Impact of hyperglycemia on early embryo development and embryopathy: *in vitro* experiments using a mouse model

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**BACKGROUND:** The aim of this study is to model the processes of early embryopathy seen in human pregnancy complicated by maternal hyperglycemia secondary to maternal diabetes using a mouse embryo culture system.

**METHODS:** Female mice were superovulated and mated in pairs. Two-cell embryos were harvested from the oviducts and cultured *in vitro* in KSOM medium (synthetic oviductal medium enriched with potassium) supplemented with 0.2, 5.56, 15.56 or 25.56 mM glucose. Cell proliferation, differentiation and apoptosis were assessed. Experiments were performed in constant, embryos exposed to a particular concentration of glucose (0.2, 5.56, 15.56 or 25.56 mM) from harvest to either Day 5 post fertilization (pf) or Day 8 pf, and fluctuating, embryos exposed to alternate high 25.56 mM and normal 5.56 mM concentrations of glucose between harvest and Day 5 pf, glycemic culture. **RESULTS:** Expected levels of blastocyst formation and hatching were seen at 0.2 and 5.56 mM concentrations of glucose but both were impaired at higher concentrations (χ², P < 0.005; P < 0.001). Total cell numbers (P < 0.002) and cell allocation to the inner cell mass (P < 0.01) were reduced, but with no evidence of enhanced apoptosis in the hyperglycemic cultures. Variation in hyperglycemic exposure of the embryos on Days 2, 3 and 4 showed no adverse effects of hyperglycemia up to 24 h, but 48 and 72 h exposures were equally embryopathic (P < 0.01). **CONCLUSIONS:** Hyperglycemic exposure for >24 h is toxic to early embryo development. These findings may explain the lower than expected implantation rates and higher than expected rates of congenital abnormality and early pregnancy loss seen in patients with diabetes, particularly those with poor diabetic control.

**Keywords:** embryopathy; hyperglycemia; *in vitro*; mouse embryo; diabetes

**Introduction**

The St Vincent Declaration of the European Association for the Study of Diabetes and the World Health Organization set a target for women with diabetes of achieving pregnancy outcomes similar to those of non-diabetic women. One area in which this target is not being achieved is the prevention of congenital malformation in the offspring. Collected series confirm that embryopathy associated with maternal diabetes remains an important clinical problem. In women with pre-existing diabetes, the rate of major malformations is 4–5 times the rate in non-diabetics. This excess has been associated with relative maternal hyperglycemia since the original observations of Miller *et al.* (1981) which reported that malformations were a feature of poor periconceptional plasma glucose control manifested by high levels of glycated hemoglobin (HbA1c) measured in the first trimester of pregnancy.

A large body of clinical data including case series and quasi-randomized experiments has demonstrated that improved periconceptional control is associated with a reduction in fetal malformation rates in women offered preconceptional counselling and enhanced diabetic control during pregnancy. The meta-analysis of Ray *et al.* (2001) reported a mean fall from 7.7 to 2.4% for major malformations, a 68% reduction. Of particular interest is the Diabetes in Early Pregnancy Study (Mills *et al.*, 1988) that reported a significant reduction in fetal malformations in women who enrolled before pregnancy and were subjected to an enhanced preconceptional diabetes control program. It was observed, however, that the malformation rate in these women was still more than twice that of non-diabetic matched controls, and surprisingly that malformations were still seen amongst the better controlled women as judged by lower individual HbA1c levels. High post-prandial peak excursions of plasma glucose can occur in apparently well-controlled subjects. The difference between pre- and post-prandial concentrations of glucose in apparently well-controlled women with diabetes can be 10-times greater compared to non-diabetics (Kyne-Grzebalski *et al.*, 1999). Since the concentration of glucose in tubal and uterine fluid is directly related to the levels in...
plasma (Leese, 1981), we have hypothesized that in well-controlled diabetic women the embryo may still be exposed to intermittently fluctuating high levels of glucose during development inside the female reproductive tract. We also hypothesized that exposure to fluctuating levels of glucose, as well as absolute hyperglycemia, may induce further stress on the preimplantation embryo, compromising development and contributing to the increased incidence of fetal malformations seen in apparently well-controlled diabetes. Since the publication of the studies analysed by Ray and colleagues, therapeutic advances in diabetes care suggest that safer, better-targeted periconceptional control programs may be devised. The first of these is the introduction of basal-bolus type self-management programs such as DAFNE (DAFNE Study Group, 2002) which might allow a safe enhancement of diabetic control at the woman’s own initiative. Secondly, and perhaps complementary to this, is the availability for use in pregnancy of new insulin analogues. These can be administered to modulate the high post-prandial peak excursions of plasma glucose seen in apparently well-controlled subjects with diabetes.

The mechanisms of diabetes-induced embryopathy have been the subject of many studies usually based on experimental diabetes in rodent models. These have shown that abnormal maternal fuel metabolism and hyperglycemia associated with diabetes serve to impair embryogenesis as early as the pre-implantation stages of development (Moley, 1999). In vivo studies have demonstrated morphological, cellular and biochemical changes in early embryos flushed from the reproductive tract of spontaneous and chemically induced diabetic mice and rats. Blastocysts (Day 4 of pregnancy) have a higher degree of nuclear fragmentation, a reduced number of inner cell mass cells (ICM) and an increased incidence of apoptosis in the ICM compared to those from non-diabetic animals (Pampfer et al., 1990, 1997; Vercheval et al., 1990; De Hertogh et al., 1991a; Moley et al., 1991, 1998a; Lea et al., 1996). Trophoblast outgrowth in vitro is also impaired (Pampfer et al., 1994a; Leunda-Casi et al., 2001). In addition, De Hertogh et al. (1991a) have also shown that the total number of embryos formed is reduced in diabetic mice. Interestingly, the administration of insulin to these animals appears to prevent diabetes-induced embryopathy although the total number of embryos is still significantly lower compared to non-diabetic mice (Moley et al., 1991; De Hertogh et al., 1991b).

In vitro experiments using rodent models have also attempted to identify the teratogenic agents and associated underlying mechanism(s) that underpin diabetic embryopathy during the preimplantation period. Critical factors include hyperglycemia, but also high levels of ketones, reactive oxygen species and tumor necrosis factor alpha (Zusman et al., 1987; Pampfer et al., 1994b, 1994c, 1995, 1997; Moley et al., 1994, 1996; Ornay et al., 1996; Wuu et al., 1998). Embryo culture experiments in the presence of high concentrations of exogenous glucose (20–52 mM) have been shown to inhibit blastocyst development, cell proliferation and cell differentiation, as well as increase apoptosis and affect intra-embryonic metabolite levels (Zusman et al., 1985; Diamond et al., 1989; Pampfer et al., 1990; De Hertogh et al., 1991a; Moley et al., 1996; Moley, 2001).

Moreover, a hyperglycemic environment in vitro is associated, paradoxically, with down regulation of Glucose Transporter 1, 2 and 3 in the blastocyst, reduced glycolytic activity, and a relative drop in intra-embryonic free glucose (Moley et al., 1998b; Leunda-Casi et al., 2001; Moley, 2001). In turn, this has been shown to increase levels of reactive oxidative species and stimulate expression of pro-apoptotic genes (p53 and Bax) (Moley et al., 1998b; Keim et al., 2001; Leunda-Casi et al., 2001) and intracellular apoptotic effectors (caspase-3 and caspase-activated deoxyribonuclease) (Hinck et al., 2001), resulting in a reduction in ICM numbers.

The minimum exposure time during the preimplantation period required to induce irreversible effects during the peri- and post-implantation stage of development is yet to be determined. Moreover, there has been little attempt to define the periods of preimplantation development that are susceptible to hyperglycemia, in particular whether this sensitivity occurs before, as well as after, activation of the embryonic genome. Prior to activation of the embryo’s own genome, which occurs at the 2–4 cell stage in the mouse, the embryo is reliant on stores of mRNA and proteins inherited from the oocyte (Braude et al., 1979; Flach et al., 1982). An understanding of the synchronization of such factors contributing to teratogenesis is important for establishing a mouse model to further investigate the underlying mechanisms associated with diabetes induced embryopathy.

Materials and Methods

Animals

Outbred mice (CD1 strain; Harlan, Loughborough, UK) were housed with a 12 h light:12 h dark photoperiod, with light commencing at 07:00 h. Food and water were available ad libitum. Females (2–3 weeks old) were superovulated by an i.p. injection of 5 IU Pregnant Mare Serum Gonadotrophin (PMSG, Intervet, UK) per mouse followed by a second i.p. injection 46 h later of 5 IU HCG (Intervet, UK). After injection with HCG the mice were mated, in pairs, with 6–8-week-old male mice (CD1 strain; Harlan, Loughborough, UK). The following morning, the female mice were separated, and this was deemed to be Day 1 post fertilization (pf). The use of animals in these procedures was approved by the South Sheffield Research Ethics Committee, and carried out in accordance with the Home Office Animals (Scientific Procedures) Act 1986.

Preimplantation embryo retrieval and culture

Female mice were killed on Day 2 pf or Day 4/5 pf to obtain 2-cell embryos or blastocysts, respectively. Two-cell embryos were retrieved by flushing the oviducts, whilst blastocyst stage embryos were retrieved by flushing the uterine horns with either warm (37°C) synthetic oviductal medium enriched with potassium (mKOSOM) or warm (37°C) synthetic oviductal medium enriched with potassium and 50% reduced amino acids (KOSOM) using a 32 gauge stainless steel hypodermic needle (Popper and Sons, NY, USA). Different culture media were used initially to determine which was the most suitable for culturing embryos to the blastocyst stage. Two-cell embryos were cultured in groups of 10–15 in 50 µl droplets of KOSOM medium supplemented with 0.2, 5.56, 15.56 or 25.56 mM D-glucose, and overlaid with mineral oil. The range of concentrations chosen taken into account the concentration of glucose measured in mouse oviduct fluid (5.10 ± 0.20 mM) (Gardner and
Leese, 1990) and in rodent diabetic serum (23.3 ± 0.5 mM) (Pampfer et al., 1997). It was postulated that embryos are exposed to glucose levels in this range within the female reproductive tract. Any increase in osmolarity caused by the addition of higher concentrations of glucose to culture medium was compensated for by adjusting the concentration of sodium chloride accordingly. Embryos were cultured in a humidified incubator gassed with 5% CO2 in air at 37°C (Martin and Leese, 1995, 1999). To prevent the build-up of toxic ammonium ions, each embryo was transferred to a fresh pre-equilibrated drop of culture medium once each day until Day 5 pf.

**Peri-implantation embryo culture**

Hatched blastocysts (Day 5pf) were cultured in groups of 10–15 on a defined extra cellular matrix of fibronectin at 37°C in 5% CO2 in air until Day 8 pf. Briefly, a four-well plate was prepared by incubating round, glass, sterile cover slips with a 10 μg/ml fibronectin (Sigma) solution in phosphate-buffered saline overnight at 4°C. Wells were washed thoroughly before transferring zona-free blastocysts to warm, pre-equilibrated KSOM medium.

**Assessment of preimplantation embryo development (Days 2–5)**

Four parameters were used to assess preimplantation embryo development *in vitro* on Day 5 pf: (i) morphological development; (ii) cell proliferation; (iii) cell death and (iv) cell differentiation.

**Morphological development**

Embryos were assessed daily for cellular morphology, including cytoplasmic granularity, fragmentation, blastomere size and shape. Embryos were also assessed daily for their developmental stage, i.e. the proportion of embryos reaching the blastocyst stage and the percentage of these hatching. A low proportion of embryos reaching blastocyst stage is indicative of compromised development.

**Cell proliferation**

On Day 5 pf, the total cell number of blastocysts was measured using the fluorochrome nuclear stain Bisbenzimide Hoechst No 33258 (Sigma) (Martin et al., 1998). Briefly, embryos were incubated in acid tyrodes solution (Sigma) to remove the zona pellucida before being washed in ethanol and incubated in 50 μg/ml bisbenzimide at 4°C overnight. Embryos were washed thoroughly in ethanol before being mounted in glycerol and examined using an Olympus BH2-RFCA fluorescence microscope (Fig. 1A). A low cell number at the blastocyst stage can be associated with reduced embryo viability (Desai et al., 2000).

**Cell death (apoptosis)**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used to determine the proportion of cells undergoing apoptosis employing the Fluorosecin-FragEL DNA Fragmentation Kit (Oncogene) (Fig. 1B). Embryos incubated in DNase I, to generate double strand breaks, were used as a positive control. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to determine total cell number. An increase in the dead cell index (number of apoptotic cells/total cell number) is associated with a decrease in embryo quality (Brison and Schult, 1997).

**Cell differentiation**

The allocation of cells to the two cell populations of the blastocyst, the ICM and trophectoderm (TE), was measured using antibody mediated complement-lysis employing the florochrome propidium iodide and bisbenzimide (Hewitson et al., 1996) (Fig. 1C). A threshold number of ICM cells are required for normal fetal development. Too few ICM cells are incompatible with normal fetal development (Van Soom et al., 1997).

**Assessment of peri-implantation embryo development (Days 6–8)**

**Morphological development**

On Day 8 pf morphological development of peri-implantation embryos was assessed as the percentage of blastocysts remaining attached to the cover slip after gentle agitation, and the proportion of blastocysts with signs of trophoblast outgrowth. The degree of trophoblast outgrowth (indicated by a flattened area of the attached embryo) was initially assessed using a semi-quantitative method. Embryos were graded as having none; slight; moderate or significant outgrowth (Fig. 2). The degree of outgrowth was also quantified by measuring the total area of spreading using image analysis equipment (Scion) (Martin et al., 1998; Martin and Leese, 1999). Trophoblast
Cell proliferation

Cell number in attached blastocysts was determined by removing and inverting cover slips onto a droplet of Vectashield with DAPI. Blastocysts were examined under fluorescent light. Cell number was approximated as cell density was high.

Cell death (apoptosis)

TUNEL was used to determine the proportion of cells undergoing apoptosis, as described earlier.

Cell differentiation

The proportion of trophoblast cells exhibiting a giant nuclear phenotype was used as a marker of differentiation (Pampfer et al., 1994a). The distribution of phagocytosed fluorescent beads was used to assess whether there was an ICM present in the embryos on Day 8 pf (Nishioka et al., 1994; Albieri et al., 1996; Grandjean et al., 1997, Rassoulzadegan et al., 2000). Briefly, embryos were cultured in the presence of fluorescent beads for 2 h and then washed in culture media to remove any beads, which had not been phagocytosed. The embryos were then fixed and mounted in DAPI and the number and distribution of beads was analysed. The presence of an ICM was indicated by an area of the attached embryo being devoid of beads. ICM, in contrast to TE/Trophoblast (TB), do not phagocytose the fluorescent beads (Fig. 3). The density of beads was used to indicate what proportion of the two discrete groups of TE cells where present, with a higher bead density indicating more cells of the mural TE type and a lower bead density indicating more cells of the polar TE type. The mural TE generates primary trophoblast giant cells and the polar TE generates the ectoplacental cone and the extra-embryonic ectoderm and later much of the fetal part of the placenta (Rassoulzadegan et al., 2000).

Determination of the teratogenic susceptible periods during preimplantation development

To determine the stage at which exposure to teratogenic glucose concentrations (as determined from the preceding experiment) is most detrimental to preimplantation development, embryos were exposed to teratogenic levels of glucose for either 24 or 48 h at differing times throughout preimplantation development. Embryos were cultured in a physiological level of glucose (5.56 mM) at all other times. Embryos exposed to 5.56 mM glucose from Day 2 to 5 pf served as controls. Embryo development was assessed on Day 5 pf using parameters as before.

Figure 2: Trophoblast outgrowth (scale bar 50 μm)

(A) No trophoblast outgrowth, the embryo is highly compacted and raised. (B) Slight trophoblast outgrowth, the embryo is highly compacted and raised with a small area of flat growth. (C) Moderate trophoblast outgrowth, the embryo is less compacted and is not as raised with an extended area of flat growth. (D) Significant trophoblast outgrowth, the embryo is predominately flat.
In the post-implantation embryo phagocytosis appears as an early marker of differentiation. Trophoderm cells possess phagocytic ability and therefore take up the fluorescent beads. ICM cells do not possess phagocytic ability and are unable to take up the fluorescent beads (scale bar 50 μm). (A) The area has not phagocytosed any fluorescent beads and is therefore ICM. (B) The area has phagocytosed the fluorescent beads and is therefore Trophoderm.

All these experiments were repeated at least five times. Positive and negative controls were included each time. Data were then pooled before analysis.

**Statistical analysis**

Statistical differences between control and diabetic distribution patterns (i.e. percentages) were analysed using chi-squared test. Mean values (e.g. total cell numbers) between the control and treatment groups were compared using unpaired Student’s t-test or one-way analysis of variance where appropriate.

**Results**

**Choice of preimplantation culture system**

Preliminary experiments were carried out to determine the optimal culture system to examine the effects of exposure of pre and peri-implantation embryos to teratogenic concentrations of glucose. Embryos were cultured from the 1- to 2-cell stage, in two different culture media, mKSOM or KSOM (containing 50% reduced concentration of amino acids).

No effect of increasing glucose concentration was observed on morphological development of 1-cell embryos to the blastocyst stage, in either mKSOM or KSOM. This was attributed to the low rate of blastocyst development. A phenomenon known as the 2-cell block which is well documented in embryos cultured from the 1-cell stage (Robi et al., 1988; Schini et al., 1988; Minami et al., 1992; Goddard et al., 1983; Liu et al., 1995) is probably responsible for the low rates of blastocyst development observed here. However, increasing concentrations of glucose (0.2–25 mM) did result in significant difference in blastocyst development when embryos were cultured from the 2-cell stage in both mKSOM and KSOM. As the rates of blastocyst development on Day 5 pf were significantly greater in KSOM [61.2% (66.3% including expanded blastocysts)] compared to mKSOM (45.6%). KSOM was selected as the medium for further experiments.

In order to establish effective in vivo and in vitro controls, Day 2 pf embryos were cultured in KSOM with 5.56 mM glucose, which is equivalent to the rodent physiological glucose concentration, until Day 5 pf and compared to in vivo embryos harvested on Day 4/5 pf. Initially, blastocysts were retrieved in the morning of Day 5 and assessed without culturing. It was found, however, that by Day 5 the majority (>90%) of blastocysts had already attached and very few embryos could be flushed from the uterine horns. In an attempt to increase the yield, blastocysts were retrieved on Day 4.5 and again assessed without culturing. Again, however, it was found that between 30 and 50% of blastocysts had already attached and only a few embryos were able to be flushed from the uterine horns. Finally, to further increase the yield, blastocysts were retrieved on Day 4 and assessed without culturing. There were no problems encountered with retrieval at this stage. All the blastocysts (Day 4, 4.5, 5 in vivo) were assessed and compared to each other and to Day 5 in vitro blastocysts. Owing to the reduced number of animals involved, Day 4 in vivo blastocysts were used as the in vivo control. Moreover, all parameters assessed on Day 5 pf embryos cultured in vitro were comparable to their in vitro counterparts on Day 4 pf (data not shown). As there was no statistical difference between Day 4 in vivo and Day 5 in vitro embryos, it was decided to use Day 5 in vitro embryos as the only control for further experiments.

**Determination of the concentrations of exogenous glucose that cause embryopathy during the pre- and peri-implantation periods**

**Effect on preimplantation development**

Exposure of preimplantation embryos to increase the concentrations of glucose resulted in significantly fewer blastocysts, and fewer blastocysts hatching, by Day 5 pf compared to control embryos (cultured in 5.56 mM glucose) (Table I).

A significant effect on cell allocation on Day 5 pf was observed. There was a significant reduction in both the number of ICM cells and the number of TE cells with the increasing concentrations of glucose on Day 5 pf. The

<table>
<thead>
<tr>
<th>Glucose concentration (mM)</th>
<th>Expanded blastocysts (%)</th>
<th>Hatching (%)</th>
<th>Total cell number</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>64.8</td>
<td>39.7</td>
<td>36</td>
<td>71.1 ± 4.4</td>
</tr>
<tr>
<td>5.56</td>
<td>64.4</td>
<td>38.7</td>
<td>36</td>
<td>54.6 ± 3.5</td>
</tr>
<tr>
<td>15.56</td>
<td>56.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td>57.4 ± 3.5</td>
</tr>
<tr>
<td>25.56</td>
<td>46.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24</td>
<td>51.3 ± 3.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Total cell number significantly different to 0.2 mM glucose culture.  
<sup>b</sup>Expanded blastocysts significantly different to 0.2 and 5.56 mM cultures (χ², P < 0.005).  
<sup>c</sup>Hatching significantly different to 0.2 and 5.56 mM cultures (χ², P < 0.001).

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**Figure 3:** Phagocytosis of fluorescent beads

In the post-implantation embryo phagocytosis appears as an early marker of differentiation. Trophoderm cells possess phagocytic ability and therefore take up the fluorescent beads. ICM cells do not possess phagocytic ability and are unable to take up the fluorescent beads (scale bar 50 μm). (A) The area has not phagocytosed any fluorescent beads and is therefore ICM. (B) The area has phagocytosed the fluorescent beads and is therefore Trophoderm.
Table II. The effect of increasing the concentration of glucose in embryo culture medium on cell allocation and ratio on Day 5 pf.

<table>
<thead>
<tr>
<th>Glucose concentration (mM)</th>
<th>n</th>
<th>Total</th>
<th>ICM</th>
<th>TE</th>
<th>Ratio (ICM:TE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>46</td>
<td>69.16 ± 3.526</td>
<td>12.63 ± 0.921</td>
<td>56.09 ± 3.381</td>
<td>0.2756 ± 0.033</td>
</tr>
<tr>
<td>5.56</td>
<td>46</td>
<td>55.51 ± 2.803</td>
<td>15.02 ± 1.106</td>
<td>40.48 ± 3.607b</td>
<td>0.4335 ± 0.042b</td>
</tr>
<tr>
<td>15.56</td>
<td>42</td>
<td>54.51 ± 2.707a</td>
<td>11.57 ± 0.920</td>
<td>42.94 ± 2.376b</td>
<td>0.2880 ± 0.027d</td>
</tr>
<tr>
<td>25.56</td>
<td>35</td>
<td>49.94 ± 2.492a</td>
<td>9.943 ± 0.895a</td>
<td>40.00 ± 2.319b</td>
<td>0.2706 ± 0.026a</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM. ICM, inner cell mass; TE, trophectoderm.

As a result, cells were grouped into ‘bands’ of cell numbers. Cell numbers were then represented as the median value for that band (e.g. 1–25 = 13, 26–50 = 38). Increasing glucose concentrations resulted in significantly fewer cells in attached blastocysts by Day 8 (Table IV). There was no significant effect on the total surface area of Day 8 blastocysts, as quantified using Scion software (Table IV). There was a significant effect on cell density however with density decreasing as glucose concentration increases (Table IV).

Increasing concentrations of glucose resulted in significantly fewer embryos containing an ICM ($\chi^2, P < 0.01$) as indicated by an area of the embryo being devoid of fluorescent beads. In 0.2 and 5.56 mM glucose cultures, 80% of embryos contained an ICM whereas in 15.56 and 25.56 mM glucose cultures only 60.5 and 31.3%, respectively, had an ICM.

The total uptake of fluorescent beads was also used to quantify the proportion of ICM to TE cells, with fewer beads per median cell number indicating less cells of the TE/TB type. Increasing concentrations of glucose were associated with an increase in the uptake of beads and an increase in bead density (Table V).

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Determination of the teratogenic susceptible periods during preimplantation development

The experiments outlined above suggest that embryopathic effects are maximal at 25.56 mM D-glucose. Mouse embryos...
were therefore exposed to 25.56 mM d-glucose for 24 and 48 h periods between Days 2 and 5 pf to examine whether relatively short and/or fluctuating exposure to supraphysiological levels of glucose can induce embryopathy.

The development of embryos cultured in the presence of 25.56 mM glucose for 24 h on Days 2, 3 or 4 (early 24 h, mid 24 h or late 24 h) was not significantly compromised when assessed on the basis of morphology, cell proliferation, differentiation and apoptosis on Day 5 pf (Tables VI and VII). However, development to the blastocyst stage was reduced following exposure to 25.56 mM glucose for a total of 48 h, this became significant when it involved exposure to fluctuating levels (Split 48; Fig. 4).

Cell proliferation was significantly reduced in embryos exposed to 25.56 mM glucose for 48 h in the ‘late’ and ‘split’ experiments, but not in the ‘early’ exposure experiments (Table VI).

In comparison, no significant effect on cell apoptosis was observed when embryos were exposed to high glucose for a total of 48 h split between Days 2 and 4 (data not shown). However, significant effects on both cell allocation and total cell number were observed (Table VI). No effect on cell allocation was observed when embryos were exposed to 25 mM glucose for 48 h in the ‘early’ and ‘late’ exposure experiments.

**Discussion**

We used an *in vitro* mouse model to investigate the effects of embryo exposure to continuous or fluctuating raised concentrations of glucose to mimic the pre- and post-prandial level changes observed in apparently well-controlled diabetes. The results of this study provide insight into the benefits from optimizing glycemic control before conception in diabetic women. The pathogenesis of hyperglycemia in diabetic teratogenesis has not previously been adequately explored in the mouse pre-implantation *in vitro* model. Previous studies have not attempted to determine which concentrations of glucose induce embryopathy during the preimplantation period and we can find only one study of the effects of glucose concentration within this context, which was restricted to measurement of intra-embryonic metabolite levels (Moley et al., 1996).

In studying our culture system, we found expected levels of expanded blastocyst formation and hatching on Day 5 both at the 0.2 mM glucose concentration and the more physiological 5.56 mM concentration. Higher levels of glycemia were associated with a progressive fall in success of both processes. Total cell numbers and allocation to an ICM were both significantly reduced in the hyperglycemic culture.

Previously reported experiments with blastocysts from mice and rats with induced diabetes suggested increased rates of apoptosis—particularly affecting the ICM—leading to increased rates of malformation and absorption (Moley et al., 1998a). In contrast, induction of the anti-apoptotic factor Bcl-2 has been reported in response to hyperglycemia (28 mM versus 6 mM glucose) and a mechanism similar to this may explain the lack of increased apoptosis seen in our particular experiment (Pampfer et al., 2001).

<table>
<thead>
<tr>
<th>Glucose concentration of medium H = 25.56 mM, L = 5.56 mM</th>
<th>Total cell number</th>
<th>ICM</th>
<th>TE</th>
<th>Ratio (ICM:TE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>Expt 1</td>
<td>Control</td>
<td>L</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Early 24 h</td>
<td>H</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Late 24 h</td>
<td>L</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>Expt 2</td>
<td>Control</td>
<td>L</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Early 48 h</td>
<td>H</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Late 48 h</td>
<td>L</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Expt 3</td>
<td>Control</td>
<td>L</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Mid 24 h</td>
<td>L</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>Split 48 h</td>
<td>H</td>
<td>L</td>
<td>H</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.

*Significantly different to Expt 2 control and early 48 h (P < 0.005).

*Significantly different to Expt 2 control (P < 0.01).

*Significantly different to Expt 2 control and early 48 h (P < 0.01).

*Significantly different to Expt 3 control (P < 0.001).

*Significantly different to Expt 3 control and mid 24 h (P < 0.01).

*Significantly different to Expt 3 control (P < 0.01).

*Significantly different to mid 24 h (P < 0.01).
Our observation that increasing glucose concentration had no significant effect on the apoptotic cells numbers or proportion of apoptotic cells is in sharp contrast to previously published studies (Keim et al., 2001; Chi et al., 2002; Jimenez et al., 2003). This could be due to differences in experimental design, e.g. Jimenez et al. (2003) cultured embryos initially in normal glucose levels then increased the glucose levels for a period of time before assessing the level of cell death whereas we cultured the cells continuously in the various glucose concentrations before assessing the level of cell death. The stage at which the level of cell death is assessed also varies between papers.

In addition to the shift from ICM to TE cells associated with increasing glucose concentrations, the hyperglycemic environment stimulates trophoblastic overgrowth in vitro. Despite this there was a reduction in median cell number and cell density with increasing glucose concentrations. The relevance of these observations to viability and disordered embryogenesis will have to await re-implantation experiments, but the dramatic reductions in the numbers of embryos with an ICM, confirmed the profound adverse effects of hyperglycemia in culture.

Most importantly in the experiments where the glucose concentration was varied over 1 or 2 days, of Days 2, 3 or 4 of culture, we have demonstrated preservation of percentage blastocyst formation on embryos exposed to only 24 h of hyperglycemia, on any of the 3 days. There was a significant reduction in successful blastocyst formation in the split hyperglycemia experiment, Days 2 and 4 only. This reduction in blastocyst formation may be due to the significant reduction in total cell number and the significant change in the ratio ICM—TE cells we observed, as there was no evidence of a significant increase in cell death.

The type of fluctuation used in the split hyperglycemia experiment may most closely mimic the environment experienced by the human embryo in poorly controlled maternal diabetes. These findings may help explain the high rates of implantation failure and early pregnancy loss seen in women with diabetes. We plan to extend these experiments to study events following embryo transfer, using embryos exposed to glycemic fluctuation, to quantify absorption and malformation rates associated with early embryopathy in the non-diabetic mouse.

**Conclusion**

This animal model offers great potential for studies of detailed time related aspects of disordered embryopathy in relation to maternal hyperglycemia in human pregnancy complicated by maternal diabetes.

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**References**


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