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Glucose and lipid on developmental competence

Title: Hyperglycaemia and lipid differentially impair mouse oocyte developmental competence

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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 ($Gft2$) and $O$-linked $\beta$-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).
The detrimental effects of hyperglycaemia on oocyte developmental competence is in part mediated by up-regulation of the hexosamine biosynthesis pathway (HBP) (Cheryl J. Schelbach 2012; Schelbach, Kind et al. 2010; Sutton-McDowall, Mitchell et al. 2006), a fuel-sensing pathway, which metabolises glucose to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). Single UDP-GlcNAc molecules modify serine or threonine amino acids of proteins, a process known as $\beta$-$O$-linked glycosylation ($O$-GlcNAcylation), which acts in an analogous manner to phosphorylation to regulate protein function (Butkinaree, Park et al. 2010). Altered $O$-GlcNAcylation is one of the primary pathologies of diabetes in somatic cells and notably the primary mechanism behind the development of insulin resistance in Type II diabetes (Marshall, Bacote et al. 1991; Yang, Ongusaha et al. 2008).

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

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

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

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

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

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

Materials and methods

Mice

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

Isolation and in vitro maturation of mouse COCs

Immature, unexpanded COCs were isolated by puncturing the antral follicles of ovaries collected 46 h post-intraperitoneal injection of 5IU pregnant mare’s serum gonadotropin (PMSG; Folligon; Intervet, Boxmeer, The Netherlands). All COCs were collected in HEPES-buffered α-MEM handling media (Life Technologies, Invitrogen, CA, USA)
supplemented with 4 mg mL\(^{-1}\) fatty acid free Bovine Serum Albumin (FAF BSA; MP Biomedicals, Solon, OH, USA). Immature COCs were cultured in groups of 30 in 1.5 mL of pre-equilibrate IVM medium, overlaid with paraffin oil (Merck, Darmstadt, Germany). Base IVM media (control) was bicarbonate-buffered \(\alpha\)-MEM supplemented with 1% fetal bovine serum (FBS; Invitrogen, Gibco, Victoria, Australia), 50 mIU mL\(^{-1}\) recombinant human follicle stimulating hormone (FSH; Puregon-Organon, Oss, The Netherlands) and 10 ng mL\(^{-1}\) recombinant human epidermal growth factor (EGF; R & D Systems). Experimental treatments were 1) Control (5.56 mM glucose); 2) high glucose (30 mM glucose); 3) glucosamine (2.5 mM GlcN plus 5.56 mM glucose); 4) lipid (40 µM lipid concentrate; GIBCO, Invitrogen, CA, USA) and 5) lipid and high glucose (40 µM lipid concentrate plus 30 mM glucose). COCs were cultured at 37 \(^\circ\)C in an atmosphere of 6% CO\(_2\), 5% O\(_2\) and 89% N\(_2\) for 8 h or 16 h. We have previously demonstrated that large culture volumes are required to maintain hyperglycaemic concentrations of glucose, due to the high metabolic rate of COCs (Frank, Sutton-McDowall et al. 2013). In this study, 30 mM was used to avoid depletion to more normoglycaemic levels during the course of maturation (Sutton-McDowall, Gilchrist et al. 2010). Lipid concentrate was diluted 1 in 25, calculated to result in a final concentration of 40 µM. See Table 1 for composition of the lipid concentrate.

Development of the lipid accumulation model

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

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

Assessment of cumulus expansion index (CEI)

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

In vitro fertilisation (IVF) and assessment of embryo development

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

sperm donors for IVF. Mice were sacrificed by cervical dislocation and the epididymides and vasa deferentia were collected into warm (37 °C) wash medium (VitroWash, Cook Australia), cleaned of excess fat and tissue and transferred into 1 mL of fertilisation media. Sperm were extracted into the medium and allowed to capacitate at 37 °C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂ for 1 h. Capacitated sperm (10 µL) was added to 90 µL fertilisation drops and COCs and sperm were co-incubated for 4 h at 37 °C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂. COCs were transferred to wash medium, cumulus cells removed mechanically by repeat pipetting. Presumptive zygotes were washed in embryo culture medium (VitroCleave, Cook Australia) and placed into culture drops (4 – 7 per 10 µL drop) at 37 °C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂. Twenty-four hours following IVF (Day 2), the fertilisation rate was assessed and 2-cells embryos were transferred onto a fresh 20 µL drop of embryo culture medium. Embryo morphology was assessed on Day 5 (the end of culture period, 96-100 h post-fertilisation). On-time embryo development was assessed on Day 2 (expected 2-cell stage) and Day 5 (blastocysts or hatching blastocysts). Seven replicates were performed, with 50 COCs per treatment group and replicate.

Immunocytochemistry

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

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

RNA isolation and real-time RT-PCR

Following 8 or 16 h IVM, groups of 90-100 COCs were collected in a minimal volume of medium, snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated from COC using TRIZol Reagent (Invitrogen, CA, USA) method, followed by DNase treatment using Ambion kit (Invitrogen, CA, USA) as per manufacturer’s instructions. RNA concentration and purity were quantified using a Nanodrop ND-1000 Spectrophotometer (Biolab, Carmel, IN) before reverse transcribing 600 ng of RNA
using random primers (Invitrogen, CA, USA) and Superscript™ III Reverse Transcriptase (Invitrogen, CA, USA) according to the manufacturer’s instructions. Ribosomal protein \textit{RP119} (QIAGEN) was used as a validated internal control for every sample. \textit{Xbp1s} primers were: \textit{Xbp1s} reverse, 5’-AGG CTT GGT GTA TAC ATG G-3’ and \textit{Xbp1s} forward, 5’-GGT CTG CTG AGT CCG CAG GAG G-3’ (Ozcan, Ergin et al. 2009), and the other primers were Quantitect Primer assays (QIAGEN). Real-time RT-PCR was performed in quadruplicate using SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) and a Rotor-Gene™ 6000 (Corbett, Valencia, CA) real-time rotary analyser. Real-time RT-PCR data were analysed using the $2^{(\Delta\Delta CT)}$ method and expressed as the fold change relative to a calibrator sample, which was included in each run. Eight replicates were performed for the control treatment and four replicates were performed for all other treatments, with 90-100 COCs per treatment group and replicate.

\textbf{Statistical analysis}

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

\textbf{Results}
Experiment 1: Lipid and hyperglycaemic mimetic, GlcN, impaired oocyte developmental competence.

Cumulus expansion indices (CEI) were examined prior to IVF. Hyperglycaemia and lipid treatments during IVM imposed no effect on cumulus expansion (P > 0.05; Table 2). On day 2, the cleavage rate of COCs treated with high glucose + lipid was significantly lower than the lipid treatment (high glucose + lipid = 81.86 ± 1.88% vs. lipid = 89.86 ± 2.06%; P < 0.05). There were no significant differences in cleavage rates between the other treatment groups. The fertilised oocytes from COCs treated with GlcN and lipid yielded fewer blastocysts on day 5 (48.49 ± 5.19% and 50.35 ± 4.93% respectively), which were significantly lower than the control (69.28 ± 3.29%; P < 0.05).

High glucose + lipid treatments trended toward a lower blastocyst rate when compared to the control (Fig. 1; P = 0.08).

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

Immunohistochemical localisation of O-GlcNAc with CTD110.6 revealed positive staining in the oocytes, with higher intensity in COCs treated with high glucose, lipid, GlcN and high glucose + lipid treatments compared to the control (Fig. 2A). Densitometry analysis of the CTD110.6 revealed similar results, where GlcN treatment significantly increased O-glycosylation positive staining within the oocyte compared to the control group (Fig. 2B; P < 0.05). Increased O-glycosylation was also observed in the oocytes treated with high glucose. In contrast, addition of lipid with high glucose diminished the extent of staining relative to glucose alone. No significant difference was observed in the cumulus cells between the treatment groups (Fig. 2B).
273

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

277

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

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

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

Discussion

There is a significant body of literature that demonstrates poor pre-implantation embryo development outcomes following hyperglycaemic or lipidemic exposure during in vivo or in vitro development. The current study elucidates some of the cellular mechanisms by which high glucose and lipid conditions contribute to reduced oocyte quality and developmental competence. In this study, we utilised a chemically defined lipid concentrate that contains various non-esterified fatty acids (NEFAs) as a substitution of using single lipids such as palmitic acid or lipid-rich follicular fluid, which were used in other studies (Aardema, Vos et al. 2011; Wu, Russell et al. 2012; Yang, Wu et al. 2012). This was because the lipid concentrate is more closely resembled the NEFA detected in women with increased body mass index (Robker, Akison et al. 2009; Valckx, De Pauw et al. 2012) and that follicular fluid contains hormones, growth factors and undefined proteins which may compromise development. Furthermore, we have investigated the role of high glucose and lipid on O-GlcNAcylation and ER-stress during and at the end of IVM. Notably, hyperglycaemic conditions increase aberrant O-linked glycosylation in the oocytes and induce the expression of ER-stress genes. Surprisingly, presence of lipid in the culture media represses the expression of HBP enzymes genes such as the rate-limiting enzyme Gfpt2 and Ogt.
The cleavage rate of COCs treated with the combination of high glucose and lipid was lower than treatment with lipid alone, whereas blastocyst development was significantly reduced following GlcN and lipid supplementation, but only tended to decrease in high glucose. This discrepancy with high glucose treatment may be explained by the difference in culture media used; a much simpler medium was utilised in our previous study (Frank, Sutton-McDowall et al. 2013). The result with lipid treatment is consistent with several other publications, demonstrating that lipid level is critical during embryo development (Leroy, Vanholder et al. 2005; Van Hoeck, Sturmey et al. 2011; Wu, Russell et al. 2012; Yang, Wu et al. 2012). Interestingly, there was a trend of decreased blastocyst development following culture with high glucose + lipid suggestive of high levels of glucose not having an additive impairment of oocyte developmental competence.

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

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

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

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

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

The induction of ER-stress is a well-characterised response of lipotoxicity (Borradaile, Han et al. 2006; Malhi and Gores 2008). Consistent with previous studies, Xbp1 and Atf4 were specifically up-regulated in COC following lipid treatment (Wu, Russell et al. 2012; Yang, Wu et al. 2012). GlcN treatment significantly increased mRNA expression of Xbp1, Atf4 and Grp78 suggesting that the COC is undergoing a distinct UPR response or that its presence influences the stress response. Interestingly, high glucose significantly increased the expression of Atf4 after 16 h of culture, which was down-regulated in the high glucose and lipid treatment, further emphasising that lipid appears to inhibit the effect induced by hyperglycaemia. In contrast, the Atf6 expression
remained unaffected by all the treatments at both time points. Activation of Atf6 requires a dissociation step from its inhibitory regulator, Binding immunoglobulin Protein (BiP). Binding of Atf6 to BiP was reported to be very stable (Shen, Snapp et al. 2005), thus could explain why there was no activation of this pathway in response to the treatments.

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

Declaration of interest
Authors have no conflict of interest.

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Glucose and lipid on developmental competence

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Glucose and lipid on developmental competence

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Glucose and lipid on developmental competence


Glucose and lipid on developmental competence


Glucose and lipid on developmental competence


Glucose and lipid on developmental competence


Glucose and lipid on developmental competence
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Glucose and lipid on developmental competence


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Glucose and lipid on developmental competence


Glucose and lipid on developmental competence

Figure legends

Figure 1. Cleavage and blastocyst development following IVM in high glucose, lipids or glucosamine (GlcN). Data are presented as the mean ± SEM. Different letters indicate significance differences (ab,xy P < 0.05). GlcN = Glucosamine.

Figure 1

![Bar graph showing cleavage and blastocyst development following IVM in high glucose, lipids or glucosamine (GlcN).](image)

Figure 2A. Localisation of β-O-linked glycosylation in COCs following IVM in the presence of high glucose and lipid. Propidium iodide (PI, red) shows nuclear staining, CTD 110.6 (green) shows β-O-glycosylation (O-GlcNAc). The images were merged (merge). Scale bars = 100 μm. GlcN = Glucosamine.
Figure 2B. Intensity of CTD110.6 after cultured with different treatments.

COCs were collected after 16 h of culture with control and glucose, fixed in 4% paraformaldehyde and incubated with CTD110.6 for $\beta$-$O$-glycosylation (O-GlcNAc) and propidium iodide (PI) for nuclear staining. Values are mean ± SEM (lighter shading represents error bars). Asterisks indicate significance differences compared to the control. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. GlcN = Glucosamine.
Figure 3. Expression of HBP enzymes after 8 and 16 hours of IVM.
COCs were collected after 8 h and 16 h of culture with different treatments. Total RNA was isolated, reverse transcribed and expression of HBP enzymes (A) Glutamine:Fructose-6-phosphate transaminase 1 (Gfpt1), (B) Glutamine:Fructose-6-phosphate transaminase 2 (Gfpt2), (C) O-linked glycosyltransferase (Ogt) and (D) hyaluronan synthase 2 (Has2) were analysed by RT-PCR. Values are mean ± SEM expressed as a fold change relative to Rpl19. Different letters indicate significant differences (ab P < 0.05). GlcN = Glucosamine.

Figure 4. Expression of ER stress marker genes after 8 and 16 hours of IVM.
COCs were collected after 8 h and 16 h of culture with different treatments. Total RNA was isolated, reverse transcribed and expression of ER stress marker genes (A) X-box-
Glucose and lipid on developmental competence

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

**Figure S1.** BODIPY lipid staining following IVM in increasing doses of lipid.

COCs were matured for 16 hours in IVM in medium containing various concentrations of defined lipid concentrate and stained with neutral lipid dye BODIPY 490/503. (A) 0
Glucose and lipid on developmental competence

µM lipid; (B) 5 µM lipid; (C) 10 µM lipid; (D) 20 µM lipid; (E) 40 µM lipid. Scale bars = 100 µm.

Figure S1
Table 1. Chemically Defined Lipid concentrate used in lipid treatment during in vitro maturation

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic Acid</td>
<td>20</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>220</td>
</tr>
<tr>
<td>DL-alpha-Tocopherol Acetate</td>
<td>70</td>
</tr>
<tr>
<td>Ethyl Alcohol 100%</td>
<td>0</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Linolenic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Myristic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Palmitoleic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Pluronic F-68</td>
<td>90000</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>10</td>
</tr>
<tr>
<td>Tween 80®</td>
<td>2200</td>
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</table>
Table 2. Cumulus expansion indices following maturation (16 h) in the indicated treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cumulus expansion index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.81 ± 0.14</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.84 ± 0.10</td>
</tr>
<tr>
<td>Lipid</td>
<td>3.68 ± 0.03</td>
</tr>
<tr>
<td>Glucose + Lipid</td>
<td>3.63 ± 0.07</td>
</tr>
<tr>
<td>GlcN</td>
<td>3.89 ± 0.07</td>
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</tbody>
</table>