Effect of varying glucose and glucosamine concentration in vitro on mouse oocyte maturation and developmental competence

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Abstract

The effects of hyper- and hypo-glycaemic conditions during the in vitro maturation of mouse cumulus-oocyte complexes on developmental competence were examined, with an emphasis on the role of the hexosamine biosynthesis pathway. A low (1 mM) glucose concentration achieved optimal oocyte competence (3-fold higher blastocyst development rate compared to high (30 mM) glucose, $P < 0.05$). In addition, glucose supplementation during only the first hour following liberation from the follicle was necessary and sufficient to support oocyte maturation and embryo development to the blastocyst stage. Glucosamine (a known hyperglycaemic mimetic and specific activator of the hexosamine pathway) was able to substitute for glucose during this first hour, indicating that flux through the hexosamine pathway is essential for oocyte competence. In the absence of glucose throughout the maturation period, glucosamine was not able to increase developmental competence, and at higher concentrations (2.5 and 5 mM) had a detrimental effect on MII rates and blastocyst development rate, compared to controls ($P < 0.05$). These experiments underscore the importance of glucose metabolic pathways during in vitro maturation and support the concept that excess flux through the hexosamine pathway has detrimental consequences.

Introduction

Maternal diabetes and conditions such as obesity, in which blood glucose levels are elevated, are associated with reduced fertility and an increased risk of pregnancy complications, including spontaneous abortions, neonatal morbidity and mortality and congenital malformations (Becerra, Khoury *et al.* 1990; Cornblath and Schwartz 1976; Farrell, Neale *et al.* 2002; Greene 1999; Sadler, Hunter *et al.* 1988). Numerous studies have examined the effect of hyperglycaemic conditions on early embryogenesis. However, even if glycaemic control is achieved during the first few weeks of pregnancy, there is still a significant risk of complications and neonatal abnormalities among diabetic women compared to normoglycaemic women (Dunne, Brydon *et al.* 1999; Lapolla, Dalfra *et al.* 2008; Ray, O'Brien *et al.* 2001). Oocytes derived from diabetic mice are known to be smaller, have delayed completion of meiotic maturation and have altered mitochondrial distribution compared to oocytes from normoglycaemic mice (Chang, Dale *et al.* 2005; Colton, Pieper *et al.* 2002; Wang, Ratchford *et al.* 2009). It is increasingly evident that the environment that the oocyte is exposed to during the peri-conception period has a significant impact on its developmental competence (defined as the ability of the oocyte to support fertilisation and subsequent embryo development) and the long-term health of the resulting offspring (Kakar, Maddocks *et al.* 2005; Virk, Li *et al.* 2010; Wahabi, Alzeidan *et al.* 2010; Wyman, Pinto *et al.* 2008). The composition of the culture medium during in vitro maturation (IVM) influences subsequent embryo development (Rose-Hellekant, Libersky-Williamson *et al.* 1998; Rose and Bavister 1992; van de Sandt, Schroeder *et al.* 1990) and the rate of live births following embryo transfer (van de Sandt, Schroeder *et al.* 1990). Glucose concentration in particular has been found to affect oocyte developmental competence in many in vitro systems of several species (Downs and Mastropolo 1994; Hashimoto, Minami *et al.* 2000; Hendryx and Wordinger 1979; Khurana and Niemann 2000; Sutton, Gilchrist *et al.* 2003) as well as in vivo (Lea, McCracken *et al.* 1996; Moley, Vaughn *et al.* 1991). During IVM, the metabolism of glucose is influenced by the concentration in the medium (Downs and Utecht 1999), and insufficient glucose limits the substrate available for nucleic acid synthesis and energy production (Downs, Humpherson *et al.* 1998) and impairs nuclear maturation (Downs and Mastropolo 1994; Sutton-

McDowall, Gilchrist *et al.* 2005) and embryo development (Ali and Sirard 2002; Eppig, Hosoe *et al.* 2000; Rose-Hellekant, Libersky-Williamson *et al.* 1998).

Meiotic maturation of mammalian oocytes involves progression from prophase I, where oocytes are arrested in the ovarian follicle, the breakdown of the germinal vesicle (GVBD) to metaphase II (MII) of meiosis when the first polar body is extruded (Eppig, Schultz *et al.* 1994; Sutton-McDowall, Gilchrist *et al.* 2010). The pentose phosphate pathway (PPP) is considered to be an important pathway for meiotic maturation (Downs, Humpherson *et al.* 1998; Downs, Humpherson *et al.* 1996), and glucose is known to increase rates of meiotic maturation in vitro (Downs, Humpherson *et al.* 1998; Downs, Humpherson *et al.* 1996; Funahashi, Koike *et al.* 2008; Sato, Iwata *et al.* 2007; Sutton-McDowall, Gilchrist *et al.* 2005).

Cumulus matrix expansion during mouse cumulus-oocyte complex (COC) maturation occurs in response to follicle-stimulating hormone (FSH), epidermal growth factor (EGF) or EGF-like peptides signalling through the EGF receptor (Jamnongjit, Gill *et al.* 2005; Kawashima, Liu *et al.* 2012; Reizel, Elbaz *et al.* 2010; Tirone, D'Alessandris *et al.* 1997), and is facilitated by the increased production of hyaluronic acid, the extracellular glycosyaminoglycan which supports the matrix as it expands (Chen, Wert *et al.* 1990; Eppig 1981; Salustri, Yanagishita *et al.* 1989). In vivo, there is strong evidence for a role for cumulus expansion in follicle rupture and ovulation (Chen, Russell *et al.* 1993; Russell and Robker 2007), with mice with defective cumulus matrix formation sub-fertile or infertile, primarily due to impairment of ovulation. Unlike in vivo, in vitro cumulus cell expansion itself is not a direct predictor of oocyte developmental competence (Ali and Sirard 2002; Luciano, Modina *et al.* 2004), however IVM conditions which promote developmental competence (such as FSH, EGF and serum supplementation) also generally promote cumulus expansion (Merriman, Whittingham *et al.* 1998; Mikkelsen, Host *et al.* 2001).

The precursor of hyaluronic acid is UDP-N-acetylglucosamine (UDP-GlcNAc), the end product of the glucose metabolic hexosamine biosynthesis pathway (HBP). In somatic cells under normoglycaemic conditions, approximately 1-3% of total glucose consumed by the cell is directed down the HBP (Marshall, Bacote *et al.*

1991; Sayeski and Kudlow 1996), however, during in vitro COC maturation, there is a significant up-regulation of HBP activity, with approximately 25% of the total glucose metabolised via this pathway (Gutnisky, Dalvit *et al.* 2007; Sutton-McDowall, Gilchrist *et al.* 2004). An alternative fate of UDP-GlcNAc is its attachment to the hydroxyl groups of serine or threonine residues of proteins, a post-translational modification referred to as β-O-linked glycosylation (O-GlcNAcylation) (Wells, Whelan *et al.* 2003). O-GlcNAcylation functions in all cell types as a link between nutrient levels and cell signalling (Zachara and Hart 2004). This link has been especially studied in detail in relation to glucose-mediated development of insulin resistance (Marshall, Bacote *et al.* 1991; Yang, Ongusaha *et al.* 2008).

The role of O-GlcNAcylation during COC maturation has only recently been investigated, but has a significant impact on oocyte developmental competence. Glucosamine supplementation during the IVM phase, used to selectively up-regulate the HBP, has no effect on nuclear maturation or cleavage rates in bovine or porcine oocytes (Sutton-McDowall, Mitchell *et al.* 2006). However, this supplementation severely impairs blastocyst development in cow, pig and mouse as well as decreasing cleavage rates in the mouse (Kimura, Iwata *et al.* 2008; Schelbach, Kind *et al.* 2010; Sutton-McDowall, Mitchell *et al.* 2006). This is accompanied by an increase in detectable O-GlcNAcylation in cow COCs using immunofluorescence (Sutton-McDowall, Mitchell *et al.* 2006).

The aim of these experiments was to establish the effects of different concentrations of glucose on mouse COCs, using meiotic maturation, cumulus expansion and embryo development as markers of oocyte developmental competence. Secondly, the contribution of the HBP to these measures was examined using glucosamine.

Methods

Mice

CBA x C57BL6 F1 hybrid mice (females 21 days old, males 6 – 8 weeks old) were maintained in the Animal House at the Medical School, University of Adelaide, under a 14:10 hour light:dark cycle with *ad libitum* access to food and water. All experimental procedures were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and approved by the University of Adelaide Animal Ethics Committee (Medical).

Media

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Media for collection and maturation was as described in Table 1, with various glucose and glucosamine concentrations (see individual experimental designs). Medium was supplemented with bovine serum albumin (BSA, ICPbio, Glenfield, New Zealand), 4 mg/mL and 3 mg/mL in collection and maturation, respectively. Maturation medium was supplemented with 50 mIU/mL recombinant human FSH (Organon, Oss, The Netherlands), and 1 mg/mL fetuin was used in both media for all embryo development experiments as it prevents the zona hardening observed in mouse oocytes under serum-free culture conditions (Schroeder, Schultz *et al.* 1990). For embryo production following maturation, the Research Vitro Wash, Fertilization and Cleave, respectively, were utilized from Cook Medical (William A. Cook Australia Pty. Ltd., QLD, Australia). All procedures after ovary and epididymides/vasa deferentia collection were performed on warming stages calibrated to maintain medium in dishes at 37°C. COC and embryo culture and fertilisation were performed in incubators at 37°C under paraffin oil (Merck, Darmstadt, Germany), in humidified air comprising 6% CO_2, 5% O_2, 89% N_2. Maturation, fertilisation and embryo culture media were pre-equilibrated for at least 4 h prior to use at 37°C in a humidified 6% CO_2 atmosphere, and collection medium pre-warmed to 37°C.

Collection of COCs and IVM

Pre-pubertal (21 - 22 days old) female mice were stimulated with 5 IU equine chorionic gonadotrophin as an intraperitoneal injection (eCG; Folligon, Intervet, Boxmeer, The Netherlands). Forty-six hours post-eCG injection, female mice were sacrificed by cervical dislocation. Ovaries were dissected out and placed into

warm (37 °C) collection medium, and follicles punctured with a 30 G needle to liberate the COCs. COCs with a morphologically normal and unexpanded cumulus cell vestment were placed into a fresh dish in collection medium, so that total time from follicle puncture to transfer into maturation medium was consistently one hour. COCs were washed in maturation medium, transferred into pre-equilibrated maturation dishes (50 µL medium/COC) and matured for up to 17 hours.

Assessment of cumulus expansion index (CEI)

After 17 h of maturation the CEI was scored using the system of scoring reported by (Vanderhyden, Caron *et al.* 1990), where 0 indicates no expansion and 4 indicates maximal expansion, including the corona radiata.

Assessment of meiotic maturation

After 17 h of maturation, COCs were denuded mechanically using a Gilson pipette and denuded oocytes were fixed for 30 min in 4 % paraformaldehyde in phosphate buffered saline (PBS). Oocytes were washed in 0.01 % BSA in PBS and transferred to 3 µM 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes. Oocytes were washed in 0.01 % BSA, collected in 4 µl wash medium and placed on a slide next to an 8 µl drop of anti-fade reagent (Prolong Gold, Invitrogen, CA, USA) and a coverslip was applied. Chromosome configuration was determined using a Nikon Eclipse TE2000-E microscope with UV laser (330-380 nm, DAPI excitation at 358 nm, emission at 461 nm) and oocytes were classified into germinal vesicle (GV), germinal vesicle breakdown (GVBD) or metaphase II (MII) stages.

In vitro fertilisation (IVF)

Male mice which had previously been assessed for mating ability (not less than 3 days prior), were used as sperm donors for IVF. Mice were sacrificed by cervical dislocation and the epididymides and vasa deferentia were collected into warm (37 °C) wash medium, cleaned of excess fat and tissue and transferred into 1 mL of fertilisation medium. Sperm were extracted into the medium and allowed to capacitate for one hour prior to addition to fertilisation drops (10 µL capacitated sperm added to 90 µL fertilisation drop). After 17 hours of maturation COCs were

washed once in fertilisation medium and 10 COCs were transferred to each fertilisation drop (including sperm). COCs and sperm were incubated together for four hours, before COCs were transferred to wash medium and cumulus cells removed mechanically using a Gilson pipette. Presumptive zygotes were washed in culture medium and placed in culture drops (4 - 7 per 10 µL drop).

Embryo culture

Approximately 25 hours post-insemination (Day 2), embryo cleavage was assessed and any embryos which had not developed to the two-cell stage were removed from culture drops. Blastocyst development was assessed on Day 5 (at approximately 102 hours post-insemination).

Experiment 1: glucose dose-response throughout IVM

The effect of increasing doses of glucose on embryo development, cumulus expansion and meiotic maturation was examined using 0, 1, 3, 10 or 30 mM glucose or 30 mM sucrose as an osmolarity control in IVM medium. These concentrations were chosen to represent a range of below physiological concentration (~0.46 mM in the mouse follicle (Harris, Gopichandran *et al.* 2005)), two relatively low concentrations (1 and 3 mM), an intermediate (10 mM) and a high concentration, 30 mM. 30 mM was chosen as the top end of the range to represent true hyperglycaemic conditions even when accounting for substrate depletion in the media. For cumulus expansion experiments, the average number of COCs scored in total per group was 215 (n = 6 replicates). Three replicates each were performed for meiotic maturation and embryo development, with an average of 25 and 24 COCs matured per treatment per replicate respectively.

Experiment 2: glucosamine dose-response throughout IVM

Glucosamine concentrations of 0, 0.5, 1, 2.5 or 5 mM in the absence of glucose were used during IVM to examine the contribution of the HBP to various measures of oocyte developmental competence. These concentrations were based on the effective dose of 2.5 mM shown in previous studies (Schelbach, Kind *et al.* 2010; Sutton-McDowall, Mitchell *et al.* 2006), and extending the range to 0 or 5 mM. A control group containing 0.5 mM glucose in collection and 5.55 mM glucose during maturation was also included based on commonly used glucose concentrations in

defined IVM media. Three replicates were performed of each experiment, with an average of 19 and 28 COCs matured per treatment per replicate for meiotic maturation and embryo development respectively.

Experiment 3: effect of ± glucose during the first hour of IVM

During preliminary experiments, it became evident that the glucose concentration in the collection media appeared to be exerting an effect of its own. The total time that COCs were in collection media was calculated and determined to be one hour consistently. From this point on timing was measured to ensure a 1 hour exposure in each experiment, and experiments to measure cumulus expansion, meiotic maturation and embryo development were set up with collection and maturation medium containing either 0 mM or 10 mM glucose. A control group using standard glucose concentrations of 0.5 mM in collection and 5.55 mM in maturation was included, as well as an osmolarity control group using maturation medium with 10 mM sucrose alone. Three replicates were performed of each experiment, with an average of 108 COCs scored per group for cumulus expansion data (n = 3 replicates) and 28 and 30 COCs matured per treatment per replicate for meiotic maturation and embryo development experiments respectively.

Experiment 4: effect of glucosamine supplementation during the first hour of IVM

Embryo development was examined after collection in medium containing 0 or 1 mM glucose ± 2.5 mM glucosamine, and maturation in medium containing 0 or 5.55 mM glucose. Four replicates were performed with an average of 24 COCs matured per treatment per replicate.

Statistical analysis

Statistics were calculated using SPSS version 18.0.2 (Predictive Analytics SoftWare (PASW), IBM, New York, U.S.A). Proportional data within each replicate were arc-sine transformed and analysed using a one-way analysis of variance (ANOVA) and comparisons made by least-significant difference (LSD) post-hoc test. ANOVAs were weighted by the number of oocytes per treatment group for each replicate. Dose-response data were also analysed by linear regression. Data which were not normally distributed (including CEI scores) were analysed using a

Kruskal-Wallis test followed by Mann-Whitney U tests if significance was found. A P value of < 0.05 was accepted as significant.

Results

Experiment 1: glucose dose-response throughout IVM

A glucose dose-response in IVM medium was established using concentrations ranging from 0 mM to 30 mM. Cumulus expansion index (CEI) was dependent on glucose concentration with increasing concentrations corresponding with increased cumulus expansion (Fig. 1, $P < 0.001$, $R^2 = 0.153$). In the absence of glucose there was almost no expansion, increasing to a CEI of approximately 2.40 ± 0.08 with low (1 mM or 3 mM) glucose concentrations ($P < 0.05$) and approximately CEI = 2.95 ± 0.09 with higher (10 mM or 30 mM) concentrations ($P < 0.05$). COCs cultured in the presence of 30 mM sucrose displayed no expansion and cumulus cells did not plate down (CEI = 0.9 ± 0.02).

An intermediate concentration of glucose was important for the completion of nuclear maturation with the lowest metaphase II (MII) rates observed in the 0 mM glucose and 30 mM sucrose groups (Table 2). There was a trend towards higher MII rates with increased glucose until 10 mM ($P < 0.1$, $R^2 = 0.329$).

There was also a clear effect of glucose concentration during IVM on blastocyst development (Fig. 2, $P < 0.05$, $R^2 = 0.469$). The absence of glucose during IVM resulted in low blastocyst development (16.3 ± 7.0 %). However, COCs matured in low glucose levels (1 mM) had an embryo development rate of 67.3 ± 8.6 %; significantly higher than 0 mM, $P < 0.01$, decreasing linearly as glucose concentration increased. 30 mM glucose produced significantly fewer blastocysts than the 1 mM group (1 mM = 67.3 ± 8.6 % vs. 30 mM = 25.4 ± 9.4 %, $P < 0.05$), and interestingly the osmolarity control group using 30 mM sucrose produced an intermediate blastocyst rate (46.1 ± 20.3). The effect of glucose concentration on cleavage rate was less clear, although the 10 mM glucose group achieved the highest cleavage rate of 93.5 ± 3.3 % (significantly higher than the 0 mM, 3 mM and 30 mM groups, $P < 0.05$).

Experiment 2: dose-response effect of glucosamine concentration throughout IVM

A glucosamine dose-response experiment was also performed to examine the contribution of the HBP during IVM. Cumulus expansion indices were unable to be determined using the Vanderhyden system (Vanderhyden, Caron *et al.* 1990), because glucosamine supplementation dramatically increased expansion of the matrix as well as 'stickiness' of the cumulus cells, resulting in dissociation from the complex. This has also been reported in a previous study where mouse COC IVM medium was supplemented with glucosamine (Schelbach, Kind *et al.* 2010).

Glucosamine significantly affected MII and GVBD rates as well as the proportion of degenerate oocytes ($P < 0.01$, $R^2 = 0.769$, 0.413 and 0.790 respectively, Table 3). The absence of glucosamine (and glucose) in IVM media resulted in a significant decrease in MII rate compared to the control group (0.5 mM glucose during collection and 5.55 mM during maturation; $P < 0.05$). Higher concentrations of glucosamine (2.5 and 5 mM) significantly decreased the proportion of oocytes that reached MII compared to the control group ($P < 0.05$) and increased the proportion of degenerated oocytes ($P < 0.05$ all other groups).

Increasing glucosamine concentration during IVM produced a trend towards decreased cleavage and blastocyst rates ($P < 0.1$, $R^2 = 0.185$ and 0.196 respectively, Fig. 3). The 5 mM group had a significantly lower cleavage rate than the 1 mM group (60.1 ± 20.2 % vs 93.7 ± 12.6 % respectively, $P < 0.05$). Glucosamine supplementation during IVM did not increase the blastocyst rate and 5 mM glucosamine significantly decreased the rate compared to the control group (8.6 ± 8.6 % vs. 58.2 ± 11.6 % respectively, $P < 0.05$). There was a significant relationship between increasing glucosamine concentration and increased levels of degenerated oocytes ($P < 0.05$, $R^2 = 0.580$). Furthermore, as seen in the meiotic maturation experiments, 5 mM glucosamine resulted in a significant increase in the proportion of oocytes degenerating (49.8 ± 22.1 %, $P < 0.05$ all other groups).

Experiment 3: effect of presence or absence of glucose during the IVM collection phase

COCs were exposed to different glucose concentrations during the collection phase and then matured ± 10 mM glucose. The concentration of glucose in both collection and maturation contributed to cumulus cell expansion (Fig. 4, $P < 0.001$). If no glucose was present during maturation, the CEI was minimal regardless of glucose in collection medium. Glucose (10 mM) during maturation only (0 mM in collection) increased the CEI significantly (0 mM = 0.14 ± 0.05 vs. 10 mM 2.75 ± 0.13, $P < 0.05$) and when glucose was present in both collection and maturation, regardless of concentration, COCs had a high CEI (3.66 ± 0.13 and 3.35 ± 0.14, collection/maturation 10 mM/10 mM and control group respectively, $P < 0.05$ vs. all other groups). There was no cumulus expansion of COCs collected in glucose and matured in 10 mM sucrose.

Nuclear maturation was not influenced by changing glucose concentrations between collection and maturation. Consistent with Experiments 1 and 2, COCs collected and matured in the absence of glucose displayed MII rates 20 % lower than the control group (59.2 ± 2.0 % vs. 81.0 ± 2.5 %, $P < 0.05$). In all other groups, approximately 80% of oocytes had reached MII by the cessation of maturation, indicating that the presence of glucose in either collection or maturation medium is sufficient for nuclear maturation.

Cleavage rates were significantly affected by the glucose concentration during maturation (Fig. 5, $p < 0.05$), with 0 mM glucose in collection and maturation media resulting in significantly lower cleavage rates than the control group (60.1 ± 3.6 % vs 81.5 ± 6.3 % in control, $p < 0.05$). An interaction ($P < 0.01$) was observed for the proportion of cleaved oocytes that developed to blastocysts. The presence of glucose was necessary during the collection phase to support subsequent embryo development, since the absence of glucose during this first hour decreased blastocyst rates compared to control (17.8 ± 3.7 % and 18.5 ± 1.1 % collection/maturation 0 mM/0 mM and 0 mM/10 mM respectively vs. control 56.9 ± 2.5 %, $p < 0.01$). If 10 mM of glucose was present during collection, this was sufficient to overcome the absence of glucose during maturation, with no

difference in blastocyst rates compared to control (46.5 ± 3.3 %), but 10 mM glucose in both collection and maturation media significantly lowered blastocyst development (25.8 ± 4.0 %, $P < 0.01$).

Experiment 4: effect of glucosamine supplementation during the IVM collection phase

Similar to our results in Experiment 3, Pantaleon et al. (2008) demonstrated that a transient exposure (1-3h) to glucose of cleavage-stage embryos is required for blastocyst development. It was also found that glucosamine could substitute for glucose in this role. Therefore, in the current study, glucosamine (2.5 mM) was provided in the absence or presence of glucose at various concentrations. Consistent with the previous experiments, when no glucose was present during collection the blastocyst rate was reduced (18.4 ± 6.9 %, Fig. 6). However, the addition of 1 mM glucose or 2.5 mM glucosamine during collection was able to significantly increase this rate (45.4 ± 10.8 % and 55.1 ± 16.5 % respectively, $p < 0.05$) when standard (5.55 mM) glucose level was subsequently used in maturation medium. The addition of both 1 mM glucose and 2.5 mM glucosamine did not have an additive effect on blastocyst rate, nor did increasing the glucose concentration in collection to 10 mM. An additional experiment was conducted and demonstrated that the total time spent in collection medium also influences embryo development in these conditions (data not shown).

Discussion

Glucose affects every aspect of COC maturation, including energy production, meiotic and cytoplasmic maturation and cumulus cell expansion (Sutton-McDowall, Gilchrist *et al.* 2010). One glucose metabolic pathway in particular, the hexosamine biosynthesis pathway (HBP), has recently been investigated in oocytes and embryos and it appears that excess flux of metabolites through this pathway adversely affects oocyte developmental competence (Kimura, Iwata *et al.* 2008; Pantaleon, Tan *et al.* 2010; Schelbach, Kind *et al.* 2010; Sutton-McDowall, Mitchell *et al.* 2006). The HBP is one metabolic fate for intracellular glucose and is also selectively accessed by glucosamine (Marshall, Nadeau *et al.* 2005), hence the aim of these experiments was to establish glucose and glucosamine dose-

response models in a mouse IVM system. The data indicate that there is a specific role of the HBP in oocyte developmental competence. Using several indicators associated with oocyte developmental competence, we have reaffirmed the dogma that glucose supplementation during IVM is necessary to support embryo development to the blastocyst stage and improves meiotic maturation rates and cumulus expansion indices. Furthermore, we have investigated the role of glucose and glucosamine supplementation during the first hour of IVM, specifically during COC collection, and found that the presence of one of these hexoses is necessary to support embryonic development. Notably, the presence of glucose during this collection period alone was sufficient to support development to the blastocyst stage, and furthermore it is more important to have glucose in the collection medium than the maturation medium. Glucosamine is able to substitute for glucose during this time provided glucose is present during maturation, suggesting a critical role for the HBP during the first stage of oocyte maturation.

The results of glucose dose-response experiments during IVM are consistent with several other publications, demonstrating that glucose is necessary during maturation for optimal embryo development and that concentrations which are too high or low can be detrimental to oocyte developmental competence (Chang, Dale *et al.* 2005; Clark, Stokes *et al.* 2011; Eppig, Hosoe *et al.* 2000; Hashimoto, Minami *et al.* 2000). The rate of meiotic maturation to the MII stage in the absence of glucose was significantly lower than in all other groups, a finding which has also been observed in other studies in the mouse (Eppig, Hosoe *et al.* 2000; Fagbohun and Downs 1992) and cow (Hashimoto, Minami *et al.* 2000).

Cumulus expansion requires the production of hyaluronic acid from UDP-GlcNAc, the end product of the HBP, and this pathway is greatly up-regulated by FSH in the maturing COC to support expansion (Sutton-McDowall, Gilchrist *et al.* 2004). Consequently, in the absence of glucose, little cumulus expansion was observed and the CEI increased with increasing amounts of glucose.

While glucose concentration during IVM had little overall effect on cleavage rates, there was a clear dose-response effect on blastocyst development. With 0 mM glucose, there was very low blastocyst development, with the highest rate

observed at the lowest concentration (1 mM glucose), then a steady decrease as the glucose concentration increased towards 30 mM. This pattern supports a similar study performed by Eppig, Hosoe et al. (2000), where 1 mM glucose during IVM was also able to sustain optimal levels of blastocyst development. However, in that study, no significant decrease in blastocyst rates was observed with higher glucose concentrations (5.5 or 27.8 mM). This may be due to a higher COC density than was used in the current study, reducing the impact of a high glucose concentration. A low fertilisation rate was found with 0 mM glucose by Eppig, Hosoe et al. (2000), which we also observed. The poor cumulus expansion observed in the 0 mM glucose group may be contributing to this, as a lack of cumulus expansion has been associated with poor fertilisation rates (Chen, Russell *et al.* 1993; Hizaki, Segi *et al.* 1999). The decrease in meiotic maturation rates in the absence of glucose, as well as possible defects in cytoplasmic maturation, may be causes of the decrease in fertilisation in this group.

Experiments performed with glucosamine supplementation during IVM revealed that in the absence of glucose, glucosamine is unable to facilitate meiotic maturation or embryo development. This is the first study to use glucosamine in the absence of glucose during IVM of mouse COCs. In previous studies in both the cow and mouse, glucosamine supplementation at 2.5 mM in IVM media in the presence of glucose resulted in a significant decrease in blastocyst development rates (Schelbach, Kind *et al.* 2010; Sutton-McDowall, Mitchell *et al.* 2006). This effect was also seen in the experiments presented here, although it was not significant until 5 mM glucosamine was used. This may be explained by a decreased flux through the HBP in the 2.5 mM glucosamine group in this study compared to the earlier studies conducted in our laboratory, in which glucose was also present. However, contrary to those reports, an increasing glucosamine concentration also caused a significant decrease in MII rates and a notable increase in degenerated oocytes. These may be caused by the inhibition of the PPP, a key pathway in meiotic maturation (Downs, Humpherson *et al.* 1996; Herrick, Lane *et al.* 2006) when the HBP is up-regulated. Glucosamine 6-phosphate, an intermediate in the HBP, is a competitive inhibitor of glucose 6-phosphate dehydrogenase (G6PDH), the first rate-limiting enzyme of the PPP (Kanji, Toews *et al.* 1976), thereby causing a decrease in PPP activity when the

HBP is significantly stimulated (Schelbach, Kind *et al.* 2010; Zhang, Liew *et al.* 2010).

In the current study, poor cumulus expansion was observed in all groups without glucose in the maturation medium. Nevertheless, it appears that while glucose during the maturation period is essential for cumulus expansion, the provision of glucose during a short (1 h) period following removal from the follicle is sufficient to stimulate meiotic completion and good cleavage and blastocyst development rates. This could also be interpreted that a sudden absence of glucose at the initiation of maturation is highly detrimental and unrecoverable for developmental competence. This has significance in the application of IVM for infertility treatment and animal breeding, where it is not uncommon to find very simple salt solutions (even un-buffered saline) being used to recover COCs.

We also found that rates of meiotic maturation completion were the same regardless of which phase of IVM glucose was present in, which suggests that PPP activity within the COC may not be sensitive to the timing of glucose provision in the current experimental system. To further investigate the possible mechanism behind this first-hour effect, glucosamine was added as a substitute for glucose during the collection phase. While glucose supplementation during collection was able to overcome the absence of glucose during maturation, glucosamine supplementation was sufficient during collection only if glucose was present during maturation. Together, the results from these experiments suggest that glucose flux within the HBP is essential during the first hour of IVM, whereas flux through other pathways is essential during IVM, but is not temporally specific.

In conclusion, our results reveal that concentration-dependent effects of glucose on the COC affect oocyte competence during spontaneous in vitro maturation. In particular, there is a requirement for glucose to flux through the HBP immediately after COCs are liberated from follicles. Further studies are required to establish the downstream targets of HBP activity.

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Component	Concentration (mM)	
	Collection	**Maturation**
Sodium chloride	95.00	115.00
Potassium chloride	5.50	6.00
Magnesium sulphate heptahydrate	1.00	0.80
Sodium dihydrogen phosphate	0.30	-
Potassium dihydrogen phosphate	-	2.00
Sodium bicarbonate	5.00	27.50
Sodium pyruvate	0.32	0.40
Sodium L-lactate	9.97	-
Calcium chloride dihydrate	1.80	1.80
Taurine	0.10	-
3-(N-Morpholino)propanesulfonic acid (MOPS)	20.00	-
Phenol red	0.01	-
Gentamicin	75 mg/L	75 mg/L
Glutamax 1*	1.0 mL/L	1.0 mL/L
Non-essential amino acids (NEAA)*	1.0 mL/L	1.0 mL/L
Essential amino acids (EAA)*	-	2.0 mL/L

Table 1 Simple collection and maturation media used for all experiments.

*Glutamax 1, NEAA (100x stock) and EAA (50x stock) all from GIBCO, Invitrogen, CA, USA.

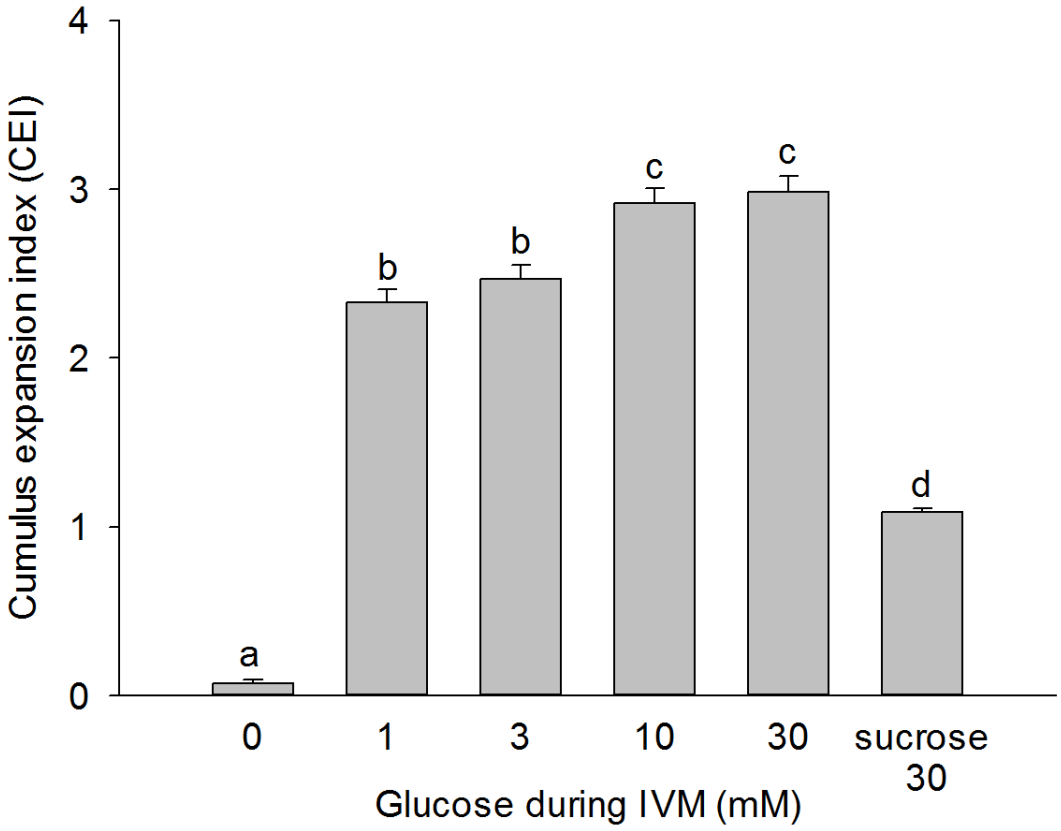

Figure 1 Cumulus expansion indices following glucose dose-response in IVM

Cumulus expansion was measured using the Vanderhyden scoring system for COCs (Vanderhyden, Caron *et al.* 1990) following IVM in media containing various glucose concentrations. Data are presented as mean ± SEM and groups with different superscripts differ significantly ($P < 0.05$).

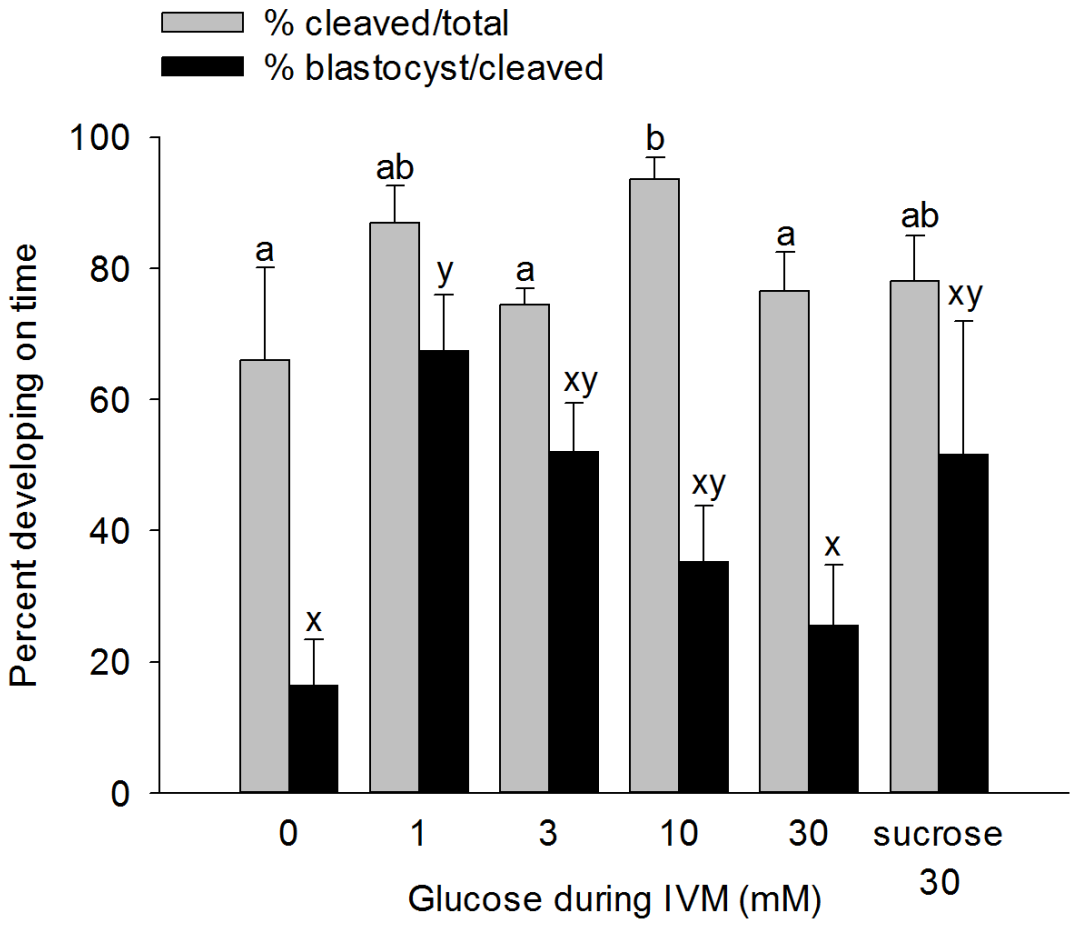

Figure 2 Cleavage and blastocyst development following glucose dose-response in IVM

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following IVM in media containing various glucose concentrations. Data are presented as mean ± SEM and groups with different superscripts differ significantly ($P < 0.05$).

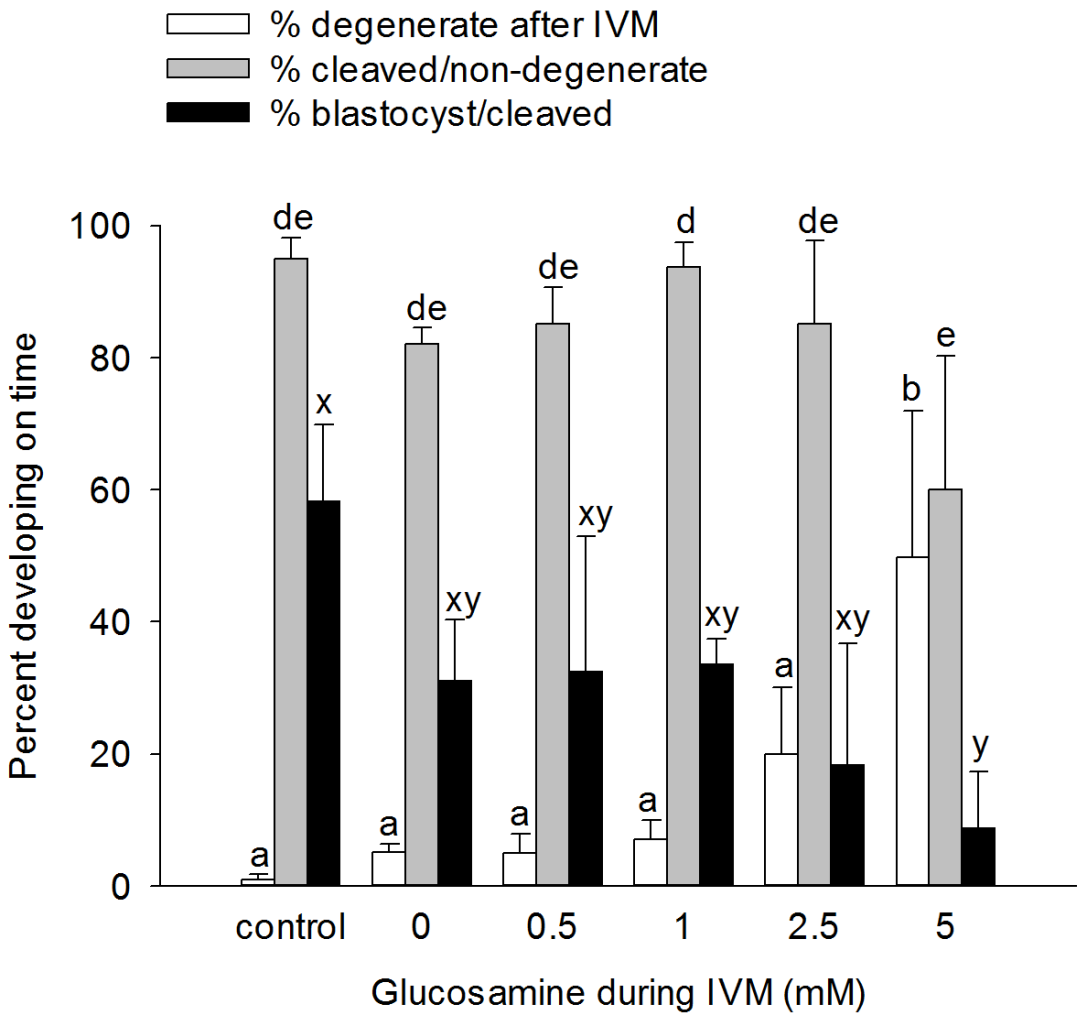

Figure 3 Cleavage and blastocyst development following glucosamine dose-response in IVM

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following IVM in media containing various glucosamine concentrations. Oocytes degenerate after fertilisation were measured as a proportion of COCs matured; cleavage rate was measured as a proportion of cleaved from those not degenerate at the end of fertilisation. Data are presented as mean ± SEM and groups with different superscripts differ significantly ($P < 0.05$).

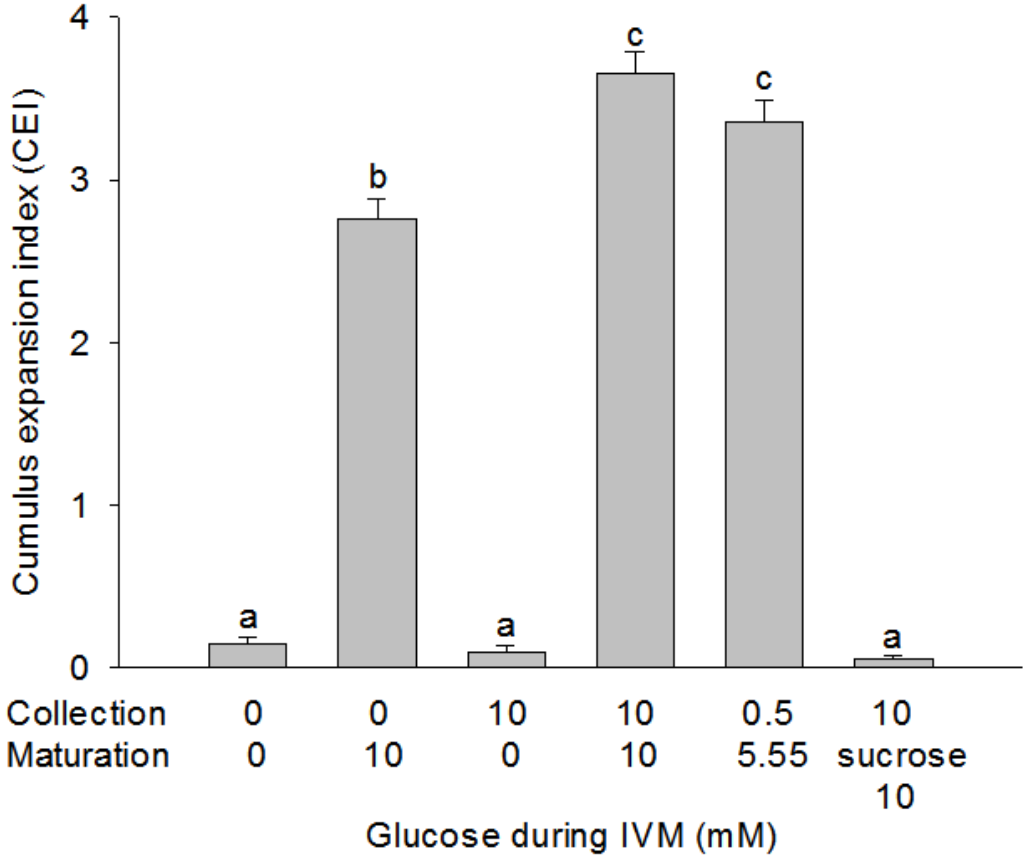

Figure 4 Cumulus expansion indices following collection and maturation in various glucose concentrations

Cumulus expansion was measured using the Vanderhyden scoring system for COCs (Vanderhyden, Caron *et al.* 1990) following collection and maturation in media containing various glucose concentrations. Data are presented as mean ± SEM and groups with different superscripts differ significantly ($P < 0.05$).

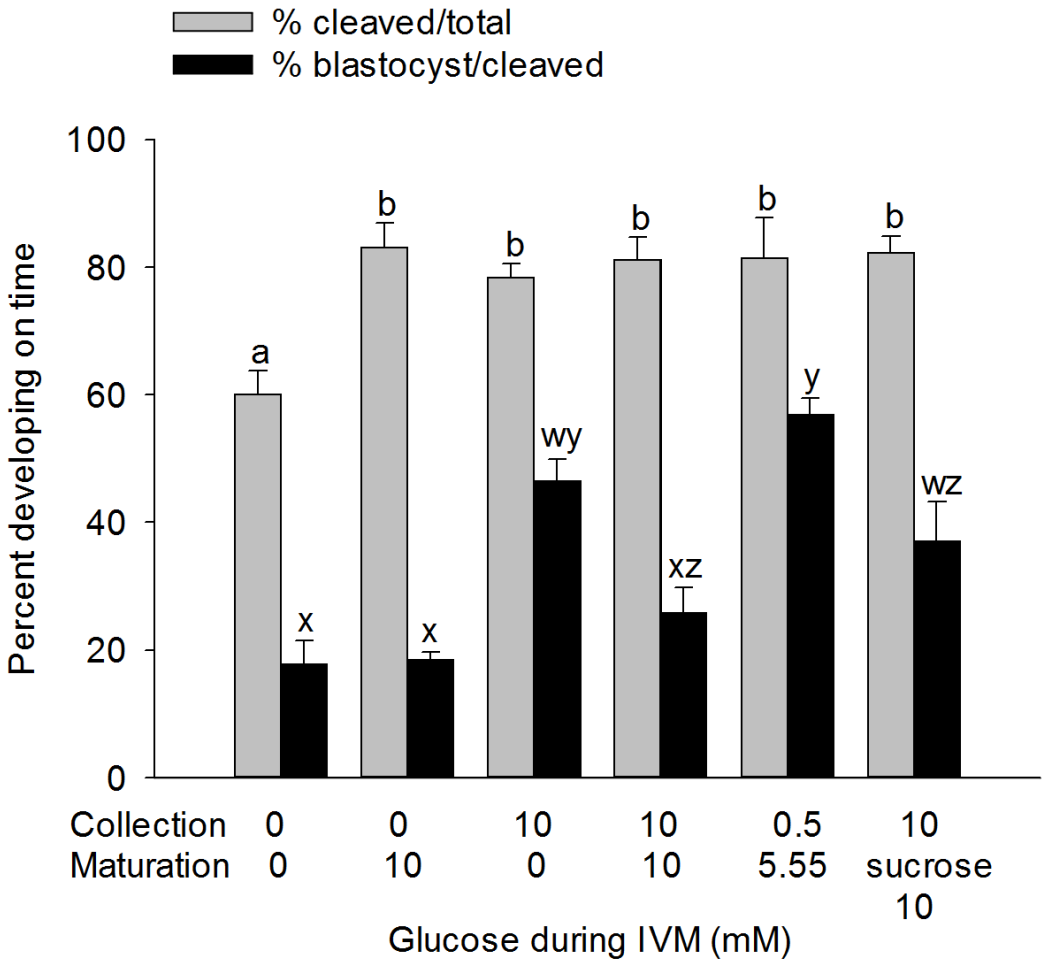

Figure 5 Cleavage and blastocyst development following collection and maturation in various glucose concentrations

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection and maturation in media containing various glucose concentrations. Data are presented as mean ± SEM and groups with different superscripts differ significantly ($P < 0.05$).

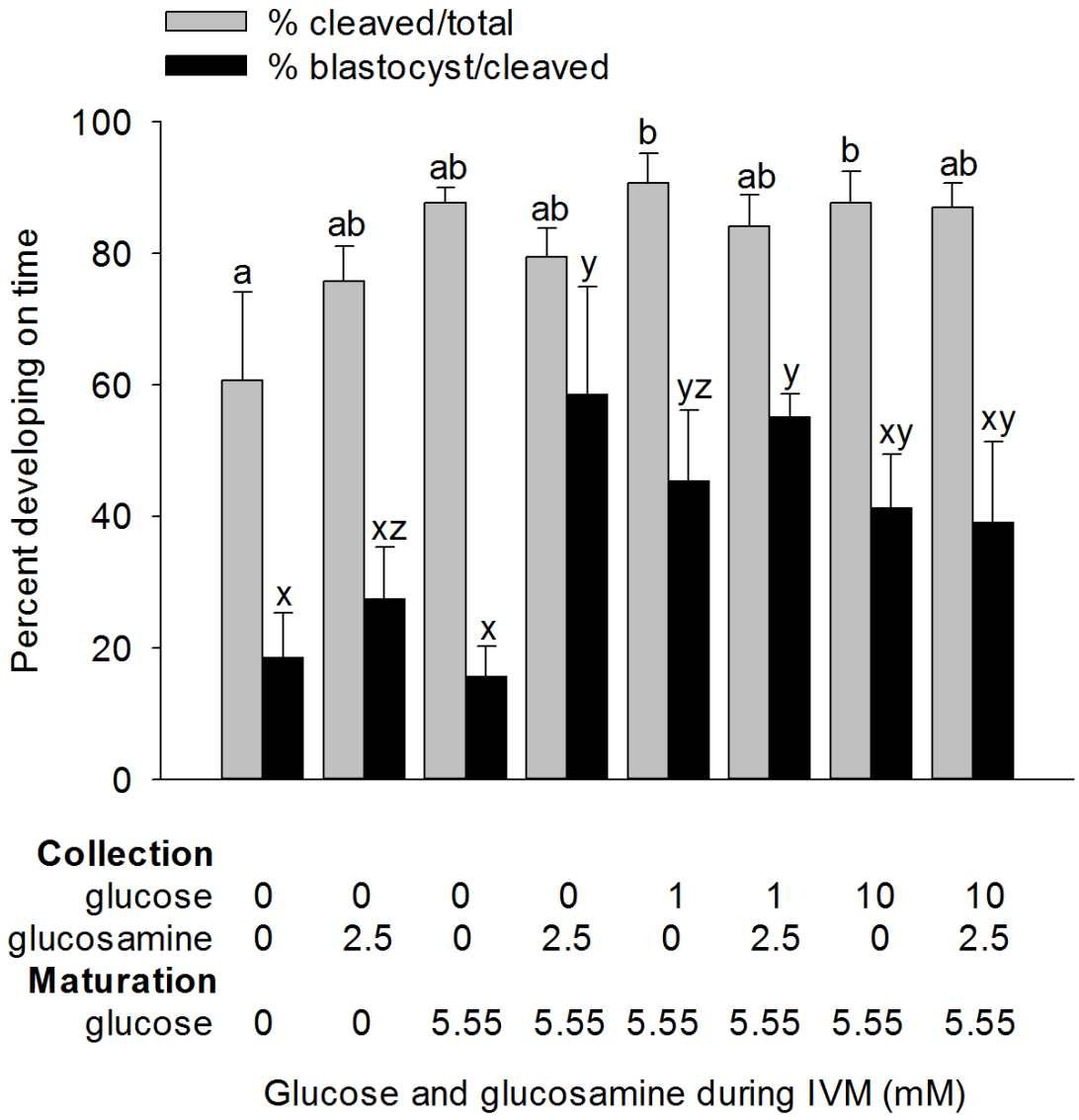

Figure 6 Cleavage and blastocyst development following collection and maturation in various glucose concentrations ± glucosamine supplementation

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection and maturation in media containing various glucose concentrations ± glucosamine supplementation (2.5 mM). Groups with different superscripts differ significantly ($P < 0.05$).