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human reproduction ORIGINAL ARTICLE *Embryology*

Combined parental obesity negatively impacts preimplantation mouse embryo development, kinetics, morphology and metabolism

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STUDY QUESTION: Does combined parental obesity, both an obese mother and father, have a greater effect on mouse preimplantation embryo development and quality than single-parent obesity?

SUMMARY ANSWER: Combined parental obesity causes a greater reduction in the blastocyst rate and a greater delay to the timing of key embryonic developmental events than single-parental obesity, as well as altering embryonic characteristics, such as zona pellucida width.

WHAT IS KNOWN ALREADY: Maternal or paternal obesity alone are known to have significant and detrimental impacts on preimplantation embryo development. Furthermore, these early embryonic perturbations can have long-term impacts on both offspring health and further generations. This is one of the first studies to examine the effects of having both an obese mother and an obese father.

STUDY DESIGN, SIZE, DURATION: A cross-sectional control versus treatment mouse study of diet-induced obesity was employed, in which 300 embryos per group were generated and studied from reciprocal matings: (i) control female and control male (Lean Parented Embryos); (ii) control female and obese male (Paternal Obese Parented Embryos); (iii) obese female and control male (Maternal Obese Parented Embryos) and (iv) obese female and obese male (Combined Obese Parented embryos). Assessments of the embryonic development rate, timing of development, morphological characteristics, metabolic gene expression, metabolism and cell lineage allocation were made at selected time points and analysed in relation to parental obesity status.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Three-week-old C57BL6 male and female mice were fed control (7% total fat) or high fat (21% total fat) diets for a minimum of 8 weeks. Females were superovulated, mated, fertilized zygotes recovered and standard mouse *in vitro* embryo culture performed. Time-lapse monitoring was undertaken to compare developmental timings and morphological characteristics (embryonic area and zona pellucida width) for embryos from all four reciprocal matings. Differential staining identified cell lineage allocation. Real-time quantitative RT–PCR (qRT–PCR) and microfluorescence were used to measure gene expression and metabolism (glucose consumption and lactate production), respectively, in embryos from Lean Parented and Combined Obese Parented matings. This research was completed in a University research laboratory.

MAIN RESULTS AND THE ROLE OF CHANCE: Blastocyst rate was reduced in Combined Obese Parented embryos when compared with both Single Obese (11% decrease for Maternal Obese Parented, $P < 0.05$; 15% for Paternal Obese Parented, $P < 0.05$) and Lean Parented embryos (25% decrease, $P < 0.01$). Time-lapse analysis of developmental kinetics highlighted a delay of 1 h at the 2–3 cell division, extending to 6 h delay by the blastocyst stage for Combined Obese Parented embryos ($P < 0.05$). A reduction in the total cell number of Combined Obese Parented blastocysts was a further manifestation of this developmental delay ($P < 0.05$). Zona pellucida width was reduced in Combined Obese Parented embryos ($P < 0.05$). Glucose consumption was increased in Combined Obese Parented embryos ($P < 0.05$), which was associated with the up-regulation of Glucose transporter 1 expression ($P < 0.05$).

LIMITATIONS AND REASON FOR CAUTION: This study was completed in fertile C57BL/6 mice using a well-defined model of diet-induced obesity in which embryos were fertilized *in vivo*. Human obesity is complex, with many causes and co-morbidities, and therefore, the impact of combined obesity would require further investigation in human settings.

WIDER IMPLICATIONS OF THE FINDINGS: This study demonstrates that combined parental obesity has a detrimental impact on mouse embryo development, a finding consistent with previous studies on individual parent obesity. Of note, the effect of combined parental obesity upon embryo development markers was greater than that of individual parental obesity. Plausibly, human embryos will be similarly impacted. The reduction in the blastocyst rate and delayed time to developmental events confirms that embryos of obese parents differ from those of lean parents. Allowance for this should therefore be incorporated into clinical practice when selecting the best embryo for the transfer of an obese couple.

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Key words: obesity / high-fat diet / zona pellucida / time-lapse

Introduction

The World Health Organization recognizes obesity as the epidemic of the 21st century, with greater than 30% of adults around the world defined as obese (BMI $\geq$30 kg/m^2; WHO, 2015; Ng *et al*., 2014). The reduction to an individual's reproductive success is one of the many impacts of obesity (Mokdad *et al*., 2003; Guh *et al*., 2009). For the non-scientific community, this would be underscored by the lowered ability of obese individuals to conceive and maintain pregnancy (Norman and Clark, 1997; Maheshwari *et al*., 2007; Feuer *et al*., 2013). Such reductions in fecundity are consistent with the increased level of aneuploidy, mitochondrial dysfunction, endoplasmic reticulum dysfunction and poor morphology observed in the oocytes and sperm of obese individuals, both human and animal (Jensen *et al*., 2004; Kort *et al*., 2006; van der Steeg *et al*., 2008; Robker *et al*., 2009; Bakos *et al*., 2011a,b; Binder *et al*., 2012a; Luzzo *et al*., 2012; Dupont *et al*., 2013; Wu *et al*., 2015). Of further concern is the ability of parental obesity to act as a 'developmental programmer' and thus impact health across multiple generations (Chavatte-Palmer *et al*., 2012; Lane *et al*., 2014).

Historically, developmental programming focused upon environmental exposures, typically post-implantation and throughout gestation (Silveira *et al*., 2007; Wadhwa *et al*., 2009). Numerous studies in humans and other species have now established that developmental programming can occur during the periconception period and lead to long-term health impacts upon offspring (Gardner *et al*., 2004a,b; Watkins *et al*., 2010; Maloney *et al*., 2011). Indeed, evidence from rodent studies reveals that maternal obesity results in reduced blastocyst rate, slower embryonic development and down-regulation of key metabolic genes, as well as negative impacts on fetal health (Bermejo-Alvarez *et al*., 2012; Binder *et al*., 2012b; Luzzo *et al*., 2012). Similarly, paternal obesity imparts negative effects on embryonic health including delayed timing of embryonic development, altered glucose metabolism of the blastocyst, perturbed fetal development as well as impacting fecundity in subsequent generations of mice (Binder *et al*., 2012a,b; Fullston *et al*., 2012; McPherson *et al*., 2014). This is likely to be mediated through mitochondrial dysfunction (maternal), microRNAs (paternal) as well as genetic and epigenetic mechanisms (Skinner, 2011; Lane *et al*., 2014). Importantly, human evidence also supports the theory that maternal or paternal obesity alters embryonic development, as demonstrated by reduced success in IVF cycles (Styne-Gross *et al*., 2005; van der Steeg *et al*., 2008; Robker *et al*., 2009; Bakos *et al*., 2011a). Of note, the studies above all focused upon the impacts of individual parental obesity; maternal obesity or paternal obesity alone, with little human data on the effects of combined parental obesity.

Obesity rates are increasing around the world, and couples of reproductive age are likely to share lifestyle choices. Thus, cases of combined parental obesity (both an obese mother and an obese father) are becoming more common. The impacts of combined parental obesity upon offspring are, however, yet to be elucidated. To date, only one study of combined parental obesity has been undertaken, which among other findings, noted poor fertilization rates for obese couples undergoing IVF in Germany (Kupka *et al*., 2011). However, this study included a number of confounding variables, including socioeconomic status and parental age. Hence, more clinical studies are required. Based on previous work on maternal and paternal obesity in both humans and animals, it is hypothesized that combined parental obesity has a greater detrimental effect on fertility, as measured by early embryo development and quality, than that of single-parental obesity (either maternal or paternal). Therefore, in this study, we determined how combined parental obesity impacted preimplantation embryo development and quality in a mouse model. The advantage of this model is that it minimizes many potential confounders evident in clinical studies. Specifically, the aims of this study were to characterize the effect of combined parental obesity on: (i) the developmental potential, morphology, kinetics and cell lineage allocation of the preimplantation embryo and (ii) the metabolism and expression of key metabolic genes in the blastocyst. In characterizing these effects, this work further highlights the importance of combined parental obesity in the preconception period, as well as indicating potential pathways in which these effects can be mitigated and ultimately improve the reproductive success of obese couples.

Materials and Methods

Experimental animals, diets and hormonal stimulation

Three-week-old male and female C57BL/6 mice (WEHI, Melbourne, Australia) were randomly assigned to either a control diet consisting of 7% total fat wet weight and 19.4% protein (AIN93G; Specialty Feeds, Perth, Australia) or a high-fat diet (HFD) consisting of 21% total fat wet weight and 19% protein (SF00-219; Specialty Feeds). Mice were fed for a minimum of 8 to a maximum of 12 weeks, at which point they were defined as control or obese, respectively, based on previous characterization (Bakos *et al*., 2011b; Mitchell *et al*., 2011; Binder *et al*., 2012b;

Fullston *et al.*, 2012). Mice were maintained in a 12 h light:12 h dark photoperiod with food and water supplied *ad libitum*. Female mice were caged in groups of four and male mice were caged individually. Body weight was recorded weekly.

Female mice were superovulated with an intraperitoneal injection of 0.25 IU/g mare serum gonadotrophin (PMSG; Folligon, Intervet, Bendigo, Australia) followed 48 h later by 0.25 IU/g human chorionic gonadotrophin (hCG; Chorulon, Intervet). Mice were mated to produce four reciprocal matings from which resultant embryos were studied: (i) control female and control male (Lean Parented Embryos); (ii) control female and obese male (Paternal Obese Parented Embryos); (iii) obese female and control male (Maternal Obese Parented Embryos) and (iv) obese female and obese male (Combined Obese Parented embryos).

Ethical approval

This study was carried out in accordance with the Australian code of practice for the care and use of animals for scientific purposes, and all protocols were approved by the Animal Ethics Committee of The University of Melbourne.

Embryo collection

Twenty-two hours post-hCG injection, pronucleate oocytes were collected in G-MOPS handling medium supplemented with 5 mg/ml human serum albumin (GMOPS+). Pronucleate oocytes were denuded of cumulus cells via incubation in GMOPS containing 300 IU/ml hyaluronidase for 20 s (bovine testes, type IV; Sigma-Aldrich, Castle Hill, NSW, Australia) followed by washing in GMOPS+. Denuded pronucleate oocytes were immediately washed in GMOPS+ and cultured as previously detailed (Gardner and Lane, 2014).

Embryo culture: time-lapse analysis

Embryo morphokinetics were assessed by time-lapse analysis using an EmbryoScope multigas incubator (Unisense, Aarhus, Denmark). Methods for mouse embryo culture in this incubator were modified from a human protocol (Meseguer *et al.*, 2011); in which, individual pronucleate oocytes were transferred to 25 μl drops of G1 medium (Gardner and Lane, 2014) in EmbryoSlide dishes (Unisense) under 1.2 ml paraffin oil (Ovoil, Vitrolife) and cultured until 72 h post-hCG under 6% CO_2, 5% O_2 and 89% N_2 at 37°C. Embryos were then transferred to pre-equilibrated G2 medium (Gardner and Lane, 2014) and cultured for a further 46 h. Images of embryo development were acquired every 7 min throughout the culture period, at five planes of view. The timings of developmental milestones were calculated post-hCG, as well as from pronuclear envelope breakdown. Resultant embryos were assessed for developmental stage before being subjected to differential staining. Timing of developmental events and specific embryo characteristics (zona pellucida width, perivitelline space and embryo area) were then determined retrospectively from stored images (EmbryoViewer; Unisense). Measurements of individual embryos were standardized to the plane of the maximum width of the polar body and measured at the pronucleate oocyte and early blastocyst stage. Data were analysed only for embryos that developed to the blastocyst stage on time (by day 4.5), as previously defined (Gardner *et al.*, 2004a,b; Wale and Gardner 2010). Only those that developed to the blastocyst stage were used for analysis, to avoid skewing results to groups with lower developmental potential and for relevance to clinical practice in which only data on transferable embryos would be considered.

Embryo culture for metabolic and gene expression analysis

Embryos for metabolic and gene expression analysis were cultured in a Sanyo 19M multigas incubator (Sanyo Corporation, Osaka, Japan) under a 6% CO_2, 5% O_2 and 89% N_2 atmosphere at 37°C (Gardner and Lane, 2014). Embryos from Lean Parental matings and Combined Parental Obesity matings were cultured individually in 5 μl pre-equilibrated G1 media under 3.5 ml paraffin oil. At 72 h post-hCG, embryos were transferred to (i) 1 μl pre-equilibrated modified G2 medium (containing glucose as the sole source of carbohydrates) under 3.5 ml paraffin oil for metabolic analysis or (ii) 5 μl pre-equilibrated G2 medium under 3.5 ml paraffin for a further 48 h before being snap frozen for subsequent gene expression analysis.

RNA extraction and reverse transcription

Total RNA was isolated from frozen blastocysts from Lean Parental matings and Combined Parental Obesity matings ($n = 40$ blastocysts/group, repeated in triplicate) using an Absolutely RNA Nanoprep Kit (Agilent Technologies, Mulgrave, Australia) according to the manufacturer's instructions (Dupont *et al.*, 2012). Briefly, RNA was bound to a column matrix and a series of salt washes removed contaminants. Isolated RNA was DNase treated to eliminate contaminating DNA according to the manufacturer's specifications (Agilent Technologies). The cDNA was synthesized from RNA using Superscript III Reverse Transcriptase (Life Technologies, Mulgrave, Australia) and Random Primers (Promega, Alexandria, Australia) according to the manufacturer's instructions (Invitrogen, Carlsbad, USA) and as described previously (Harvey *et al.*, 2004).

Quantitative RT–PCR gene expression analysis

Real-time quantitative RT–PCR (qRT–PCR) was performed on a ViiA™7 thermocycler (Applied Biosystems, Mulgrave, Australia) as described previously (Harvey *et al.*, 2004). Primers were designed using Primer Express (Applied Biosystems) and were synthesized by Geneworks (Adelaide, Australia). Primer specificity and efficiency were calculated using dilutions of liver cDNA samples, followed by embryo cDNA samples. The genes investigated were Glucose transporter 1 (*Glut1*, also known as *Slc2a1*), Pyruvate kinase muscle isozyme 2 (*Pkm2*), Peroxisome proliferator-activated receptor gamma (*Pparγ*), Insulin growth-like factor 2 receptor (*Igf2r*) and *Glut3* (also known as *Slc2a3*). Primer sequences and product sizes are detailed in Table I.

Real-time qRT–PCR was performed in triplicate 10 μl reactions containing 1X SYBR Green Master Mix (Invitrogen) and 500 nM forward and reverse primers. cDNA was diluted in nuclease-free water (Life Technologies) to a concentration equivalent to cDNA generated from 0.25 embryo/μl. Reactions were run according to the following parameters; 50°C for 5 min, 95°C for 10 min, then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. The cycle threshold (Ct) was calculated for each sample using the ViiA™7 software. Dissociation curves, to detect non-specific amplification, were generated for all reactions. No template samples containing water substituted in place of cDNA were included in all assays to confirm the absence of non-specific amplification products, as were minus RT samples to confirm the absence of DNA contamination. Raw Ct values were analysed using the delta CT method in the Q-Gene software package (Muller *et al.*, 2002; Simon, 2003) normalized to that of 18S rRNA. 18S rRNA was confirmed as an appropriate housekeeper, against a group of candidate housekeeper genes, using the NormFinder software package (Andersen *et al.*, 2004). Data were then expressed as a fold change relative to the Lean Parented embryo group.

Assessment of glucose consumption and lactate production

Individual embryo glucose consumption and lactate production was assessed in a cohort of embryos from Lean Parental matings and Combined Parental Obesity matings. Compacted morula were placed into G2-modified

Table I Primer sequences and associated details used for real-time qRT–PCR studies.

Gene	Accession number	Forward primer (5′→3′)	Reverse primer (3′→5′)	Product length	Reference (if applicable)
18s rRNA	NR_003278.3	GAACGGCTACCACATCCAA	CCTGTATTGTTATTTTTCGTCACTACCT	91	Kind *et al.* (2005)
Pkm2	NM_011099.3	TCTTCCCTGTGCTGTGTAA	CCACCCGGTCAGCACAAT	140	Not applicable
Igf2r	NM_010515.2	CTTGCCCTCCAGAAACGGAT	TGCTACACCACAGTTTCGCT	111	Not applicable
Glut1	NM_011400.3	CCAGCTGGGAATCGTCGTT	CAAGTCTGCATTGCCCATGAT	76	Not applicable
Glut3	NM_011400.3	CGGTGATAGTCCTTAAGCCTTCT	ATGGGGTCACCTTGCTTGTC	146	Not applicable
Pparγ	NM_008904.2	TATGGAGTGACATAGAGTGTGCT	GTCGCTACACCACTTCAATCC	143	Not applicable

Table II Body weight (b.w), weight change and body fat of 3-week-old male ($n = 15$) and female ($n = 48$) mice fed a control (7% total fat) or high-fat (21% total fat) diet for a minimum period of 8 weeks.

Measure	Control diet male	High-fat diet males	*P*-value	Control diet females	High-fat diet females	*P*-value
Initial weight (g)	10.74 ± 0.49	10.1 ± 0.51	NS	12.3 ± 0.25	12.1 ± 0.24	NS
Final weight (g)	33.1 ± 1.28	37.9 ± 1.48	<0.05	21.3 ± 0.24	25.1 ± 0.35	<0.001
Weight gain (g)	22.4 ± 1.01	27.7 ± 1.05	<0.01	9.17 ± 0.30	12.9 ± 0.42	<0.001
Proportion fat (%b.w.)	2.58 ± 0.45	4.37 ± 0.51	<0.05	2.26 ± 0.21	3.50 ± 0.14	<0.001

media, containing 0.5 mM glucose as the sole carbohydrate source, and without lactate at 72 h post-hCG for a period of 24 h. Spent medium of resultant early blastocysts (with early blastocyst defined as a stage from cavity formation to less than half the total embryo volume comprising the blastocoel) was analysed by microfluorescence as described previously (Gardner and Leese, 1990; Lane and Gardner, 1998). Subsequently, the total cell number was determined via staining in 0.1 mg/ml Bisbenzimide (Hoechst, 33342; Sigma-Aldrich) in 10% v/v ethanol for 30 min, washed in GMOPS+ for 5 min then mounted in glycerol on glass slides under coverslips (Thermo Fisher, Scoresby, Australia). Cell numbers were visualized and photographed using a fluorescent microscope (Nikon Eclipse TS100) equipped with a Nikon Digital Sight DS-L2 camera (Nikon, Tokyo, Japan). Cell numbers were retrospectively determined manually using ImageJ Version 1.47 (Schneider *et al.*, 2012). Metabolic measurements were expressed on a per embryo basis, as well as per cell per hour for each individual embryo to account for variation in the embryonic cell number.

Assessment of blastocyst cell allocation

Allocation of cells to the inner cell mass (ICM) and the trophectoderm (TE) of blastocysts was determined via differential nuclear staining, as described previously (Hardy *et al.*, 1989). Briefly, blastocysts were placed in 0.5% pronase (Sigma-Aldrich) until the zona pellucida disbanded, followed by washing in GMOPS+ for 5 min. Embryos were then incubated in 10 mM 2,4,6-trinitrobenzene sulfonic acid (Sigma-Aldrich) for 10 min then washed in GMOPS+ for 5 min, before a 10 min incubation in 0.1 mg/ml anti-dinitrophenol (Sigma-Aldrich). Blastocysts were subsequently washed for 5 min in GMOPS+, then incubated in 10% v/v guinea pig serum with 25 mg/ml propidium iodide (IMVS, Adelaide, Australia) for 5 min. Blastocysts were transferred to 0.1 mg/ml Bisbenzimide (Sigma-Aldrich; Hoechst, 33342) in 10% v/v ethanol for 15 min, washed in GMOPS+ and finally mounted in glycerol on glass slides under coverslips (Thermo Fisher). Cells were visualized, photographed and counted as outlined for total cell number determination above.

Statistical analyses

All data were assessed for normal distribution via the Shapiro–Wilk test. Animal body weight gain was analysed using a two-tailed *t*-test. Day 5 blastocyst rate was arc-sine transformed prior to analysis. Metabolic data were measured as pmol/embryo/h and also normalized for cell number prior to analysis. Gene expression levels were normalized to that of the Lean Parented embryo group. Time-lapse data were analysed relative to hours post-hCG and also normalized to individual embryo pronuclear envelope breakdown to mitigate possible effects of the timing of mating. The effect of parental obesity on embryo measurements (zona pellucida width, perivitelline space and embryo area), timing of developmental events and cell allocation were analysed by ANOVA using a PROC MIXED procedure employing a Tukey's *post hoc* analysis to identify differences between groups. Culture replicate was included in the model as a random factor. Metabolic data, total cell counts and gene expression data were analysed using two-tailed *t*-tests. All analyses were performed in SPSS Version 20 (IBM, Armonk, USA). Data are presented as a mean ± SEM unless otherwise stated. Significance was determined at the level of $P < 0.05$.

Results

Impact of dietary fat on parental weight gain

Female mice fed a HFD gained significantly more weight after an 8-week feeding period compared with control fed mice, as well as having significantly increased peritoneal fat deposits ($P < 0.001$, Table II). Similarly, male mice fed a high-fat diet for 8 weeks gained significantly more weight than mice on control diets and had increased peritoneal fat deposit weight ($P < 0.01$, Table II).

Impact of parental obesity upon mating and fertilization success

The presence of a copulatory plug (indicative of mating success) was significantly lower in the Combined Obesity Parental matings (34 ± 6%) compared with Lean Parental matings (68 ± 6%, $P < 0.001$). There was no significant difference between Paternal Obese mating success (63 ± 6%) or Maternal Obese mating success (54 ± 6%) compared with Lean Parental matings ($P > 0.05$). The number of oocytes ovulated per female (including fertilized, unfertilized and degenerate) was significantly reduced in the females mated in Maternal Obese matings (12.6 ± 1.95, $P < 0.05$) and Combined Obese Parental matings (11.7 ± 0.73, $P < 0.01$), but was not significantly reduced in those of Paternal Obese mating (14.2 ± 0.9), compared with those of Lean parental mating (16.6 ± 1.1). The number of fertilized eggs per female was also significantly reduced in the females of Maternal Obese matings (9.2 ± 1.6, $P < 0.05$) and of Combined Obese Parental matings (8.4 ± 0.7, $P < 0.001$) compared with females of Lean Parental matings (12.7 ± 0.7). The number of fertilized eggs per female in Paternal Obese matings (11.4 ± 1.2) did not differ significantly from Lean Parental matings.

Obesity effects on developmental potential

Compared with Lean Parented embryo development rates, the percentage of fertilized zygotes reaching the blastocyst stage was significantly reduced in groups in which one parent was obese (Paternal Obese Parented 15% decrease, Maternal Obese Parented 11% decrease, $P < 0.05$; Fig. 1). Similarly, compared with the Lean Parented embryo development rates, blastocyst development from Combined Obese Parented embryos was further decreased (27% decrease, $P < 0.01$). Development rates of Combined Obese Parented embryos did not differ from embryos that had one obese parent ($P > 0.1$). This reduction in the blastocyst rate did not correlate with a block at a specific developmental stage rather development failure occurred throughout the culture period.

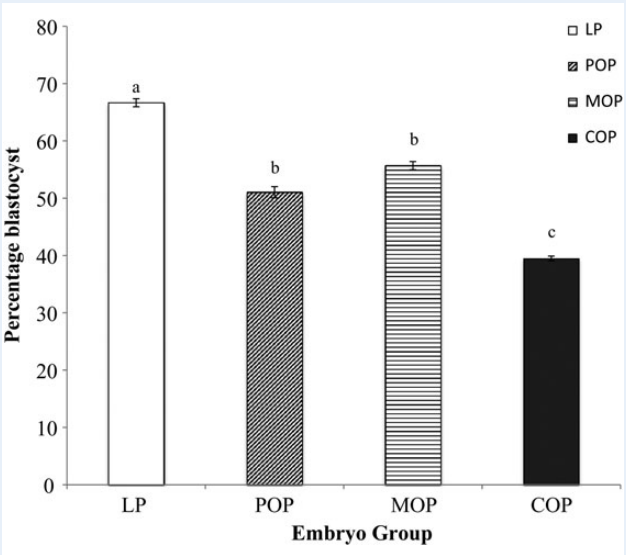

Figure 1 The percentage of fertilized zygotes that reach the blastocyst stage for each of the four reciprocal mating groups; LP = Lean Parented embryos ($n = 188$), POP = Paternal Obese Parented embryos($n = 96$), MOP = Maternal Obese Parented embryos($n = 96$) and COP = Combined Obese Parented embryos ($n = 157$). Different superscript letters show significant differences ($P < 0.05$) between groups. Data are expressed as the mean ± SEM.

Obesity effects on developmental kinetics

Analysis of developmental timings relative to hours post-hCG administration demonstrated a delay of 1 h by pronuclear envelope breakdown (tPNB, $P < 0.05$) in Maternal Obese Parented, Paternal Obese Parented and Combined Obese Parented compared with Lean Parented embryos. Paternal Obese Parented and Maternal Obese Parented embryos did not show significant delays in developmental timing compared with Lean Parented embryos after the morula stage ($P > 0.1$). The delay in developmental timings evident in Combined Parental Obese Parented embryos extended to 7 h at the time of blastocoel formation (tSB, $P < 0.01$; Fig. 2a) relative to those of Lean Parented embryos.

When developmental timings were expressed relative to individual embryo pronuclear envelope breakdown, rather than hours post-hCG to remove any potential cofounding effects in the time of mating, delays in development to the 2-cell stage were not evident (t2, $P > 0.1$). Combined Obese Parented embryos displayed a 1 h delay at the 4-cell division (t4, $P < 0.05$), which extended to a 6 h delay at the tSB ($P < 0.01$; Fig. 2b) relative to Lean Parented embryos. Timing of the first cleavage division did not differ significantly from Maternal Obese Parented or Paternal Obese Parented embryos ($P > 0.1$). Significant differences between Combined Obese Parented embryos and Maternal Obese Parented or Paternal Obese Parented embryos appeared from the 5-cell division onwards and persisted until the tSB ($P < 0.05$).

Obesity effects on blastocyst cell lineage allocation

No differences in ICM cell number or the ICM:TE ratio were observed between any of the groups (Fig. 3a and c). TE and total cell numbers were reduced in both Maternal Obese Parented and Combined Obese Parented embryos relative to Lean Parented embryos ($P < 0.05$; Fig. 3b and d).

Metabolic analysis

Glucose consumption and lactate production when expressed per embryo were not different between Lean Parented and Combined Obese Parented embryos (Fig. 4a). Total cell number was lower in Combined Obese Parented than Lean Parented embryos ($P < 0.01$, Fig. 4b). Glucose consumption, when normalized for the total embryo cell number, was higher ($P < 0.01$) in embryos from Combined Obese Parented embryos compared with Lean Parented embryos (Fig. 4c). Lactate production did not differ between the two groups ($P > 0.1$). Glycolytic rate, the % of glucose converted to lactate, calculated on the basis that 2 mole of lactate is produced per 1 mole of glucose consumed by the embryo (Gardner and Leese, 1990), was higher in Combined Obese Parented embryos (52%) compared with Lean Parented embryos (42%); however, this difference was not significant (Fig. 4d, $P > 0.1$).

Quantitative gene expression analysis in response to combined parental obesity

Glut1 expression was increased in Combined Obese Parented relative to Lean Parented embryos ($P < 0.05$, Fig. 5a). Pyruvate kinase muscle

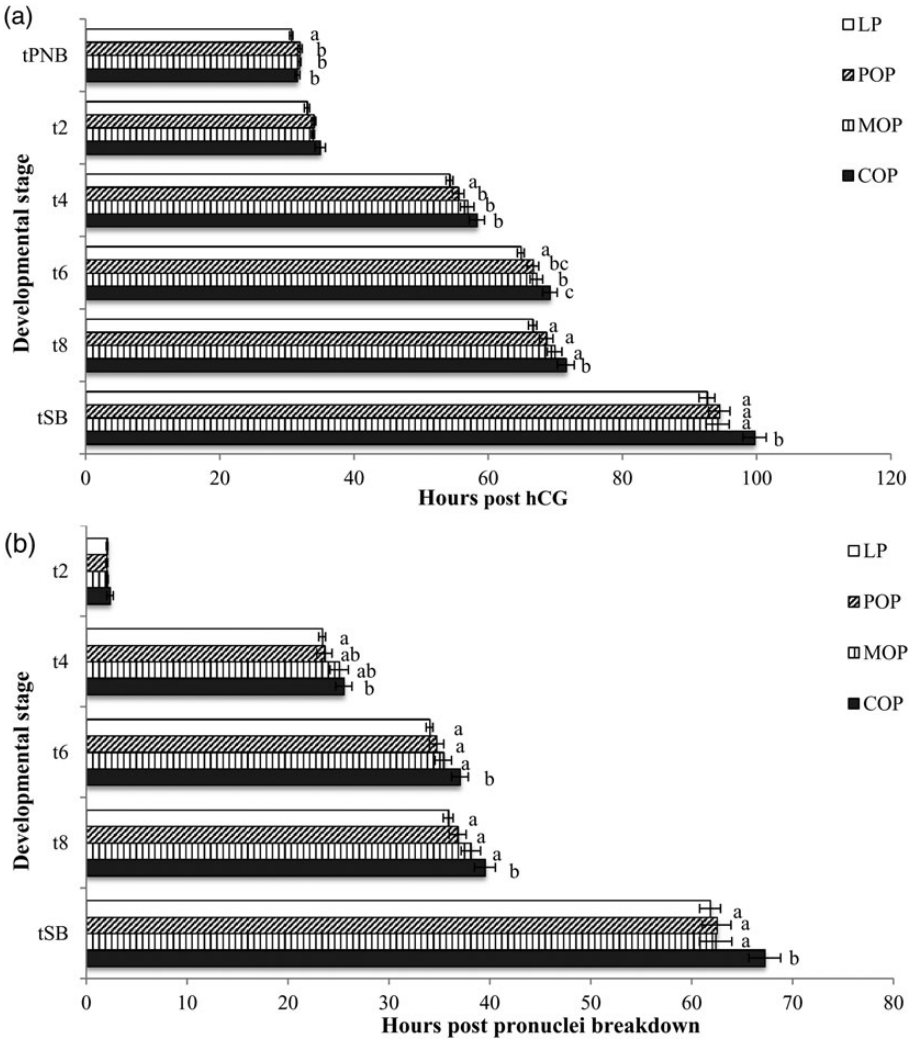

Figure 2 Timing of major developmental events, expressed in hours post-hCG injection (**a**) and hours post pronuclear envelope breakdown (**b**). tPNB = timing of pronuclei breakdown, t2 = timing of 2-cell division, t4 = timing of 4-cell division, t6 = timing of 6 cell division, t8 = timing of 8-cell division, tSB = time blastocoel starts formation. LP = Lean Parented embryos, POP = Paternal Obese Parented embryos, MOP = Maternal Obese Parented embryos and COP = Combined Obese Parented embryos. Different superscript letters indicate a significant difference ($P < 0.05$) between groups. $n > 60$ embryos per group. Data are expressed as the mean ± SEM.

isozyme 2 (*Pkm2*) expression was significantly increased in Combined Obese Parented relative to Lean Parented embryos ($P < 0.01$, Fig. 5b). *Pparγ* showed a trend towards increased expression in the Combined Obese Parented compared with Lean Parented embryos ($P < 0.08$, Fig. 5c). No differences between groups were evident in insulin-like growth factor two receptor (*Igf2r*) or glucose transporter three (*Glut3*) expression (Fig. 5d and e), respectively.

Obesity alters zona pellucida characteristics

At the pronucleate oocyte stage, zona pellucida width was reduced in Combined Obese Parented embryos compared with Lean Parented embryos (Fig. 6, $P < 0.05$). Paternal Obese Parented embryos showed no difference in zona pellucida width from Lean Parented embryos. Maternal Obese Parented embryos did not demonstrate a difference in zona pellucida width from Lean Parented or Combined Obese Parented embryos ($P > 0.1$). There was no difference in measurements of the total embryo area and width of the peri-vitelline space between any groups ($P < 0.1$). For measurements taken at the blastocyst stage, there was a reduction in zona pellucida width for all groups compared with the pronucleate oocyte stage ($P < 0.05$).

Discussion

Developmental rate and kinetics are reduced by the obese state of parents

The number of 2-cell embryos that developed on time to the blastocyst stage was significantly reduced across all groups with an obese parent compared with the Lean Parented embryos, and this reduction was more pronounced in Combined Obese Parented embryos. A reduction

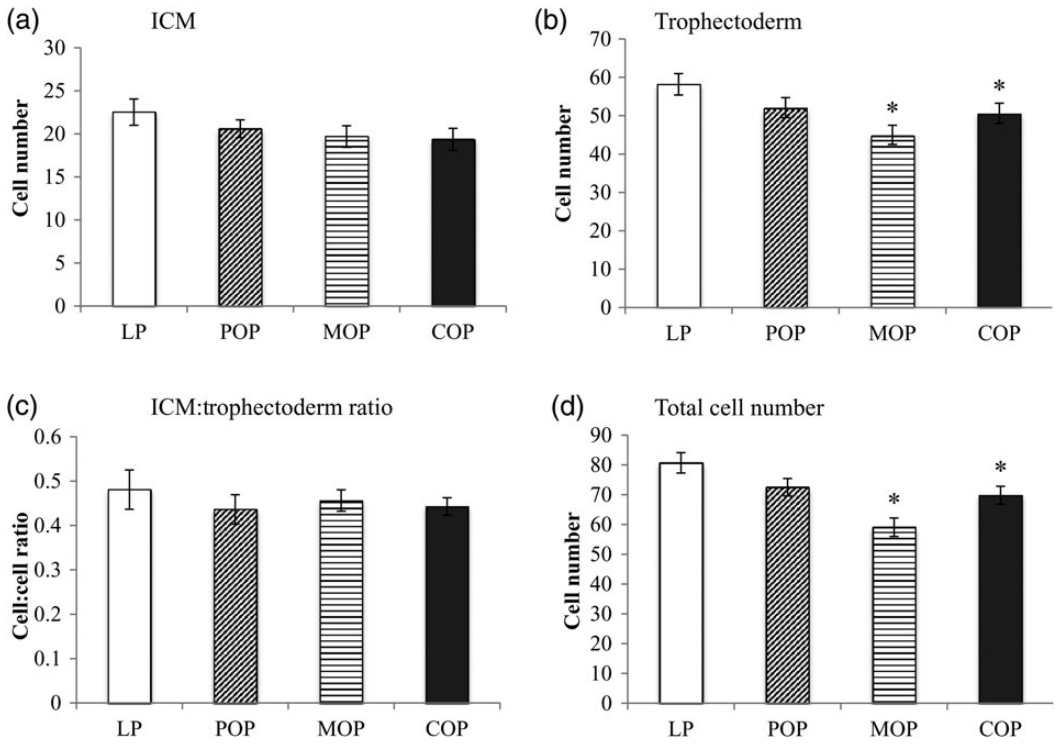

Figure 3 Mean cell counts and ratios to show lineage specification for all embryos that developed to the blastocyst stage (day 5) at the expected time for each of the four groups. (**a**) ICM cell counts, (**b**) TE cell counts, (**c**) ICM to TE cell ratio and (**d**) total cell number. Lean Parented embryos (LP, open bars), Paternal Obese Parented embryos (POP, diagonal hashed bars), Maternal Obese Parented embryos (MOP, horizontal hashed bars) and Combined Obese Parented embryos (COP, shaded bars). Asterisk denotes significantly ($P < 0.05$) different from LP, $n > 60$ embryos per group. Data are expressed as the mean ± SEM.

in developmental competence of embryos produced when both parents are obese is consistent with previous studies on individual parent obesity in both animals and humans (Bakos *et al.*, 2011a, b; Binder *et al.*, 2012b; Luzzo *et al.*, 2012) and is likely caused by a number of factors, contributed from both the mother and the father, as reviewed by Lane *et al.* (2014). Furthermore, a reduction in ovulation and fertilization rate was evident in the females of Maternal Obese matings and Combined Obese Parental matings, suggesting that obesity impacts the developmental competency of these oocytes. One of the principal issues appears to be that oocytes from obese female mice and women show increased levels of aneuploidy, mitochondrial and endoplasmic reticulum stress and apoptosis. These conditions may lead to embryonic developmental arrest and may not permit blastocyst formation (Hardy *et al.*, 2001; Acton *et al.*, 2004; Igosheva *et al.*, 2010; Luzzo *et al.*, 2012; Wu *et al.*, 2015). Human embryos of obese couples may show a similar reduction in the blastocyst rate, as observed in human paternal obese parented embryos (Bakos *et al.*, 2011a). There may also be a greater reduction in human blastocyst rates based on additional confounding factors, such as subfertility and other comorbidities, which would translate to a decreased potential for implantation and successful pregnancy.

Notably, in the current study for those embryos of Combined Obese Parented matings that did develop to the blastocyst stage, development was at a slower rate than that of Lean Parented embryos, with an initial delay of 1 h at the 3-cell division (data not shown), which accumulated to 6 h by the initiation of blastocoel formation. This delay is greater than previously seen in individual parental obesity studies, which reported differences of 1 h or less throughout development in IVF and mated models of embryonic development (Binder *et al.*, 2012a,b). Human studies of the impacts of parental obesity and timing of embryonic development require further investigation, as these studies remain limited, with conflicting reports (Bellver *et al.*, 2013; Leary *et al.*, 2015), potentially due to small sample sizes. The combined contributions of oocytes and sperm from obese parents may lead to the increase in developmental times and/or greater variation in the time at which embryos reach expected developmental time points. This may therefore have clinical implications when standardizing embryo developmental timings across patients with a range of BMIs, as well as for selecting the best embryo to transfer. The present study employed a mated model, thus variation in mating time may also contribute to the delay in embryonic development. However, in the present study, timings were also calculated from pronuclei disappearance to account for any differences in time of mating. Irrespective of this correction, delays in embryonic developmental timing were still evident. Furthermore, the length of time between 2- and 3-cell divisions, which is independent of mating time, was increased in the Combined Obese Parented embryos. Importantly in ART clinics, this length of time has previously been determined as a marker for embryo quality in two independent human studies, with increased time giving embryos a 'negative score' in the algorithm regarding transferrable quality embryos (Wong *et al.*, 2010; Meseguer *et al.*,

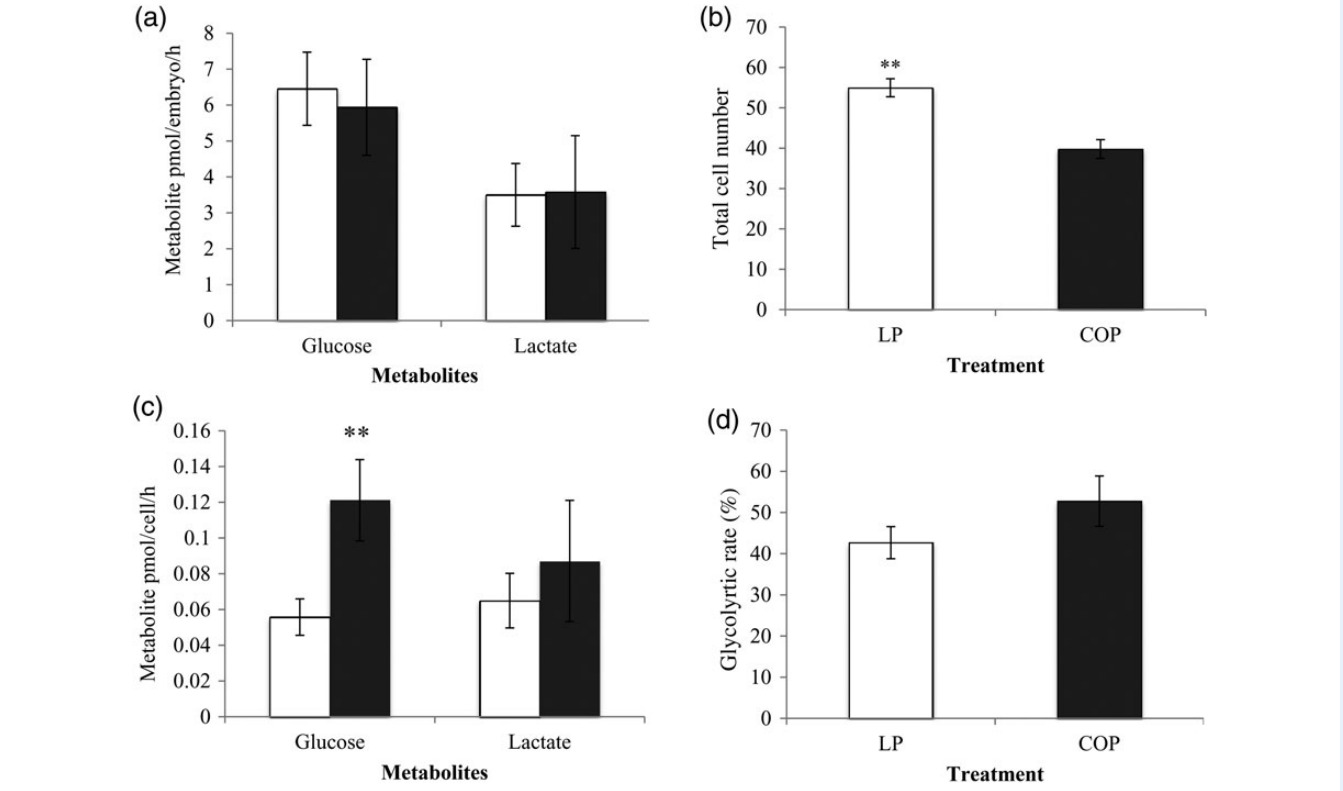

Figure 4 (**a**) Glucose and lactate concentration in spent culture media expressed h/embryo, (**b**) total cell number [day 4 early blastocysts (<half the embryo comprising of the blastocoel)], (**c**) Glucose and lactate concentration in spent culture media expressed /h/cell, (**d**) glycolytic rate (% glucose converted to lactate) of Lean Parented blastocysts (LP, open bars) and Combined Obese Parented (COP, shaded bars) blastocysts. $^{**}P < 0.01$, $n > 20$ embryos per group. Data are expressed as the mean ± SEM.

2011). Therefore, the innate ability of the resulting zygote to develop appears compromised independent of differences in time of mating.

The exposure to reproductive tract fluids in this model may also contribute to the alteration in embryonic developmental timing. Seminal fluid is known to have increased triglyceride concentrations in obese animals and humans, and it is likely female reproductive fluids would show similar changes in response to diet (Martini *et al.*, 2010; Binder *et al.*, 2015; Bromfield *et al.*, 2014). The increase in fat content may alter the viscosity and density of the various reproductive fluids, impacting the ability of sperm to swim towards the ampulla thereby extending the time to fertilization and extending the timing of development seen in the present study (De Celis *et al.*, 2000; Gulaya *et al.*, 2001). Individual parental obesity embryos show a slight delay in their developmental timings, although this does not differ significantly from Lean Parented embryos. Importantly, this demonstrates that an effect on embryonic development is evident when either maternal or paternal obesity is present. The increased, and significant, delay in Combined Obese Parental embryos may reflect the suboptimal interaction between high-fat male and female reproductive fluids, as well as with the embryo.

On time development of embryos has been associated with increased pregnancy rates in humans (Meseguer *et al.*, 2012; Wong *et al.*, 2013; Rubio *et al.*, 2014). However, this theory remains contentious, highlighting the needs for further prospective studies (Kaser and Racowsky, 2014; Kirkegaard *et al.*, 2015). Delayed embryonic development may have an impact on implantation, considering that implantation is dependent on an intricate dialogue between the embryo and the uterus, and involves a number of factors from both the uterus and the embryo (Wang and Dey, 2006). As the Combined Obese Parented embryos are delayed on day 4 (earlier developmental stage than required for implantation), they may not be developmentally competent for implantation, and this may translate in a reduced ability for implantation to occur (Paria *et al.*, 1993). Furthermore, Combined Obese Parented embryos have reduced TE cells, which can translate to poor implantation and placentation (Hardy *et al.*, 1989; Cross *et al.*, 1994). This is consistent with previous mouse studies that demonstrate slower developmental timing, caused by parental obesity or other means, and has been associated with decreased implantation rates (Mitchell *et al.*, 2011; Binder *et al.*, 2012a). It would be of interest to investigate the composition of oviductal and uterine fluid, such as triglyceride levels, and how these interact with key hormones, as potentially this would alter the physiology of the tract and receptivity to embryonic implantation. The understanding of how delayed embryonic development interacts with an altered maternal uterine environment is an area that could potentially improve reproductive outcomes in obese couples.

Combined parental obesity alters glucose metabolism and metabolic gene expression

Obese and lean individuals are considered to have vastly different metabolic states and employ different metabolic pathways, and this concept should be extended to embryos. Combined Obese Parented blastocysts

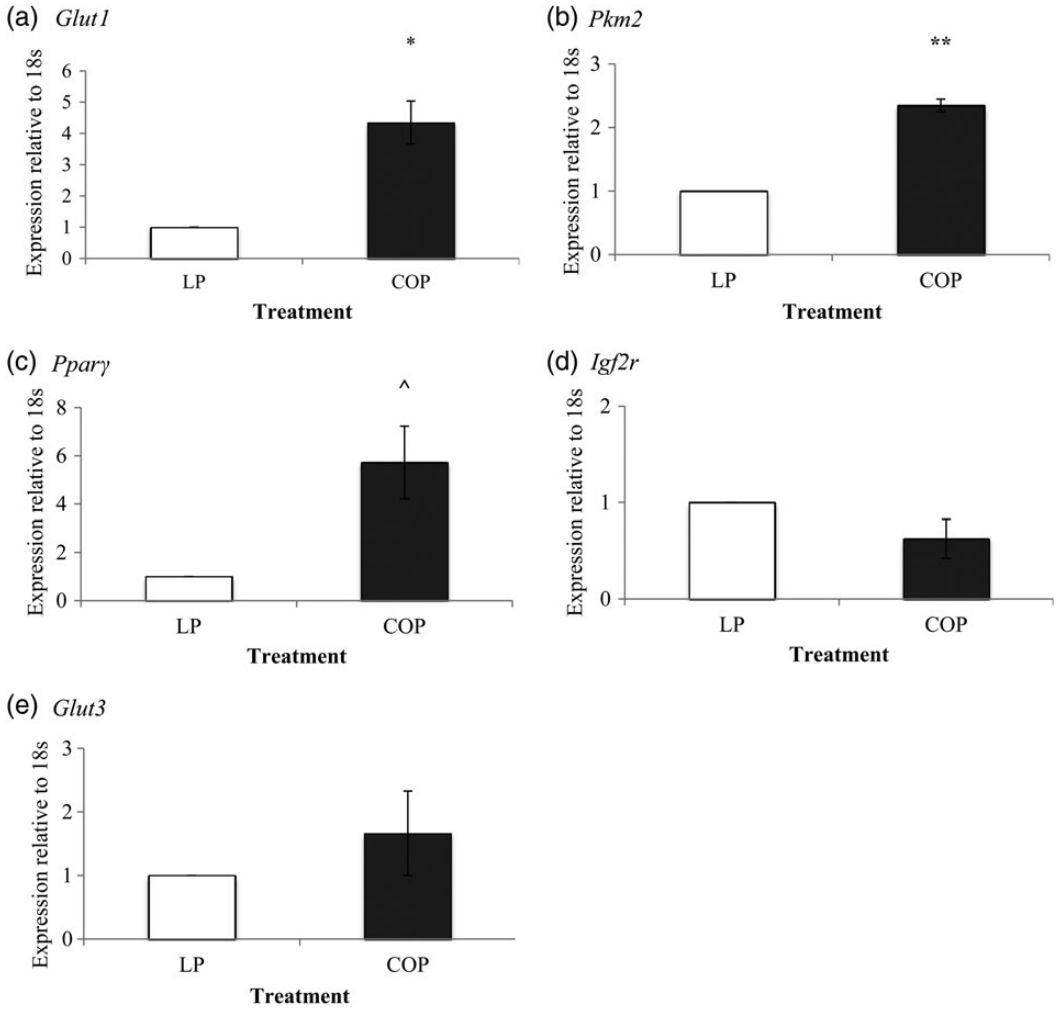

Figure 5 Mean expression of (**a**) *Glut1*, (**b**) *Pkm2*, (**c**) *Pparγ*, (**d**) *Igf2r* and (**e**) *Glut3* relative to 18S rRNA. Expression from Combined Obese Parented (COP) embryos is reflected by shaded bars. Values for COP embryos are normalized to the expression of embryos from the Lean Parented (LP) embryo group (open bars) set at 1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\wedge}P < 0.08$, $n = 3$ replicates of >40 embryos per group. Data are expressed as the mean ± SEM.

exhibited an increased uptake of glucose from media compared with Lean Parented blastocysts, when expressed per cell. Previous studies have demonstrated that maternal obesity in the mouse leads to increased glucose uptake per cell (Binder *et al.*, 2012b). Increased glucose uptake may suggest increased glycolysis, a common and essential metabolic pathway for the blastocyst (Leese and Barton, 1984; Gardner and Wale, 2013). However, lactate output, the final product of glycolysis, remained unchanged from Lean Parented embryos, suggesting that the excess glucose is being metabolized via an alternative pathway, such as the Pentose Phosphate Pathway (PPP). Potentially, the PPP is used as the concomitant production of NADPH can mitigate the effects of oxidative stress seen in the gametes and embryos of obese parents (McCord, 2000; Igosheva *et al.*, 2010; Bakos *et al.*, 2011b; Binder *et al.*, 2012b). Alternatively, glucose could be being metabolized through the hexosamine biosynthesis pathway, which supports amine sugar synthesis while also having key roles in growth and the development of insulin resistance (Sutton-McDowall *et al.*, 2010). In contrast to the data presented, a recent human study on maternal obesity reported a decrease in total blastocyst glucose consumption with increasing BMI. However, these findings, based on seven patients, determined blastocysts from women with a BMI >25 kg/m^2 were smaller in diameter and had significantly fewer cells (Leary *et al.*, 2015). Unlike the current study, it is also postulated that metabolic manifestations associated with obesity, in terms of perturbed glucose and insulin homeostasis, would also be evident in these obese patients further impacting embryo physiology. The data presented in this study indicate that more detailed metabolomic analyses of Combined Obese Parented embryos are warranted.

The assessment of metabolic gene expression levels in blastocysts of Combined Obese Parented embryos revealed a significant increase in *Glut1* and *Pkm2* and a trend towards increased *Pparγ* expression. In the blastocyst, *Glut1* is localized to apical membranes of the TE and has been shown to have a key role in detecting and responding to the surrounding glucose environment (Pantaleon and Kaye, 1998). The observed increase in *Glut1* expression and increased glucose uptake in this study may indicate metabolic stress in the Combined Obese Parented embryos. Although increased glucose uptake has been linked to increased developmental competence in blastocysts, glucose uptake must be regulated within a narrow range to ensure viability (Gardner

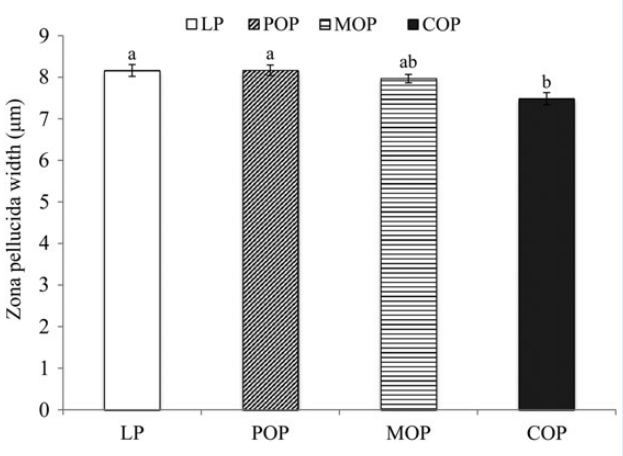

Figure 6 Zona pellucida width (μm) at the two-pronucleate stage. LP, Lean Parented embryos; POP, Paternal Obese Parented embryos; MOP, Maternal Obese Parented embryos; COP, Combined Obese Parented embryos. Different superscript letters denote a significant difference ($P < 0.05$) between groups in zona pellucida width $n > 30$ embryos per group. Data are expressed as the mean ± SEM.

and Wale, 2013). Up-regulation of *Glut1* may also drive the increased expression of *Pkm2* evident in the Combined Obese Parented blastocyst. *Pkm2* is essential to the aerobic conversion of phosphenolpyruvate to pyruvate and is essential for biosynthesis in proliferative cell types, such as cancer embryonic cells (Gupta and Bamezai, 2010; Krisher and Prather, 2012; Gardner and Wale, 2013; Gardner and Harvey, 2015). Increased *Pkm2* expression does not result in an increase in glycolysis, even in the presence of high glucose uptake, to allow for increased biosynthesis for key embryonic components such as nucleic acids, which occurs via the alternate PPP (Krisher and Prather, 2012; Gardner and Wale, 2013). The trend towards increased *Ppar γ* expression, which is activated by fatty acids, may also indicate metabolic stress, as it plays a key role in glucose homeostasis (Minge *et al.*, 2008).

The model of diet-induced obesity employed in the present study is free from the many metabolic complications evident in humans. The short feeding period (8 weeks) does not affect fasting plasma glucose levels (Bakos *et al.*, 2011a,b; Mitchell *et al.*, 2011; Palmer *et al.*, 2012) or glucose or insulin tolerance (Fullston *et al.*, 2013). Similar models of diet-induced obesity, involving higher fat diet, generational feeding and additional environmental stressors, do demonstrate prediabetic symptoms in mice (Winzell and Ahrén, 2004; Sun *et al.*, 2009; Groover *et al.*, 2013; Hillian *et al.*, 2013; Ge *et al.*, 2014). Indeed, the obese state is associated with hyperglycaemia and increased free fatty acids (Boden, 2008; Martyn *et al.*, 2008), which have negative impacts on early mammalian embryo development (Van Hoeck *et al.*, 2011; Cagnone *et al.*, 2012). Therefore, in the more complex metabolic state of obese humans, there is likely to be a greater impact upon early embryonic development. The exposure of oocytes, sperm and the embryo to the more complex metabolic environment in humans may thus lead to a greater up-regulation of these metabolic genes in the embryo than found in this study. This could have great impacts on gene, and protein function, resulting in further perturbed embryonic phenotypes and potentially adult disease.

Embryo characteristics are altered by obesity

This is the first study to demonstrate that obesity has an impact on zona pellucida width. Combined Obese Parented embryos exhibited a significant reduction in zona pellucida width. Zona pellucida width is further reduced by the contribution of parental obesity in Combined Obese Parented embryos, suggesting that there is a further paternal contribution. Previous studies have associated higher zona integrity with increased implantation (Montag *et al.*, 2008). Further studies are required to determine how obesity impacts zona pellucida width with zona pellucida gene expression and glycoprotein cross-linking potential candidates. The role of the zona pellucida and perturbations to its structure during embryonic development is interesting and warrants further investigation, especially as more individuals seek ART (Ferraretti *et al.*, 2013).

Conclusion

The present study demonstrates that combined parental obesity prior to and at the time of conception, results in a detrimental impact upon blastocyst developmental potential, consistent with previous studies of individual parent obesity. Zona pellucida width was shown, for the first time, to be thinner in Combined Obese Parented embryos and may play a critical role in modulating embryonic development and the embryos interaction with its surrounding environment. Glucose metabolism was perturbed in Combined Obese Parented embryos, with significantly more glucose being taken up per cell than that of Lean Parented embryos. Furthermore, key genes in the regulation of metabolism were up-regulated in Combined Obese Parented embryos, indicative of oxidative stress. Persistent changes to this gene expression could confer increased adult disease risk. Although long-term programming cannot be confirmed in this current study, as future studies with embryo transfers are required, it is clear that combined parental obesity negatively impacts preimplantation embryonic development. Previous studies indicate early embryonic changes do program long-term health of the individual (Sinclair and Singh, 2007; Lane *et al.*, 2014). This study is limited to mouse embryos; however, human embryos may be similarly impacted by combined parental obesity. As more individuals become obese, issues surrounding early embryonic development will become more pertinent to ART protocols. Therefore, promoting early intervention for parents, as well as identifying and incorporating variability in embryonic parameters as a result of parental obesity will aid the development of appropriate, customized ART protocols.

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Authors' roles

All authors contributed to the design of the research study; B.J.F. performed the experiments; all authors contributed to the analysis of the data; D.K.G. provided funding; all authors contributed to the writing of the manuscript.

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Conflict of interest

M.P.G. currently holds the position of Merck Serono Lecturer of Reproductive Biology. D.K.G. received research funds from Vitrolife AB Sweden. The other authors of this manuscript have nothing to declare and no conflicts of interest.

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