IBMX + forskolin: 135, 128, 149, 162; IBMX + forskolin + heparin: 143, 133, 157, 140). Data points represent mean + SEM of three to eight replicates. *Significantly different ($P<0.05$) to –heparin condition (A) and control group (B); $^{\#}$significantly different ($P<0.05$) to IBMX + forskolin group (B).

Figure 3. (A) Effect of heparin type in pre-*in vitro* maturation (IVM) with and without

3-isobutyl-1-methylxanthine (IBMX) and forskolin on germinal vesicle breakdown (GVBD). (B) Effect of heparin dose in pre-IVM medium containing IBMX and forskolin on subsequent mouse oocyte GVBD. Mouse cumulus-oocyte-complexes (COCs) were collected and held in each treatment medium for 1 h (pre-IVM), then washed, cultured in treatment-free IVM medium for 1 h, then denuded and assessed for GVBD. Data points represent mean + SEM of four (A) and three (B) replicates. Columns from left to right represent the following number of COCs (A: 102, 111, 93, 125, 82 and 104; B: 119, 62, 101 and 77). *Significantly different ($P<0.05$) to –heparin condition (A), and means within a graph with different letters are significantly different ($P<0.05$) (B).

Figure 4. Effect of heparin in pre-*in vitro* maturation (IVM) with and without 3-isobutyl-1-methylxanthine (IBMX) and forskolin on mouse oocyte ATP content (A) and mitochondrial activity (B, C) at the end of pre-IVM. Mouse cumulus-oocyte-complexes (COCs) were collected; without culture (0 h control), or COCs were held in each treatment medium for 1 h (pre-IVM), including in one treatment where COCs were exposed to IBMX + heparin for 15 mins (IH – 15 min) followed by IBMX + forskolin without heparin for 45 mins (IF – 45 mins). Thereafter COCs were denuded, washed, and either immediately snap frozen for ATP measurement (A), or cultured for a further 15 mins with JC-1 before assessment of mitochondrial function (B, C). In (A) a total of 600 oocytes in each treatment were assay in 50 oocytes/test tube. (B) Columns from left to right represent the following number of oocytes (41, 25, 46, 28, 66, 44, 39, 60 and 55). Data points represent mean +SEM of five (A) and three (B) replicate experiments. Means with no common superscripts are significantly different ($P<0.05$).

Figure 5. Effect of heparin in pre-*in vitro* maturation (IVM) with and without 3-isobutyl-1-methylxanthine

(IBMX) and forskolin on the subsequent meiotic and developmental competence of mouse oocytes. Mouse cumulus-oocyte-complexes (COCs) were collected and held in each treatment medium for 1 h (pre-IVM), including in one treatment where COCs were exposed to IBMX + heparin for 15 min (IH – 15 min) followed by IBMX + forskolin without heparin for 45 min (IF – 45 min). After pre-IVM COCs were washed, cultured in treatment-free IVM medium for 18 h, and then either; denuded and assessed for meiotic maturation (A), or COCs were fertilized *in vitro* and cultured for 5 days (B-D). Blastocyst quality was assessed with differential staining by cell number of ICM (E) and ratio of ICM/total blastocyst cell number (F). Columns of (A - D) from left to right represent the following number of oocytes (109, 127, 130, 137, 119 and 134), and columns of (E) and (F) from left to right represent the following number of analyzed blastocysts (23, 19, 29, 19 ,22 and 23). Data points represent mean +SEM of four replicates. Means with no common superscripts are significantly different ($P<0.05$).

Figure 1

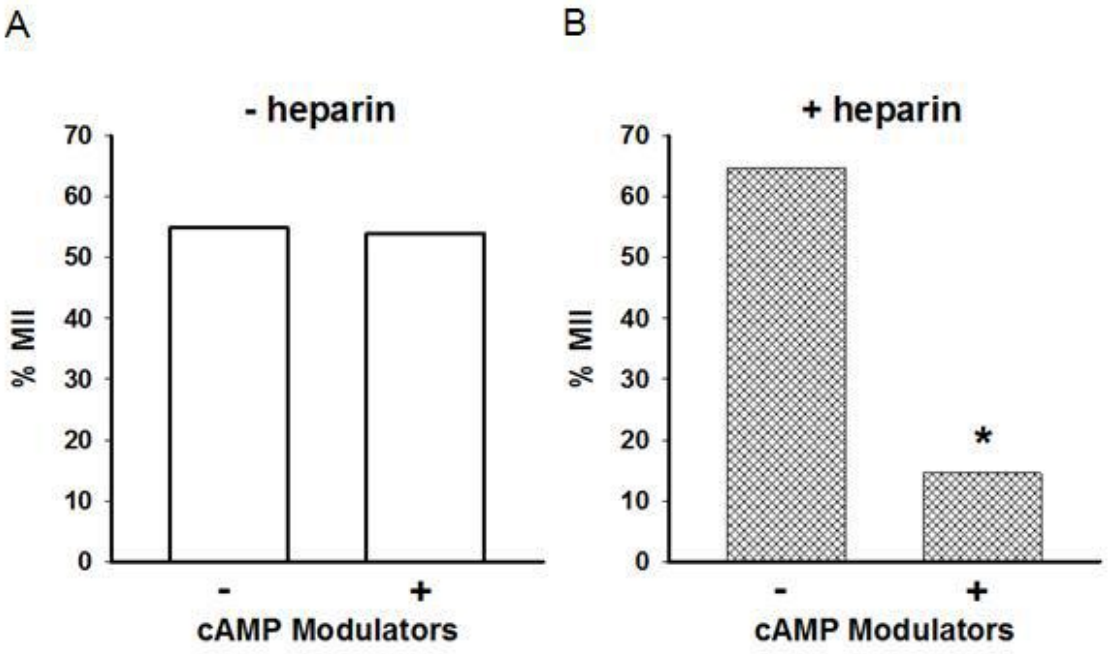

A
- heparin
% MII
70
60
50
40
30
20
10
0
-
+
cAMP Modulators
B
+ heparin
% MII
70
60
50
40
30
20
10
0
*
-
+
cAMP Modulators

Figure 2

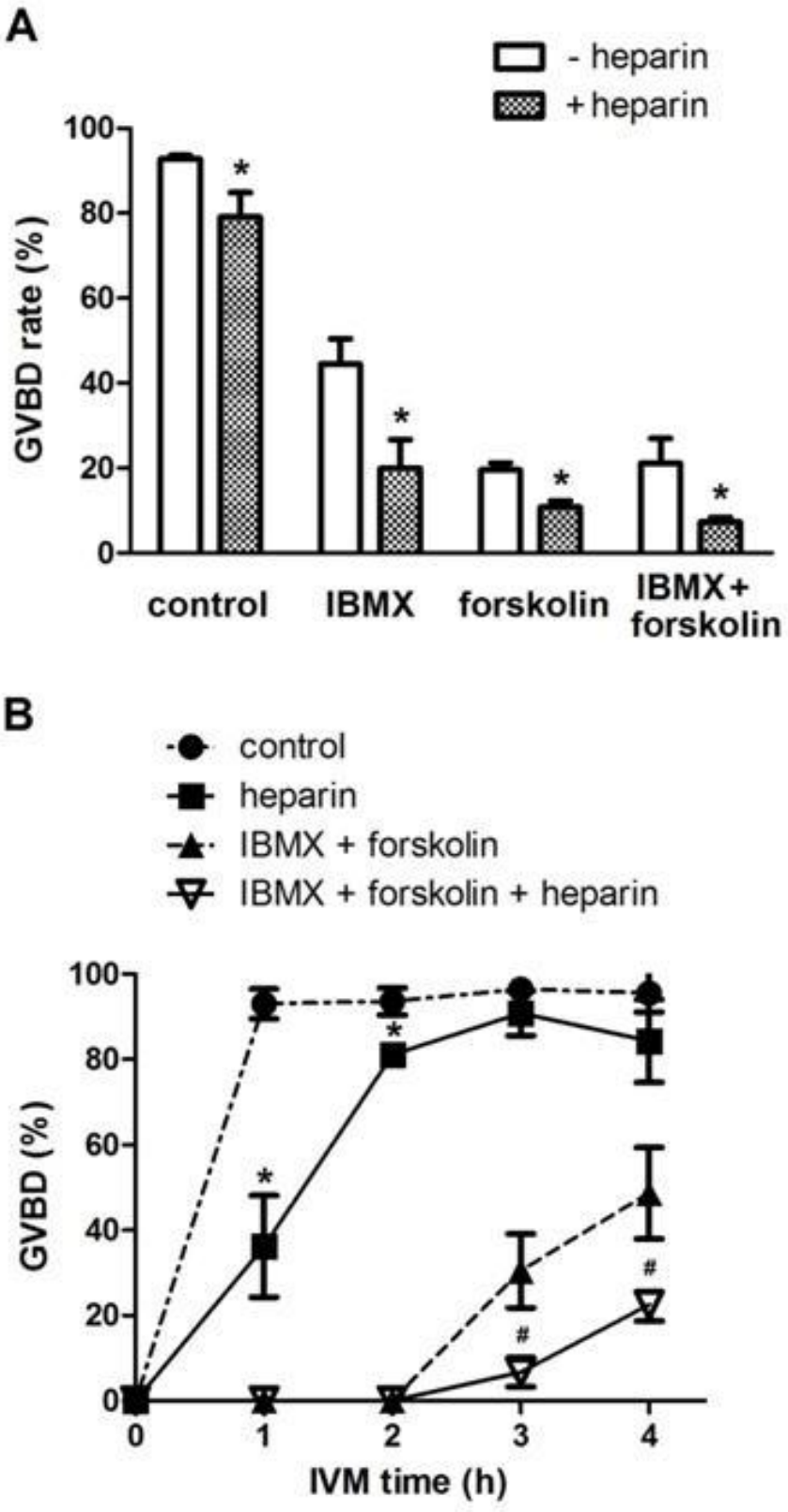

A
- heparin
+ heparin
GVBD rate (%)
100
80
60
40
20
0
*
control
IBMX
forskolin
IBMX+
forskolin
B
control
heparin
IBMX + forskolin
IBMX + forskolin + heparin
GVBD (%)
100
80
60
40
20
0
*
#
0
1
2
3
4
IVM time (h)

Figure 3

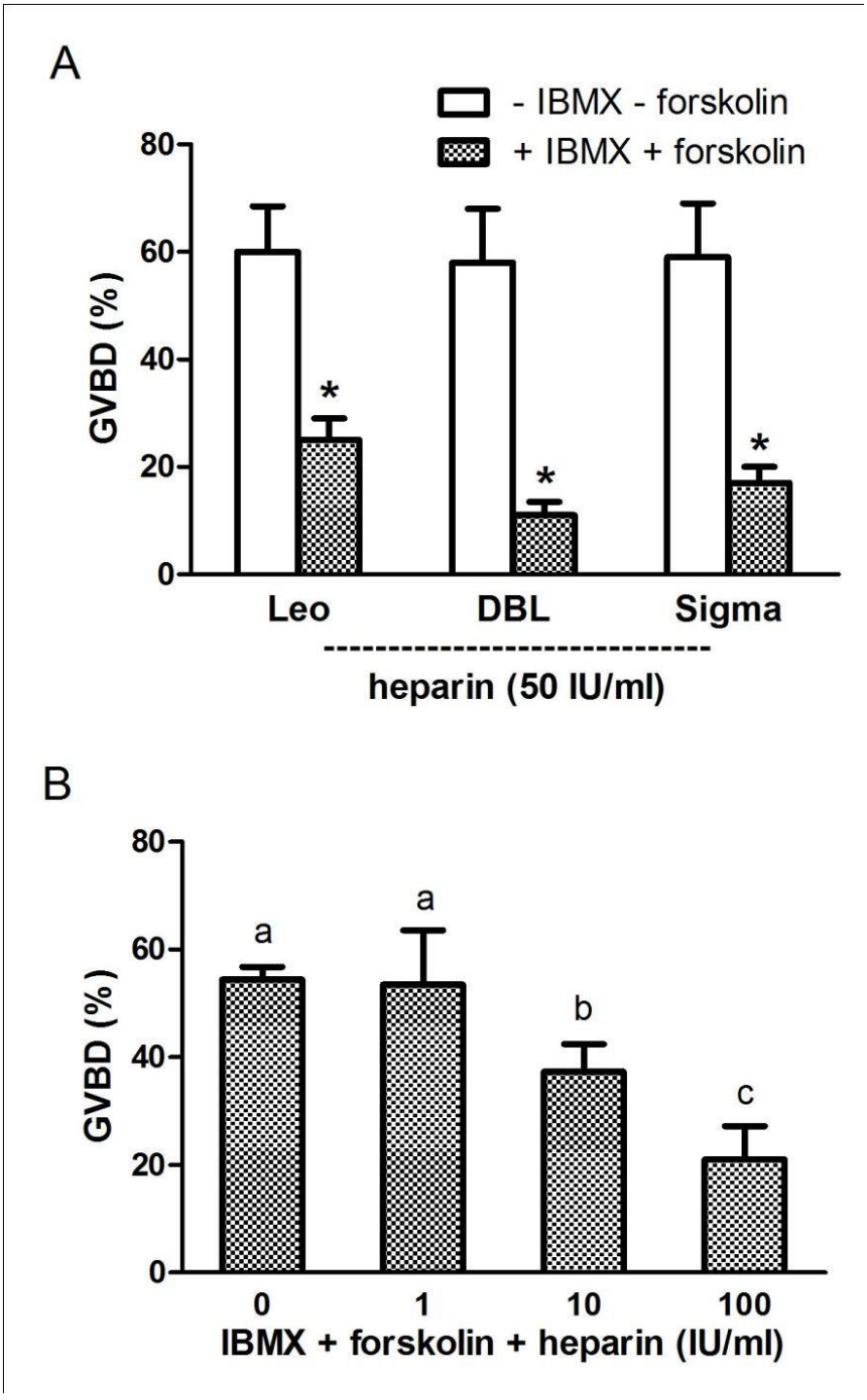
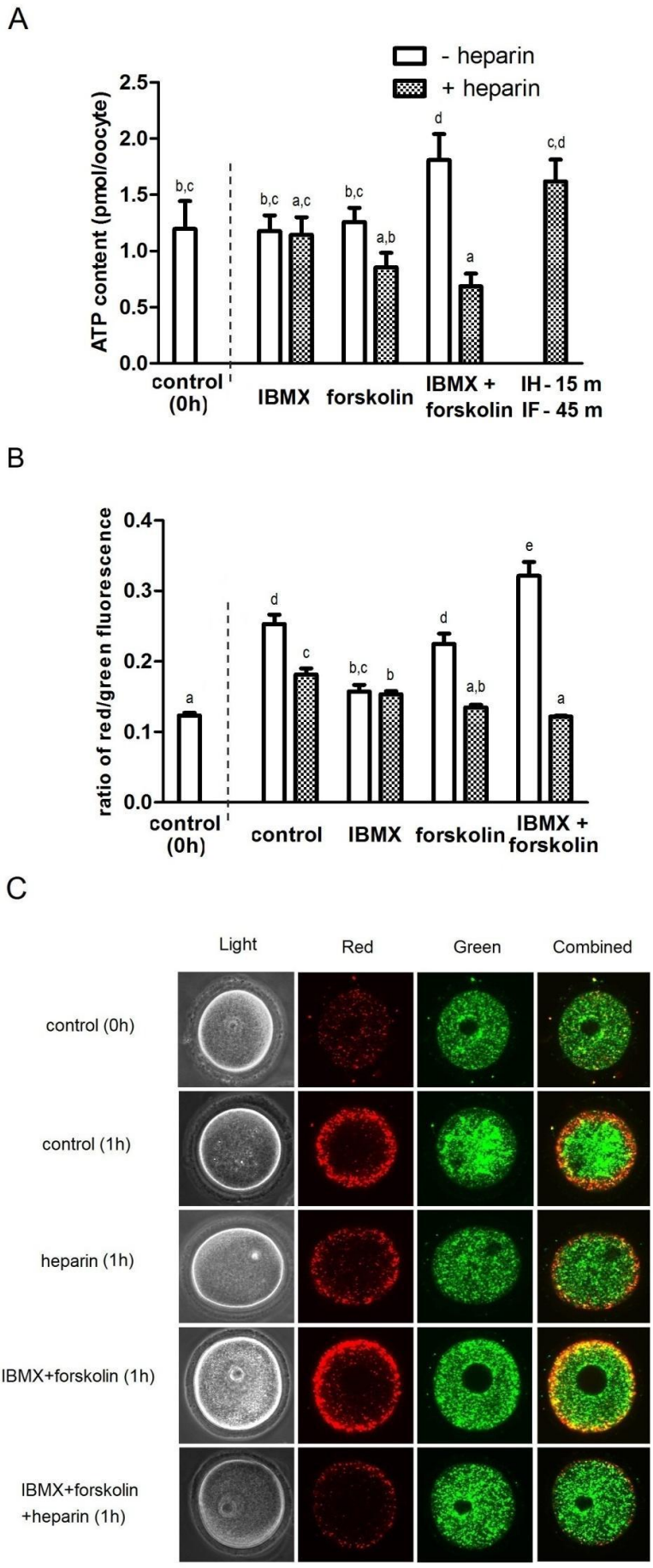

A
- IBMX - forskolin
+ IBMX + forskolin
GVBD (%)
80
60
40
20
0
*
*
*
Leo
DBL
Sigma
heparin (50 IU/ml)
B
GVBD (%)
80
60
40
20
0
a
a
b
c
0
1
10
100
IBMX + forskolin + heparin (IU/ml)

Figure 4


A
- heparin
+ heparin
ATP content (pmol/oocyte)
2.5
2.0
1.5
1.0
0.5
0.0
b,c
b,c
a,c
b,c
a,b
d
a
c,d
control (0h)
IBMX
forskolin
IBMX + forskolin
IH - 15 m
IF - 45 m
B
ratio of red/green fluorescence
0.4
0.3
0.2
0.1
0.0
a
d
c
b,c
b
d
a,b
e
a
control (0h)
control
IBMX
forskolin
IBMX + forskolin
C
Light
Red
Green
Combined
control (0h)
control (1h)
heparin (1h)
IBMX+forskolin (1h)
IBMX+forskolin +heparin (1h)

133 **Figure 5**

134