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Siew L. Wong, Linda L. Wu, Rebecca L. Robker, Jeremy G. Thompson and Melanie L. Sutton McDowall

Hyperglycaemia and lipid differentially impair mouse oocyte developmental competence

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1 Title: Hyperglycaemia and lipid differentially impair mouse oocyte developmental
2 competence

3

4 Siew L Wong [1], Linda L Wu [1], Rebecca L Robker [1], Jeremy G Thompson [1, 2], Melanie
5 L Sutton-McDowall [1, 2, 3]

6

7 [1] Robinson Research Institute, School of Paediatrics and Reproductive Health, The
8 University of Adelaide, Adelaide, SA 5005, Australia

9

10 [2] Australian Research Council (ARC) Centre of Excellence for Nanoscale BioPhotonics,
11 Institute of Photonics and Advanced Sensing

12

13 [3] Corresponding author, Email: melanie.mcdowall@adelaide.edu.au

Abstract

Maternal diabetes and obesity are characterised by elevated blood glucose, insulin and lipids, resulting in up-regulation of specific fuel sensing and stress signalling pathways. We have previously demonstrated that separately, up-regulation of the hexosamine biosynthetic pathway (HBP, under hyperglycaemic conditions) and endoplasmic reticulum (ER) stress (due to hyperlipidemia) pathways reduce blastocyst development and alter oocyte metabolism. In order to begin to understand how both glucose and lipid metabolic disruptions influence oocyte developmental competence, this study exposed mouse cumulus-oocyte complexes to hyperglycaemia (30 mM) and/or lipid (40 μM) and examined the effect on embryo development. The presence of glucosamine (a hyperglycaemic mimetic) or increased lipid during *in vitro* maturation severely perturbed blastocyst development ($P<0.05$). Hyperglycaemia, GlcN, and hyperglycaemia + lipid treatments significantly increased HBP activity, increasing total *O*-linked glycosylation (*O*-GlcNAcylation) of proteins ($P<0.0001$). All the treatments also induced ER stress pathways, indicated by the expression of specific ER stress genes. The expression of HBP enzymes glutamine:fructose-6-phosphate amidotransferase 2 (*Gfpt2)* and O-linked β-N-acetylglucosaminyltransferase (*Ogt*) were repressed following lipid treatment ($P<0.001$). These findings partially implicate the mechanism of *O*-GlcNAcylation and ER-stress as likely contributors to compromised fertility of obese women.

Keywords: Cumulus-oocyte complex, embryo, hexosamine biosynthesis pathway, endoplasmic reticulum stress, hyperglycaemia, hyperlipidemia

Introduction

Maternal obesity, a condition associated with elevated plasma insulin, glucose, and lipid, is a global health problem that affects an increasing number of women of reproductive age (AIHW 2012). Obesity is also a contributory factor to Type II diabetes, characterised by hyperglycaemia (elevated blood glucose level) and hyperlipidemia. These conditions have been associated with higher risk of sub-fertility and pregnancy complications; including increased risk of anovulation, preeclampsia, miscarriage, and spontaneous abortion (Jungheim and Moley 2010). They are also associated with increased incidence of congenital anomalies, macrosomia and stillborns (Sirimi and Goulis 2010). Moreover, these complications extend beyond neonatal health, into childhood and adulthood (Dabelea and Crume 2011; Heerwagen, Miller *et al.* 2010; O'Reilly and Reynolds 2013).

The peri-conception period (including the final stages of oocyte development and fertilisation) is particularly sensitive to the maternal metabolic environment. For example, zygotes collected from oviducts of diabetic mice and subsequently transferred to normoglycemic recipients have retarded fetal growth and increased fetal abnormalities (Wyman, Pinto *et al.* 2008). Hyperglycaemia during oocyte maturation and early development is associated with altered intracellular parameters, such as perturbed meiotic maturation and disrupted mitochondrial distribution (Chang, Dale *et al.* 2005; Colton, Pieper *et al.* 2002), as well as apoptosis in follicular and granulosa cells (Chang, Dale *et al.* 2005).

61 The detrimental effects of hyperglycaemia on oocyte developmental competence is in
62 part mediated by up-regulation of the hexosamine biosynthesis pathway (HBP) (Cheryl
63 J. Schelbach 2012; Schelbach, Kind *et al.* 2010; Sutton-McDowall, Mitchell *et al.*
64 2006), a fuel-sensing pathway, which metabolises glucose to uridine diphosphate-N-
65 acetylglucosamine (UDP-GlcNAc). Single UDP-GlcNAc molecules modify serine or
66 threonine amino acids of proteins, a process known as β-*O*-linked glycosylation (*O*-
67 GlcNAcylation), which acts in an analogous manner to phosphorylation to regulate
68 protein function (Butkinaree, Park *et al.* 2010). Altered *O*-GlcNAcylation is one of the
69 primary pathologies of diabetes in somatic cells and notably the primary mechanism
70 behind the development of insulin resistance in Type II diabetes (Marshall, Bacote *et al.*
71 1991; Yang, Ongusaha *et al.* 2008).

72

73 Glucosamine (GlcN), a known hyperglycaemic mimetic, can be metabolised via the
74 HBP by bypassing the rate-limiting enzyme of the HBP, glutamine:fructose-6-
75 phosphate amidotransferase (GFPT) (Nelson, Robinson *et al.* 2000; Uldry, Ibberson *et*
76 *al.* 2002) hence it is a potent stimulator of HBP pathway activity. We have previously
77 demonstrated that GlcN supplementation during IVM results in increased *O*-
78 GlcNAcylation in mouse COCs (Frank, Sutton-McDowall *et al.* 2014a) and perturbed
79 oocyte developmental competence in cow, pig and mouse and decreased cleavage rates
80 in the mouse (Cheryl J. Schelbach 2012; Frank, Sutton-McDowall *et al.* 2013; Kimura,
81 Iwata *et al.* 2008; Sutton-McDowall, Mitchell *et al.* 2006).

82

83 Hyperlipidemia is also known to cause numerous perturbations in oocyte structure and
84 developmental competence. Female mice fed a high-fat-diet (HFD) have higher rates of

85 anovulation, smaller and fewer mature oocytes, increased lipid accumulation, altered
86 mitochondrial activity and decreased rates of oocyte nuclear maturation and fertilisation
87 compared to oocytes derived from control mice (Igosheva, Abramov *et al.* 2010;
88 Jungheim, Schoeller *et al.* 2010; Luzzo, Wang *et al.* 2012; Wu, Dunning *et al.* 2010).
89 Increased lipid accumulation induces lipotoxicity, which causes damage to cellular
90 organelles, particularly mitochondria and endoplasmic reticulum (ER). A biomarker of
91 lipotoxicity is the ER-stress, characterised by the accumulation of misfolded proteins
92 and consequently triggering the unfolded protein response (UPR) (Alhusaini, McGee *et*
93 *al.* 2010; Wu, Norman *et al.* 2011).

94

95 UPR is an attempt by the cell to slow protein production and improve protein folding,
96 characterized by the induction of several genes, including the markers activating
97 transcription factor 4 (*Atf4*), activating transcription factor 6 (*Atf6*) and glucose-
98 regulated protein 78 (*Grp78*) (Malhotra and Kaufman 2007). Failure of the UPR will
99 eventually initiate the lipotoxicity pathways and potentially culminate in apoptotic cell
100 death. COCs from mice fed a HFD were shown to have increased expression of ER
101 stress marker genes *Atf4* and *Grp78*, similar to those matured in lipid-rich follicular
102 fluid (Wu, Dunning *et al.* 2010; Yang, Wu *et al.* 2012). Similar events happen in
103 women, with increased *Atf4* expression observed in granulosa cells of obese women
104 (Wu, Dunning *et al.* 2010).

105

106 A link between the HBP and ER-stress has been suggested in somatic cells (Lombardi,
107 Ulianich *et al.* 2012; Sage, Walter *et al.* 2010; Srinivasan, Tatu *et al.* 2009), with
108 increasing activity through the HBP increasing the transcript and translocation of ER-

109 stress markers. However, little is known about how hyperglycaemia and lipid
110 conditions impact reproductive function, in particular pre-implantation embryo
111 development. Furthermore, it is also unclear if these metabolic perturbations operate
112 through the same or different mechanisms. The aim of this study was to examine the
113 impact of hyperglycaemia and lipid supplementation on mouse oocyte developmental
114 competence. We hypothesised that combination of lipid and high glucose leads to the
115 accumulation of HBP product, UDP-GlcNAc, by increasing β-*O*-linked glycosylation
116 and activating the ER-stress pathway.

117

118 **Materials and methods**

119 **Mice**

120 Female CBA F1 mice were obtained at 21 days old from the University of Adelaide
121 (Waite campus) and kept in the Animal House at the Medical School, the University of
122 Adelaide, North Terrace campus, under a 14h:10h light:dark cycle with *ad libitum*
123 access to food and water. All animal experiments were approved by the University of
124 Adelaide's Animal Ethics Committee (Medical) and were conducted in accordance with
125 the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

126

127 **Isolation and *in vitro* maturation of mouse COCs**

128 Immature, unexpanded COCs were isolated by puncturing the antral follicles of ovaries
129 collected 46 h post-intraperitoneal injection of 5IU pregnant mare's serum gonadotropin
130 (PMSG; Folligon; Intervet, Boxmeer, The Netherlands). All COCs were collected in
131 HEPES-buffered α-MEM handling media (Life Technologies, Invitrogen, CA, USA)

132 supplemented with 4 mg mL^{-1} fatty acid free Bovine Serum Albumin (FAF BSA; MP
133 Biomedicals, Solon, OH, USA). Immature COCs were cultured in groups of 30 in 1.5
134 mL of pre-equilibrate IVM medium, overlaid with paraffin oil (Merck, Darmstadt,
135 Germany). Base IVM media (control) was bicarbonate-buffered α-MEM supplemented
136 with 1% fetal bovine serum (FBS; Invitrogen, Gibco, Victoria, Australia), 50 mIU mL^{-1}
137 recombinant human follicle stimulating hormone (FSH; Puregon-Organon, Oss, The
138 Netherlands) and 10 ng mL^{-1} recombinant human epidermal growth factor (EGF; R &
139 D Systems). Experimental treatments were 1) Control (5.56 mM glucose); 2) high
140 glucose (30 mM glucose); 3) glucosamine (2.5 mM GlcN plus 5.56 mM glucose); 4)
141 lipid (40 μM lipid concentrate; GIBCO, Invitrogen, CA, USA) and 5) lipid and high
142 glucose (40 μM lipid concentrate plus 30 mM glucose). COCs were cultured at 37 °C in
143 an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 for 8 h or 16 h. We have previously
144 demonstrated that large culture volumes are required to maintain hyperglycaemic
145 concentrations of glucose, due to the high metabolic rate of COCs (Frank, Sutton-
146 McDowall *et al.* 2013). In this study, 30 mM was used to avoid depletion to more
147 normoglycaemic levels during the course of maturation (Sutton-McDowall, Gilchrist *et*
148 *al.* 2010). Lipid concentrate was diluted 1 in 25, calculated to result in a final
149 concentration of 40 μM. See Table 1 for composition of the lipid concentrate.

150

151 **Development of the lipid accumulation model**

152 A dose response experiment was performed to determine the optimum concentration of
153 the lipid concentrate (0, 5, 10 20, 40μM). After 16 h of culture, COCs were fixed in 4%
154 paraformaldehyde in phosphate buffered saline (PBS) for 1 h, washed in PBS and
155 transferred to 1 μg mL^{-1} of the neutral lipid stain BODIPY 493/503 (Life Technologies,

156 Invitrogen, CA, USA) in PBS for 1 h in the dark at room temperature. COCs were
157 washed in PBS for 5 min and mounted on coverslips in 3 μL of DAKO fluorescent
158 mounting media (Dako North America Inc, CA, USA). Images were captured using a
159 Fluoview FV10i confocal microscope (Olympus; Tokyo, Japan) using a green laser
160 (excitation: 480 nm, emission: 515 nm) and identical magnification, image and laser
161 settings were used throughout experiments (See Fig. S1 available as Supplementary
162 Material to this paper). Two replicates were performed.

163

164 **Assessment of cumulus expansion index (CEI)**

165 Cumulus expansion was assessed after 16 h of culture by an independent assessor,
166 blinded to treatments, using a scale previously described (Vanderhyden, Caron *et al.*
167 1990). Briefly, a score of 0 indicated no expansion of cumulus cells; +1 = the outer
168 most layers of cumulus cells expanded; +2 = expansion of the entire outer half of
169 cumulus cells; +3 = all layers expanded except the corona radiatae, and +4 = maximal
170 expansion of all layers of cumulus cells. For each treatment group, a mean cumulus
171 expansion index (CEI) (0.0-4.0) was calculated. Three replicates were performed,
172 averaging 25 COCs per treatment group and replicate.

173

174 ***In vitro* fertilisation (IVF) and assessment of embryo development**

175 Following 16 h of maturation, COCs were washed once in fertilisation medium
176 (VitroFert, Cook Australia, Brisbane, Australia) and COCs were transferred to pre-
177 equilibrated fertilisation drops overlaid with paraffin oil. Male mice, which had
178 previously been assessed for mating ability (not less than 3 days prior), were used as

179 sperm donors for IVF. Mice were sacrificed by cervical dislocation and the
180 epididymides and vasa deferentia were collected into warm (37 °C) wash medium
181 (VitroWash, Cook Australia), cleaned of excess fat and tissue and transferred into 1 mL
182 of fertilisation media. Sperm were extracted into the medium and allowed to capacitate
183 at 37 °C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 for 1 h. Capacitated sperm
184 (10 µL) was added to 90 µL fertilisation drops and COCs and sperm were co-incubated
185 for 4 h at 37 °C in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2. COCs were
186 transferred to wash medium, cumulus cells removed mechanically by repeat pipetting.
187 Presumptive zygotes were washed in embryo culture medium (VitroCleave, Cook
188 Australia) and placed into culture drops (4 – 7 per 10 µL drop) at 37 °C in an
189 atmosphere of 6% CO_2, 5% O_2 and 89% N_2. Twenty-four hours following IVF (Day 2),
190 the fertilisation rate was assessed and 2-cells embryos were transferred onto a fresh 20
191 µL drop of embryo culture medium. Embryo morphology was assessed on Day 5 (the
192 end of culture period, 96-100 h post-fertilisation). On-time embryo development was
193 assessed on Day 2 (expected 2-cell stage) and Day 5 (blastocysts or hatching
194 blastocysts). Seven replicates were performed, with 50 COCs per treatment group and
195 replicate.

196

197 **Immunocytochemistry**

198 Following IVM (16 h), COCs were fixed in 4% paraformaldehyde in PBS overnight.
199 Whole COCs were adhered on Cell-Tak (BD Biosciences)-coated slides and
200 immunohistochemically stained using the primary antibody, CTD110.6 (anti O-GlcNAc
201 antibody; Covance, NJ, USA) for O-GlcNAc and propidium iodide (PI) for nuclear
202 staining. Briefly, COCs were permeabilised for 30 min in 0.25 % Triton X-100 (United

203 States Biochemical Corp., OH, USA), blocked for 2 h using 10% goat serum in PBS
204 (Jackson Immuno, PA, USA) in PBS and incubated overnight at 4˚C with 1:250
205 CTD110.6 in blocking solution at. On day 2, COCs were washed and incubated for 2 h
206 at room temperature with 1:250 Alexa Fluor 488 goat anti-mouse IgM (Invitrogen, CA,
207 USA) in blocking solution, washed twice in PBS/PVP and 30 minutes with PI, and
208 mounted under a coverslip in 3 μL of DAKO fluorescent mounting media (Dako).
209 Fluorescence intensity and localisation was examined using Fluoview FV10i confocal
210 microscope using the Alexa Fluor 488 filter for CTD110.6 (excitation: 488 nm,
211 emission: 519 nm) and the PI filter (excitation: 488nm, emission: 617 nm). Three
212 replicates were performed, with 10 COCs per treatment group. Localisation and
213 intensity of CTD110.6 positive staining across the COC was determined using Image J
214 software by placing a box across the oocyte and cumulus cells image and measuring
215 pixel intensity. The mean fluorescence intensity in each pixel column was reported and
216 mean ± SEM was calculated. The data was then represented graphically as intensity of
217 fluorescence over pixel widths (Wu, Dunning *et al.* 2010). Total *O*-linked-glycosylated
218 protein was determined as the sum of total fluorescence in the boxed area.

219

220 **RNA isolation and real-time RT-PCR**

221 Following 8 or 16 h IVM, groups of 90-100 COCs were collected in a minimal volume
222 of medium, snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated
223 from COC using TRIzol Reagent (Invitrogen, CA, USA) method, followed by DNase
224 treatment using Ambion kit (Invitrogen, CA, USA) as per manufacturer's instructions.
225 RNA concentration and purity were quantified using a Nanodrop ND-1000
226 Spectrophotometer (Biolab, Carmel, IN) before reverse transcribing 600 ng of RNA

227 using random primers (Invitrogen, CA, USA) and Superscript™ III Reverse
228 Transcriptase (Invitrogen, CA, USA) according to the manufacturer's instructions.
229 Ribosomal protein *RPl19* (QIAGEN) was used as a validated internal control for every
230 sample. *Xbp1s* primers were: *Xbp1s* reverse, 5'-AGG CTT GGT GTA TAC ATG G-3'
231 and *Xbp1s* forward, 5'-GGT CTG CTG AGT CCG CAG GAG G-3' (Ozcan, Ergin *et al.*
232 2009), and the other primers were Quantitect Primer assays (QIAGEN). Real-time RT-
233 PCR was performed in quadruplicate using SYBR green PCR Master Mix (Applied
234 Biosystems, Foster City, CA) and a Rotor-Gene™ 6000 (Corbett, Valencia, CA) real-
235 time rotary analyser. Real-time RT-PCR data were analysed using the $2^{-(\Delta CT)}$ method
236 and expressed as the fold change relative to a calibrator sample, which was included in
237 each run. Eight replicates were performed for the control treatment and four replicates
238 were performed for all other treatments, with 90-100 COCs per treatment group and
239 replicate.

240

241 **Statistical analysis**

242 Statistical analysis was performed using Graph Pad Prism version 5.0 (GraphPad
243 Software, La Jolla California USA). Differences between treatment groups were tested
244 using a general linear model, followed by Bonferroni *post hoc* tests. Cleavage and
245 blastocyst rates were arcsine transformed prior to analyses. Outcome parameters are
246 reported as mean ± SEM. A *P* value of < 0.05 was considered statistically significant.

247

248 **Results**

249 **Experiment 1: Lipid and hyperglycaemic mimetic, GlcN, impaired oocyte**
250 **developmental competence.**

251 Cumulus expansion indices (CEI) were examined prior to IVF. Hyperglycaemia and
252 lipid treatments during IVM imposed no effect on cumulus expansion ($P > 0.05$; Table
253 2). On day 2, the cleavage rate of COCs treated with high glucose + lipid was
254 significantly lower than the lipid treatment (high glucose + lipid = 81.86 ± 1.88% vs.
255 lipid = 89.86 ± 2.06%; $P < 0.05$). There were no significant differences in cleavage
256 rates between the other treatment groups. The fertilised oocytes from COCs treated with
257 GlcN and lipid yielded fewer blastocysts on day 5 (48.49 ± 5.19% and 50.35 ± 4.93%
258 respectively), which were significantly lower than the control (69.28 ± 3.29%; $P < 0.05$).
259 High glucose + lipid treatments trended toward a lower blastocyst rate when compared
260 to the control (Fig. 1; $P = 0.08$).

261

262 **Experiment 4: *O*-linked glycosylation is primarily localised in the oocyte in a**
263 **mouse model of hyperglycaemia and lipid treatment**

264 Immunohistochemical localisation of *O*-GlcNAc with CTD110.6 revealed positive
265 staining in the oocytes, with higher intensity in COCs treated with high glucose, lipid,
266 GlcN and high glucose + lipid treatments compared to the control (Fig.2A).
267 Densitometry analysis of the CTD110.6 revealed similar results, where GlcN treatment
268 significantly increased *O*-glycosylation positive staining within the oocyte compared to
269 the control group (Fig. 2B; $P < 0.05$). Increased *O*-glycosylation was also observed in
270 the oocytes treated with high glucose. In contrast, addition of lipid with high glucose
271 diminished the extent of staining relative to glucose alone. No significant difference
272 was observed in the cumulus cells between the treatment groups (Fig. 2B).

273

274 **Experiment 5: Expression of hexosamine biosynthetic pathway (HBP) enzymes**
275 **and endoplasmic reticulum (ER) stress genes following exposure to**
276 **hyperglycaemia and lipids**

277 The mRNA expression of HBP enzymes glutamine:fructose-6-phosphate
278 amidotransferase 1 (*Gfpt1), Gfpt2,* O-linked β-N-acetylglucosaminyltransferase (*Ogt*)
279 and hyaluronan synthase 2 (*Has2*) were determined following exposure to high glucose
280 and lipid treatments. After 8 h of maturation, GlcN treatment resulted in 2.2-fold
281 increase in *Gfpt1*, 2.8-fold increase in *Gfpt2* and 4-fold increase in *Has2* expressions
282 compared to the control group (Fig. 3A-B, 3D). There were no other significant
283 differences in gene expression between the other treatment groups (Fig. 3). After 16 h,
284 there was a corresponding increased expression of *Gfpt1* (1.8-fold) and *Gfpt2* (1.4-fold)
285 (Fig. 3A-B) for the GlcN treatment. Interestingly, addition of lipid to the culture
286 medium significantly decreased the expression of *Ogt* (an enzyme that adds the O-
287 GlcNAcylation to proteins) and *Gfpt2* when compared to the control (Fig. 3B-C).

288

289 After 8 h of maturation, supplementation of GlcN in the culture media resulted in
290 increased expression of ER-stress gene *Grp78* (3-fold), whereas lipid treatment
291 significantly increased *Atf4* expression (Fig. 4A-C). After 16 h, a similar trend was
292 observed in the gene expression level with increased *Xbp1* (1.8-fold) and *Grp78* (2.6-
293 fold) following GlcN treatment (Fig. 4A, 5C). Lipid and high glucose + lipid treated
294 COC also significantly increased the expression of *Xbp1* (1.8-fold). At this time point,
295 COCs treated with GlcN (2.3-fold) and high glucose (1.5-fold) were found to have up-
296 regulated the expression of *Atf4*. Interestingly, *Atf4* expression was significantly

297 reduced when lipid was added to the high glucose culture medium. There were no
298 significant differences in Atf6 gene expression between treatment groups (Fig.4D).

299

300 **Discussion**

301 There is a significant body of literature that demonstrates poor pre-implantation embryo
302 development outcomes following hyperglycaemic or lipidemic exposure during *in vivo*
303 or *in* vitro development. The current study elucidates some of the cellular mechanisms
304 by which high glucose and lipid conditions contribute to reduced oocyte quality and
305 developmental competence. In this study, we utilised a chemically defined lipid
306 concentrate that contains various non-esterified fatty acids (NEFAs) as a substitution of
307 using single lipids such as palmitic acid or lipid-rich follicular fluid, which were used in
308 other studies (Aardema, Vos *et al.* 2011; Wu, Russell *et al.* 2012; Yang, Wu *et al.* 2012).
309 This was because the lipid concentrate is more closely resembled the NEFA detected in
310 women with increased body mass index (Robker, Akison *et al.* 2009; Valckx, De Pauw
311 *et al.* 2012) and that follicular fluid contains hormones, growth factors and undefined
312 proteins which may compromise development. Furthermore, we have investigated the
313 role of high glucose and lipid on O-GlcNAcylation and ER-stress during and at the end
314 of IVM. Notably, hyperglycaemic conditions increase aberrant *O*-linked glycosylation
315 in the oocytes and induce the expression of ER-stress genes. Surprisingly, presence of
316 lipid in the culture media represses the expression of HBP enzymes genes such as the
317 rate-limiting enzyme *Gfpt2* and *Ogt*.

318

319 The cleavage rate of COCs treated with the combination of high glucose and lipid was
320 lower than treatment with lipid alone, whereas blastocyst development was significantly
321 reduced following GlcN and lipid supplementation, but only tended to decrease in high
322 glucose. This discrepancy with high glucose treatment may be explained by the
323 difference in culture media used; a much simpler medium was utilised in our previous
324 study (Frank, Sutton-McDowall *et al.* 2013). The result with lipid treatment is
325 consistent with several other publications, demonstrating that lipid level is critical
326 during embryo development (Leroy, Vanholder *et al.* 2005; Van Hoeck, Sturmey *et al.*
327 2011; Wu, Russell *et al.* 2012; Yang, Wu *et al.* 2012). Interestingly, there was a trend of
328 decreased blastocyst development following culture with high glucose + lipid
329 suggestive of high levels of glucose not having an additive impairment of oocyte
330 developmental competence.

331

332 Increased β-*O*-GlcNAcylation of proteins is a hallmark of increased HBP activity,
333 induced by either hyper- or hypoglycemia. Following IVM, high glucose or GlcN
334 significantly increased β-*O*-GlcNAcylation levels, in line with GlcN being a potent
335 stimulator of HBP UDP-GlcNAc production (approximately 40 times, as measured in
336 adipocytes (Marshall, Bacote *et al.* 1991)). Excess flux with either glucose or GlcN
337 through HBP has previously shown to reduce embryo development and this
338 phenomenon was reversed using an OGT inhibitor, reflecting their relative potential to
339 stimulate HBP and UDP-GlcNAc production (Frank, Sutton-McDowall *et al.* 2014a;
340 Frank, Sutton-McDowall *et al.* 2014b; Pantaleon, Tan *et al.* 2010). The addition of lipid
341 to the medium did not increase *O*-GlcNAcylation levels. This phenomenon could be
342 explained by the fact that opposing interactions between unsaturated fatty acids (such as

343 oleic acid) are able to inhibit the pro-apoptotic effect of their counterpart-saturated
344 palmitic and stearic acid (Nemcova-Furstova, James *et al.* 2011).

345

346 There were no additive effects of high glucose and lipid on β-*O*-GlcNAcylation. Indeed,
347 intensity within the oocyte the in lipid and high glucose group was overall reduced
348 compared to high glucose treated COCs. Under the *in vitro* conditions used, lipid
349 appears to play a role in down-regulating cellular responses to hyperglycaemia.
350 Hyperglycaemia is a characteristic of both types of diabetes, yet hyperlipidemia is only
351 associated with Type II diabetes. Although the current study utilises an *in vitro* model,
352 we have also found that systemic administration of GlcN during the peri-compaction
353 period had significant effects on fetal survival and abnormalities, which were most
354 evident in lean rather than obese mice, indicating the possibility that high lipid and
355 glucose levels may interact and regulate fuel-sensing pathways, specifically the HBP
356 (Schelbach, Robker *et al.* 2013).

357

358 Compared to the control group, GlcN treatment significantly increased mRNA
359 expression of the rate-limiting enzyme of HBP, *Gfpt1* and *Gfpt2* after 8 and 16 h of
360 culture. While high glucose treatment did not affect the gene expression of HBP
361 enzymes (possibly due to glucose regulation of these enzymes being at the substrate
362 availability and post-translational level), expression of *Gfpt2* and *Ogt* (the enzyme that
363 modifies proteins with GlcNAc) were down regulated in the lipid group compared to
364 the control, supportive of the notion that lipids interact with glucose sensing pathways.
365 Previously, elevated fatty acids have been shown to interfere with glucose by inhibiting
366 glucose-induced insulin secretion and β-cell oxidation in rat pancreatic islets (Sako and

367 Grill 1990; Zhou and Grill 1994). Moreover, free fatty acids supplementation decreases
368 the expression of glucose transporter type 2 (SLC2A2) and glucokinase (Gremlich,
369 Bonny *et al.* 1997). Therefore, down-regulation of HBP enzymes in the presence of
370 lipids may be due to impaired glucose transporter activities, reducing glucose
371 transportation down the pathway and decreasing the production of UDP-GlcNAc, the
372 end product of HBP. Alternatively, the level of UDP-GlcNAc may also be limited by
373 the known feedback inhibition of *Gfpt* by UDP-GlcNAc (Kornfeld 1967). All these
374 interactions of lipid with glucose further supported the idea that nutrient-sensing
375 pathways are also interconnected in the ovary/COC/ovarian cells. While O-
376 GlcNAcylation staining revealed maximal staining in the oocyte, should the differences
377 in gene expression are due to changes in the oocytes or the surrounding cumulus cells.
378 Oocyte exhibits low transcriptional activity, however, bidirectional communication
379 between oocyte and cumulus cells are essential for oocyte viability (Albertini,
380 Combelles *et al.* 2001; Eppig 1991). So, alteration in cumulus cells should reflect in the
381 oocytes.

382

383 The induction of ER-stress is a well-characterised response of lipotoxicity (Borradaile,
384 Han *et al.* 2006; Malhi and Gores 2008). Consistent with previous studies, *Xbp1* and
385 *Atf4* were specifically up-regulated in COC following lipid treatment (Wu, Russell *et al.*
386 2012; Yang, Wu *et al.* 2012). GlcN treatment significantly increased mRNA expression
387 of *Xbp1*, *Atf4* and *Grp78* suggesting that the COC is undergoing a distinct UPR
388 response or that its presence influences the stress response. Interestingly, high glucose
389 significantly increased the expression of *Atf4* after 16 h of culture, which was down-
390 regulated in the high glucose and lipid treatment, further emphasising that lipid appears
391 to inhibit the effect induced by hyperglycaemia. In contrast, the *Atf6* expression

392 remained unaffected by all the treatments at both time points. Activation of *Atf6*
393 requires a dissociation step from its inhibitory regulator, Binding immunoglobulin
394 Protein (BiP). Binding of *Atf6* to BiP was reported to be very stable (Shen, Snapp *et al.*
395 2005), thus could explain why there was no activation of this pathway in response to the
396 treatments.

397

398 In summary, the current findings demonstrated that lipid at level of 40 μM (and GlcN)
399 induced ER stress and that high glucose (and GlcN) increase O-GlcNAcylation but that
400 the two treatments do not potentiate each other in these pathways. Furthermore, this
401 study also implicates the possibility of lipid down-regulating the detrimental
402 consequences of hyperglycaemia on oocyte health. This study also implicates the
403 mechanism of O-GlcNAcylation and ER stress as likely contributors to the reduced
404 fertility observed in obese women. This provides new leads for further investigation
405 into possible treatment strategies and interventions which may improve pregnancy and
406 fetal outcomes in obese women and its associated comorbidities such as diabetic
407 women.

408

409 **Declaration of interest**

410 Authors have no conflict of interest.

411

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417

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422

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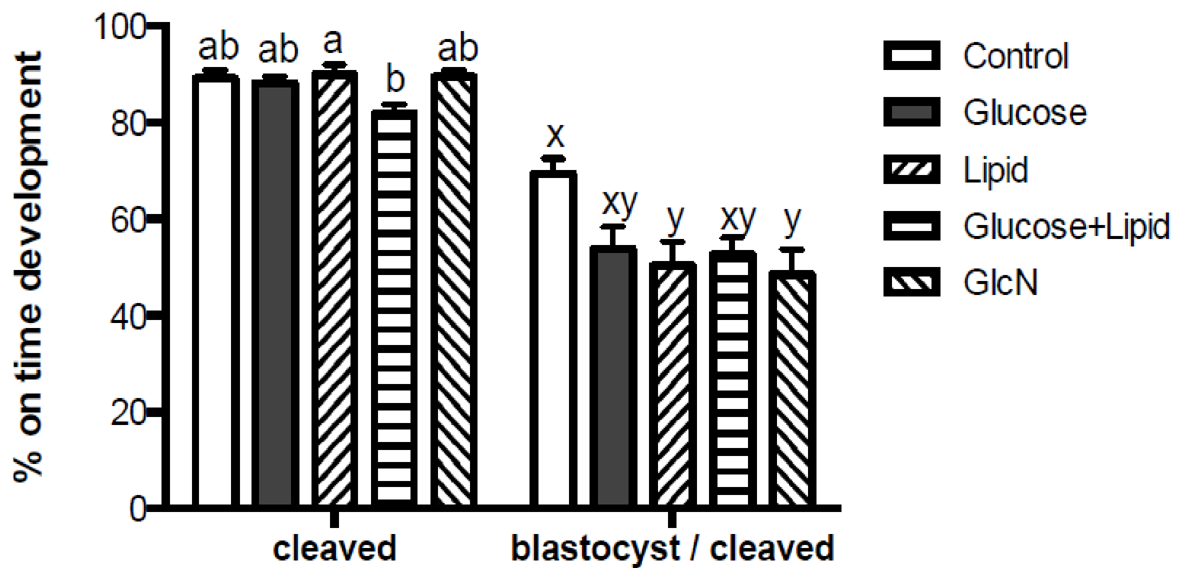
640

641 **Figure legends**

642 **Figure 1. Cleavage and blastocyst development following IVM in high glucose,**
643 **lipids or glucosamine (GlcN).** Data are presented as the mean + SEM. *Different letters*
644 indicate significance differences (^{ab,xy} $P < 0.05$). GlcN = Glucosamine.



646 **Figure 2A. Localisation of β-O-linked glycosylation in COCs following IVM in the**
647 **presence of high glucose and lipid**. Propidium iodide (PI, red) shows nuclear staining,
648 CTD 110.6 (green) shows β-*O*-glycosylation (O-GlcNAc). The images were merged
649 (merge). Scale bars = 100 μm. GlcN = Glucosamine.

Figure 2A

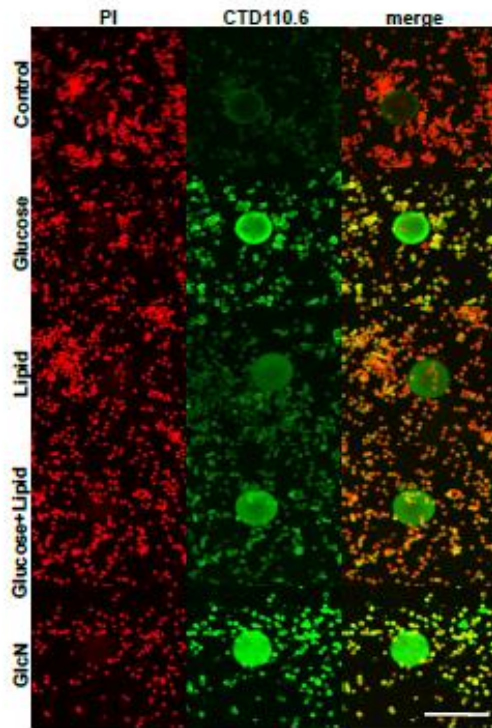
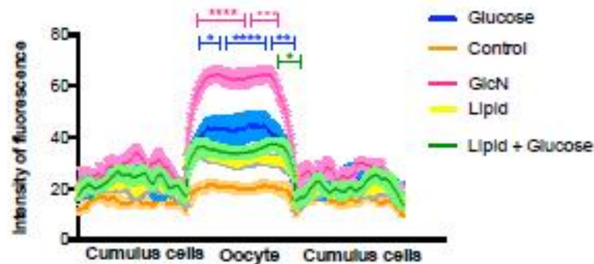


Figure 2B



650

651 **Figure 2B. Intensity of CTD110.6 after cultured with different treatments.**

652 COCs were collected after 16 h of culture with control and glucose, fixed in 4%
653 paraformaldehyde and incubated with CTD110.6 for β-*O*-glycosylation (O-GlcNAc)
654 and propidium iodide (PI) for nuclear staining. Values are mean ± SEM (lighter shading
655 represents error bars). Asterisks indicate significance differences compared to the
656 control. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. GlcN = Glucosamine.

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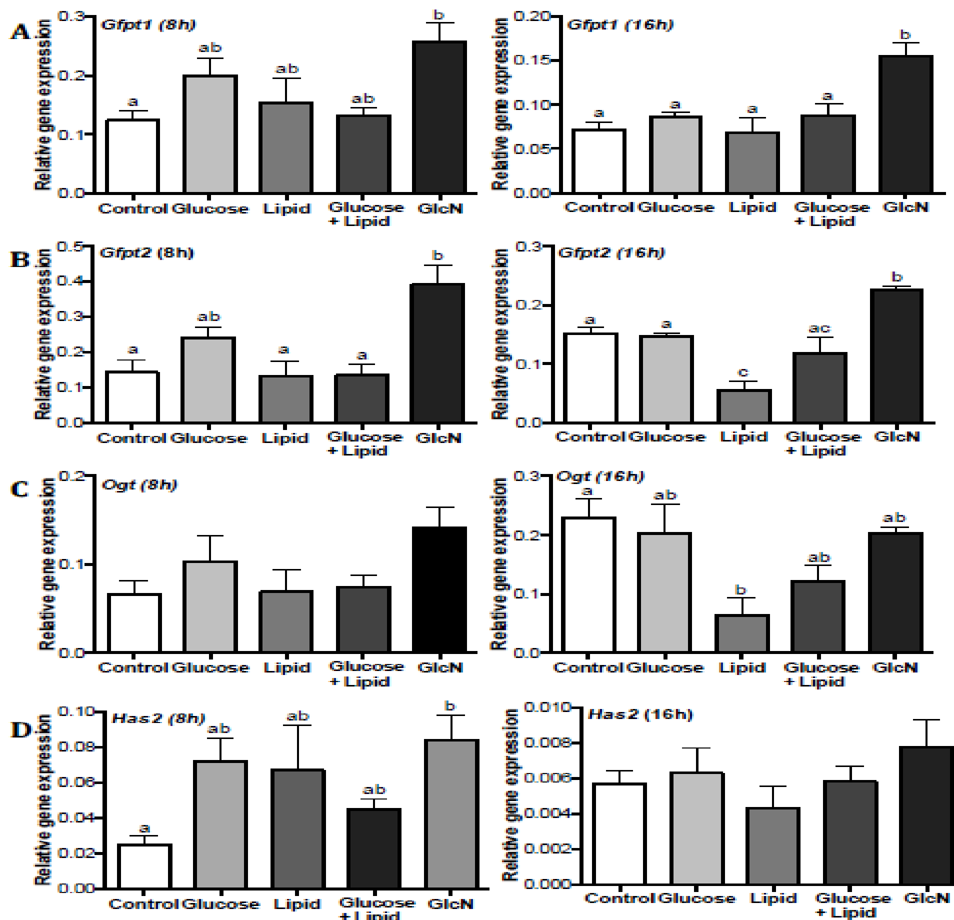
659 **Figure 3. Expression of HBP enzymes after 8 and 16 hours of IVM.**

660 COCs were collected after 8 h and 16 h of culture with different treatments. Total RNA
661 was isolated, reverse transcribed and expression of HBP enzymes (A)
662 Glutamine:Fructose-6-phosphate transaminase 1 (*Gfpt1*), (B) Glutamine:Fructose-6-
663 phosphate transaminase 2 (*Gfpt2*), (C) *O*-linked glycosyltransferase (*Ogt*) and (D)
664 hyaluronan synthase 2 (*Has2*) (D) were analysed by RT-PCR. Values are mean ± SEM
665 expressed as a fold change relative to *Rpl19*. *Different letters* indicate significant
666 differences ([ab] $P < 0.05$). GlcN = Glucosamine.

Figure 3



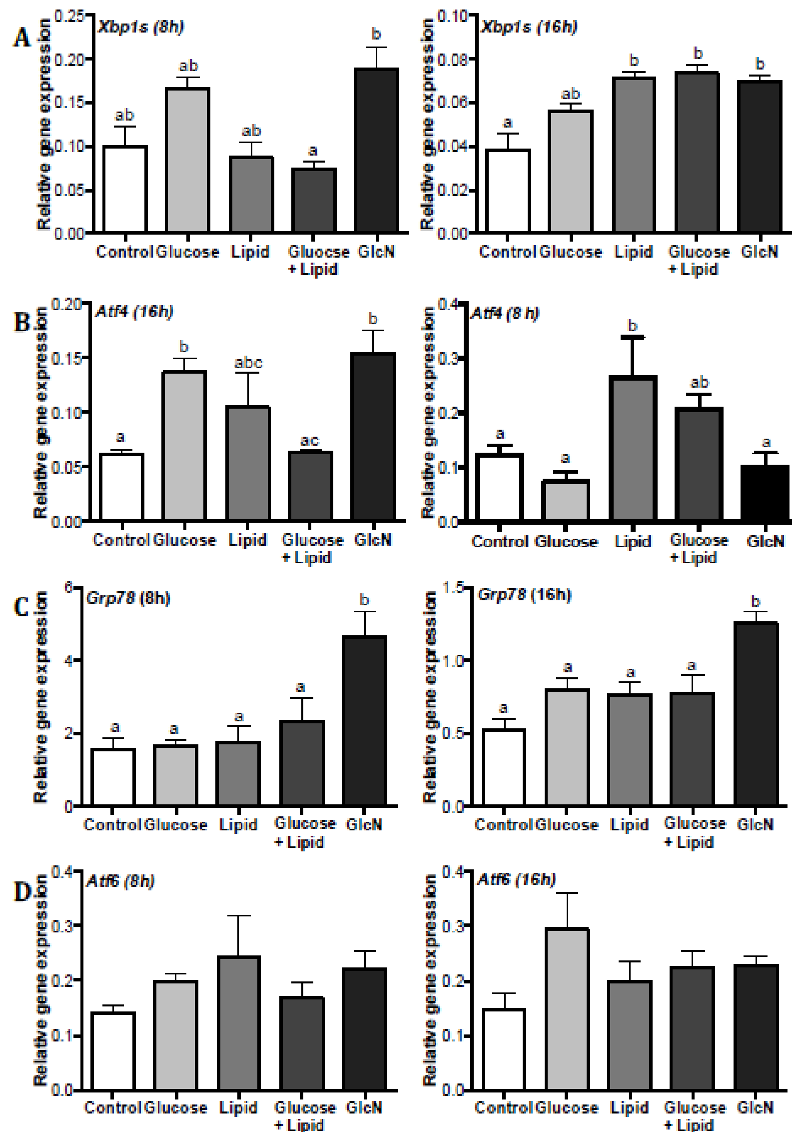
667

668 **Figure 4. Expression of ER stress marker genes after 8 and 16 hours of IVM.**

669 COCs were collected after 8 h and 16 h of culture with different treatments. Total RNA
670 was isolated, reverse transcribed and expression of ER stress marker genes (A) X-box-

671 binding protein-1 (*Xbp1*), (B) Activating transcription factor 4 (*Atf4*), (C) glucose-
672 regulated protein 78 (*Grp78*) and (D) Activating transcription factor 4 (*Atf6*) were
673 analysed by RT-PCR. Values are mean±SEM expressed as a fold change relative to
674 *Rpl19*. *Different letters* indicates significant differences by one-way ANOVA,
675 Bonferroni post hoc test.

Figure 4



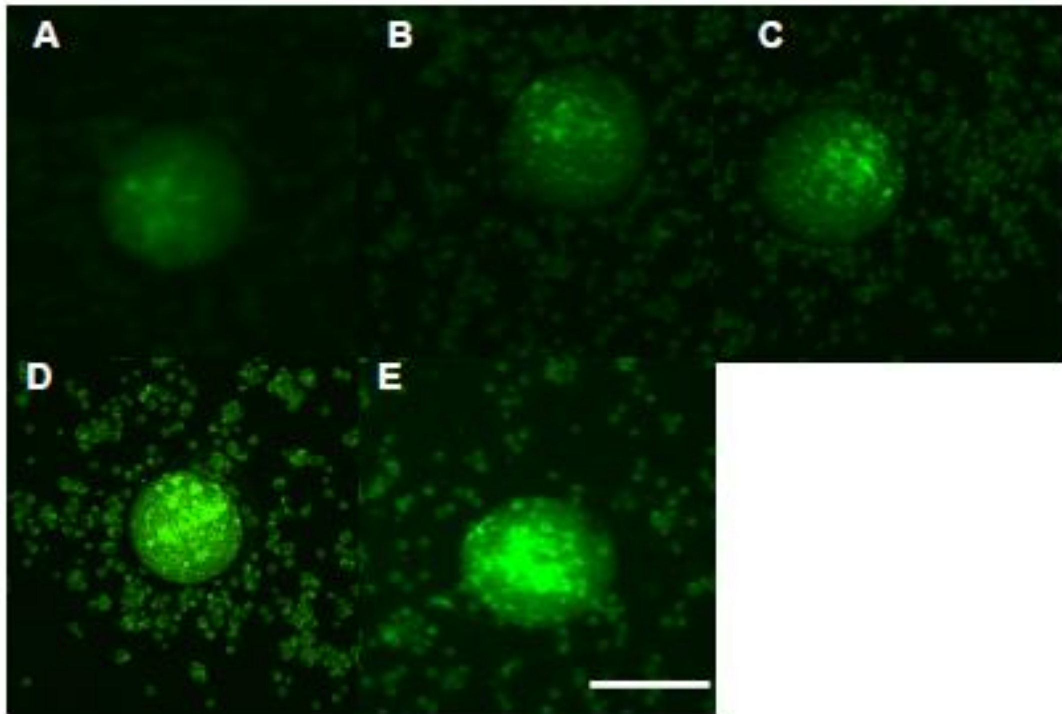
676

677 **Figure S1. BODIPY lipid staining following IVM in increasing doses of lipid.**
678 COCs were matured for 16 hours in IVM in medium containing various concentrations
679 of defined lipid concentrate and stained with neutral lipid dye BODIPY 490/503. (A) 0

680 μM lipid; (B) 5 μM lipid; (C) 10 μM lipid; (D) 20 μM lipid; (E) 40 μM lipid. Scale bars

681 = 100 μm.

Figure S1



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684 **Table 1. Chemically Defined Lipid concentrate used in lipid treatment during *in vitro***
685 **maturation**

Components	Concentration (mg/L)
Arachidonic Acid	20
Cholesterol	220
DL-alpha-Tocopherol Acetate	70
Ethyl Alcohol 100%	0
Linoleic Acid	10
Linolenic Acid	10
Myristic Acid	10
Oleic Acid	10
Palmitic Acid	10
Palmitoleic Acid	10
Pluronic F-68	90000
Stearic Acid	10
Tween 80®	2200

686

687 **Table 2. Cumulus expansion indices following maturation (16 h) in the inidictaed**

688 **treatments.**

Treatment	Cumulus expansion index
Control	3.81 $\pm$ 0.14
Glucose	3.84 $\pm$ 0.10
Lipid	3.68 $\pm$ 0.03
Glucose + Lipid	3.63 $\pm$ 0.07
GlcN	3.89 $\pm$ 0.07

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