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# Effects of gestational diabetes on human placental glucose uptake, transfer, and utilisation

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## Abstract

*Aims/hypothesis*. Gestational diabetes is associated with complications for the offspring before, during and after delivery. Poor maternal glucose control, however, is a weak predictor of these complications. Given its position at the interface of the maternal and fetal circulations, the placenta possibly plays a crucial part in protecting the fetus from adverse effects from the maternal diabetic milieu. We hypothesised that gestational diabetes may result in changes in placental function, particularly with respect to the uptake, transfer, and/or utilisation of glucose. We aimed to examine glucose transport and utilisation in intact human placental lobules from women with gestational diabetes and those from normal pregnancies.
*Method*. Dual perfusion of an isolated placental lobule was done on placentae from diet treated gestational diabetic ($n = 7$) and normal pregnant patients ($n = 9$) using maternal glucose concentrations of 4, 8, 16 and 24 mmol/l in random order over a 4-h experiment. Results were expressed in $\mu mol \cdot min^{-1} \cdot g^{-1}$.
*Results*. D-glucose uptake from the maternal circulation (control 0.492 vs gestational diabetes mellitus 0.248, at 8 mmol/l maternal glucose), D-glucose utilisation by the placenta (0.255 vs 0.129), D-glucose transfer to the fetal circulation (direct 0.979 vs 0.402; net transfer 0.269 vs 0.118) and L-lactate maternal release into both the fetal (0.052 vs 0.042) and maternal (0.255 vs 0.129) circulation were significantly reduced during in vitro perfusion of placentae from patients with gestational diabetic pregnancies. Transfer of $^3$H-L-glucose also significantly reduced in the diabetic group (8.1 % vs 2.6 %).
*Conclusion/interpretation*. These results suggest that placental transport and metabolism of D-glucose is altered during gestational diabetes. [Diabetologia (2000) 43: 576–582]

**Keywords** Gestational diabetes, human placenta, glucose metabolism, in vitro perfusion.

Both pregestational [1] and gestational diabetes mellitus [2, 3] can lead to neonatal macrosomia which is associated with fetal death, prematurity, birth trauma, and neonatal respiratory distress syndrome, hypoglycaemia, polycythaemia, hyperbilirubinaemia, hypocalcaemia, hypomagnesaemia and cardiomyopathy [4–6]. For macrosomic neonates, birth weight has not been consistently shown to positively correlate with the degree of control of maternal blood glucose [7, 8], suggesting that factors other than or in addition to maternal blood glucose are associated with adverse clinical outcomes during diabetes in pregnancy.

Offspring from pregnant women with diabetes are more likely to develop diabetes mellitus and obesity later in life. This observation and evidence that development of diabetes is more closely related to maternal than paternal health, suggests that the intrauterine environment is possibly of importance [9, 10].

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*Abbreviations*: GLUT1 Glucose transporter protein 1; GDM gestational diabetes mellitus; GTT glucose tolerance test

One intrauterine factor affecting metabolic outcomes could be placental function; however, there have been few studies of placental transport function in diabetes. We hypothesised that in diabetes there are changes in placental function, in particular with respect to the uptake, transfer and/or utilisation of glucose. We tested this hypothesis using intact human placental lobules perfused in vitro with physiological solutions via the fetal circulation and intervillous space.

Materno-fetal transfer of nutrients occurs by way of the placental barrier, of which the syncytiotrophoblast is an integral part. The syncytiotrophoblast interacts metabolically with both the maternal and fetal compartments, and has a high capacity for glycolysis, glycogenesis and glycogenolysis [11]. The syncytiotrophoblast, therefore, should have considerable capacity for nutrient buffering. This has been shown to occur at least to some degree for glucose metabolism in non-diabetic human perfused placentae [12].

The glucose transporter GLUT1 is the major mediator of materno-fetal transport of glucose across the human placenta. The transport of glucose via GLUT1 does not depend on the presence of insulin, and increases with increasing glucose concentrations over a physiological range. Saturation of the transporter occurs at high glucose concentrations. Maternal hyperglycaemia is therefore likely to result in increased transfer of glucose across the syncytiotrophoblast to the fetus. This transfer could be altered, however, by changes in GLUT activity and/or syncytiotrophoblast consumption of glucose by glycolysis and/or glycogen synthesis. Indeed, changes in glycolytic, gluconeogenic and NADP-generating enzymes have been reported in diabetes [13]. Therefore it is important when assessing placental handling of glucose to consider not only transport, but also placental utilisation of glucose.

Our study aimed to examine glucose transport and utilisation in intact human placental lobules from women with gestational diabetes and those from normal pregnancies.

## Materials and methods

The experimental procedures undertaken in this study were approved by the Royal Women's Hospital Research and Ethics Committees in accordance with the guidelines of the National Health and Medical Research Council of Australia. Written, informed consent was obtained from all patients participating in the study.

*Placental collection.* Placentae were collected at term following normal vaginal delivery or Caesarean section from women who were classified as those with gestational diabetes mellitus (GDM), ($n = 7$) or normal non-diabetic pregnancy (Control), ($n = 9$). Patients were tested for GDM with a glucose tolerance test (GTT) done in the morning after an overnight fast and using a 75-g glucose load. A diagnosis of GDM was made, if the fasting plasma glucose concentration was greater than or equal to 5 · 5 mmol/l and/or the 2-h plasma glucose concentration after the GTT was greater than or equal to 7.0 mmol/l [14]. Blood glucose concentrations of patients with GDM in this study were maintained within normoglycaemic concentrations by dietary intervention alone.

*Placental perfusion.* Bilateral perfusion of a single placental lobule with a modified Krebs solution was started within 20 min of delivery. The dually perfused isolated placental lobule technique has been extensively used and characterised [12, 15–18,]. The perfusion technique used was a modification of a previously described method [19] and is explained here in full. The membranes covering the chorionic plate and excess blood were removed from the maternal surface. A suitable fetal vein and artery pair on the surface of the chorionic plate leading to an intact peripheral lobule were cannulated (1.3 and 2.1 mm cannulae, respectively) and perfused by way of the artery using a Masterflex perfusion pump (model 7554.00 with pump head 7013; Cole-Parmer Instruments, Vernon Hills, Illinois, USA) at a constant flow rate of 5 ml/min. The Krebs solution, which contained the following (mmol/l): NaCl 97.0, $NaHCO_3$ 24.4, KCl 3.0, $KH_2PO_4$ 1.2, $CaCl_2.2H_2O$ 1.89, $MgSO_4.7H_2O$ 1.0, D-glucose 3.0 (pH 7.4), was equilibrated with 95 % oxygen and 5 % carbon dioxide. The placenta was then placed on a perspex frame with the chorionic surface facing upwards and placed within a laboratory incubator (Thermoline EI40; Thermoline Scientific, Wetherill Park, NSW, Australia) and maintained at 37 °C. The lobule being perfused was positioned centrally over a hole in the frame allowing access to the maternal-facing surface of the lobule from below. At this point, the blood draining from the lobule was inspected for any evidence of Krebs solution leaking from the fetal circulation. If Krebs was present, the lobule was rejected and the experiment terminated. The maternal component of the lobule was also continually perfused with Krebs solution with varying D-glucose concentrations at a flow rate of 10 ml/min using two pieces of plastic tubing inserted through the basal plate into the intervillous space. Fetal arterial inflow perfusion pressure was measured by a Statham transducer (P23AC; Gulton-Stratham, Costa Mesa, Calif., USA) and Maclab4 and Chart software (version 3.2, ADInstruments, Castle Hill, NSW, Australia). At the end of the experiment the perfused lobule was dissected out and weighed.

The viability of perfused tissues has been assessed previously in our laboratory by measuring lactate production and glucose consumption in the fetal circulation [19]. These did not change notably over a 5-h perfusion period. After the initial cannulation period, the fetal arterial perfusion pressure remained constant. If the fetal arterial perfusion pressure rose noticeably the experiment was discontinued until the pressure returned to baseline. Maternal and fetal effluent flow rates were measured throughout the experiment and the experiment terminated if the fetal flow rate dropped by more than 5 %. As the fetal flow rate was held constant and the pressure didn't vary, it was assumed that the perfused area remained constant.

*Glucose utilisation studies:* The D-glucose concentration of the fetal circulation perfusate was maintained at 3.0 mmol/l throughout the experiment. The D-glucose concentration of the maternal perfusate was maintained at 4 mmol/l for the first half hour of perfusion, while the lobule was cleared of blood. The maternal D-glucose concentration was then changed to 4, 8, 16 or 24 mmol/l in random order. These concentrations represent euglycaemia, mild, high and extreme hyperglycaemia. After 30 min of perfusion at each D-glucose concentration, $^{14}C$(U)-D-glucose (37 kBq/ml) and $^{3}H$-L-glucose (37 kBq/ml)

were simultaneously infused into the maternal perfusate at 0.1 ml/min for 21 min. This time was shown during preliminary experiments to be sufficient for the rate of $^{14}$C-D-glucose and $^{3}$H-L-glucose transfer to the fetal effluent to reach equilibrium. Samples of fetal and maternal effluent were collected in 1-min fractions for the final 3 min of infusion. Infusion was stopped once the samples were collected and the next maternal glucose concentration perfused. This procedure was repeated until samples were collected for each of the above four maternal D-glucose concentrations for each placenta. The perfusion of each maternal glucose concentration took no longer than 51 min and the entire experiment was completed within 4.5 h. The samples collected for each maternal glucose concentration were centrifuged immediately at 3000 rpm for five minutes, to remove any trace of blood, and assayed fresh for D-glucose and L-lactate using a YSI 2300 analyser (YSI, Yellow Springs, Ohio, USA). The concentrations of $^{14}$C-D-glucose and $^{3}$H-L-glucose in the sample were determined by liquid scintillation spectroscopy (Wallac 1409 Liquid Scintillation Counter; Pharmacia, Wallac Oy, Turku, Finland) and converted to disintegrations per min (DPM; Wallac Multicalc Advanced version 2.60, Pharmacia).

The following rates were calculated (all expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$):

1. Net uptake rate of D-glucose from the maternal perfusate: $\{([G]_{MP}-[G]_{ME}) \times V_M\}/W$
2. Net transfer rate of D-glucose to the fetal effluent: $\{([G]_{FE}-[G]_{FP}) \times V_F\}/W$
3. Net utilisation rate of D-glucose by the placenta: Equation 1 – Equation 2
4. Direct transfer rate of D-glucose to the fetal effluent: $\{G^{C14}{}_{FE} \times [G]_{FP} \times V_F\}/\{G^{C14}{}_{MP} \times W\}$
5. Release of lactate into the maternal effluent: $(L_{ME} \times V_M)/W$
6. Release of lactate into the fetal effluent: $(L_{FE} \times V_F)/W$
7. Rate of diffusion of $^{3}$H-L-glucose to the fetal effluent: $G^{L}{}_{FE}/G^{L}{}_{MP}$

where $[G]_{MP}$ is the maternal perfusate D-glucose concentration (μmol/ml); $[G]_{ME}$ is the maternal effluent D-glucose concentration (μmol/ml); $[G]_{FP}$ is the fetal perfusate D-glucose concentration (μmol/ml); $[G]_{FE}$ is the fetal effluent D-glucose concentration (μmol/ml); $V_M$ is the maternal perfusion rate (ml/min); $V_F$ is the fetal perfusion rate (ml/min); W is the perfused lobule weight (grams wet weight); $G^{C14}{}_{FE}$ is the $^{14}$C-D-glucose activity in the fetal effluent (dpm/μmol); $G^{C14}{}_{MA}$ is the $^{14}$C-D-glucose activity in the maternal perfusate (dpm/μmol); $L_{ME}$ is the lactate concentration in the maternal effluent (μmol/ml); $L_{FE}$ is the lactate concentration in the fetal effluent (μmol/ml); $G^{L}{}_{FE}$ is the $^{3}$H-L-glucose activity in the fetal effluent (dpm/μmol); $G^{L}{}_{MP}$ is the $^{3}$H-L-glucose in the maternal effluent (dpm/μmol).

To determine whether quantifiable concentrations of $^{14}$C-L-lactate were being released from the placenta, $^{14}$C (U)-D-glucose (1 μCi/ml) and $^{3}$H-L-glucose (1 μCi/ml) were simultaneously infused into the maternal perfusate at 0.1 ml/min for 90 min. The infusate was allowed to equilibrate for 45 min after which the maternal effluent (450 ml) and fetal effluent (225 ml) were collected. Separation of glucose and lactate in this sample was done using an anion exchange column (Bio-Rad AG 1-X8 in acetate form, Bio-Rad, Hercules, Calif., USA). The bound lactate was eluted with 4 mol/l acetic acid, evaporated and resuspended in 200 μl deionised water. This sample was then analysed by HPLC for the presence of $^{14}$C-L-lactate on an Aminex HPX-87H organic acid analysis column (flow rate 0.6 ml/min, isocratic eluant 1.25 mmol/l $H_2SO_4$). Fractions were collected at 1-min intervals and the radioactivity measured.

**Table 1.** Characteristics of pregnant GDM and control subjects whose placentae were studied, presented as means ± SD

| Category | Control Pregnancy | Gestational Diabetes | *p* values |
|---|---|---|---|
| *n* | 9 | 7 | |
| Maternal age | 30.1 (2.1) | 34.4 (1.7) | NS |
| Maternal weight | 62.2 (2.5) | 62.4 (7.7) | NS |
| Gestation | 39.3 (0.4) | 38.3 (0.5) | NS |
| Gravidity | 2.7 (0.6) | 4.4 (1.2) | NS |
| Parity | 2.3 (0.4) | 3.1 (1.0) | NS |
| Infant weight (g) | 3404 (85) | 3238 (90) | NS |
| Placental weight (g) | 642 (33) | 781 (82) | NS |
| Perfused lobule (g) | 23.3 (3.6) | 23.8 (4.0) | NS |
| Perfusion pressure: during first | 38 (2.5) | 38.1 (2.2) | NS |
| radioisotope infusion during fourth radioisotope infusion | 39.3 (3.0) | 38.9 (2.6) | NS |

*Statistical analysis.* Statistical analysis was done by Statgraphics for Windows 3.1 (Manugistics, Rockville, Maryland, USA). Data are presented as means ± SEM. Significant differences between means were determined using two way analysis of variance (ANOVA) with the Bonferroni correction factor. The data was also tested for fit to the General Linear Model. A value of $p < 0.05$ was considered statistically significant.

*Drugs and chemicals.* All chemicals for the Krebs solution were obtained from British Drug Houses (Merck, Kilsyth, Victoria, Australia) except magnesium sulfate, which was from Sigma (St Louis, Montana, USA) and potassium dihydrogen phosphate from ICN Biochemicals (Cleveland, Ohio, USA). $^{3}$H-L-glucose (0.1 Ci/ml), $^{14}$C(U)-D-lactate and $^{14}$C(U)-D-glucose (0.1 mCi/ml) were obtained from Dupont NEN (Boston, Massachusetts, USA).

## Results

Table 1 shows the characteristics of the pregnancies of the control and GDM patients from whom placental samples were obtained for the study. Where possible, patients in the two groups were matched for gestation, parity, gravidity, and neonatal weight. There was a trend for the GDM group to have larger placentae, although this was not statistically significant. There was no statistically significant difference in weight of the perfused placental lobules between the two groups. There was no difference in the perfusion pressures between the two groups. In addition, the mean perfusion pressure was not significantly different during the first and fourth infusion time between the two groups.

Mean net uptake of D-glucose from the maternal perfusate was proportional to maternal glucose concentration ($r^2 = 0.65$, $p < 0.0001$). Mean net uptake was significantly reduced in placentae from GDM patients compared with control subjects (Fig. 1,

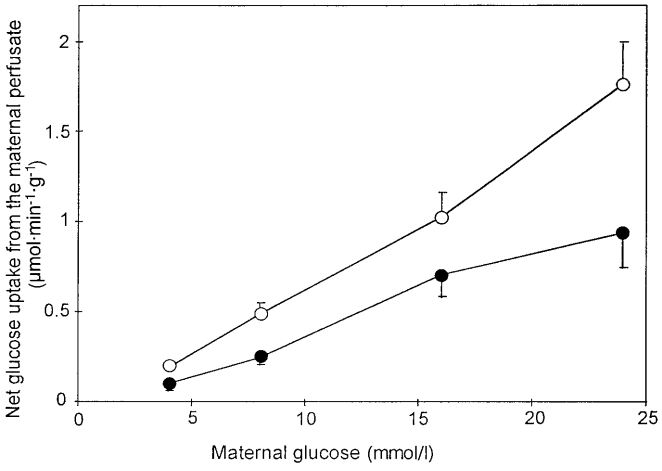


**Fig. 1.** Net D-glucose uptake from the maternal perfusate of human in vitro perfused placentae (μmol · min$^{-1}$ · g$^{-1}$) at maternal D-glucose concentrations of 4, 8, 16 and 24 mmol/l. Placentae from GDM pregnancies (closed circles) had significantly reduced uptake compared with non-diabetic controls (open circles; ANOVA $p = 0.0005$)

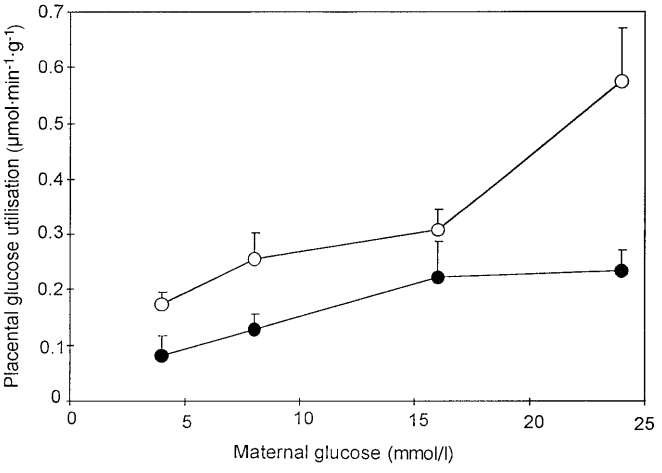


**Fig. 3.** Net D-glucose utilisation by human in vitro perfused placentae (μmol · min$^{-1}$ · g$^{-1}$) at maternal D-glucose concentrations of 4, 8, 16 and 24 mmol/l. Placentae from GDM pregnancies (closed circles) had significantly reduced utilisation compared with non-diabetic controls (open circles; ANOVA $p = 0.0002$)

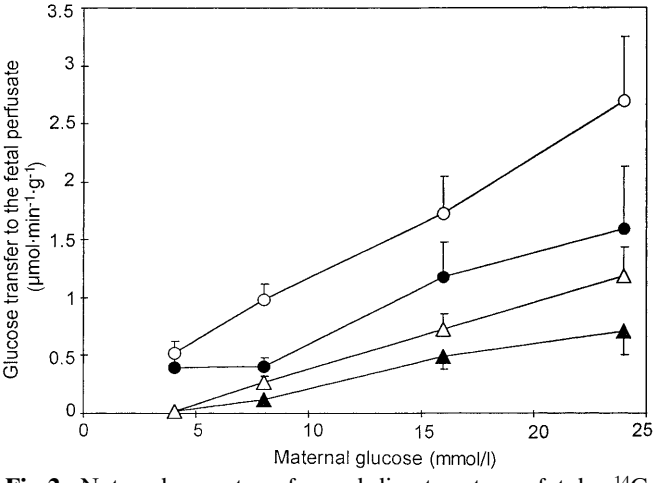


**Fig. 2.** Net D-glucose transfer and direct materno-fetal D-$^{14}$C-glucose transfer to the fetal circulation of human in vitro perfused placentae (μmol · min$^{-1}$ · g$^{-1}$) at maternal D-glucose concentrations of 4, 8, 16 and 24 mmol/l. Placentae from GDM pregnancies (closed circles) had significantly reduced net transfer compared with non-diabetic controls (open circles; ANOVA $p = 0.0327$). Placentae from GDM pregnancies (closed triangles) had significantly reduced direct transfer compared with non-diabetic controls (open triangles; ANOVA $p = 0.0147$)

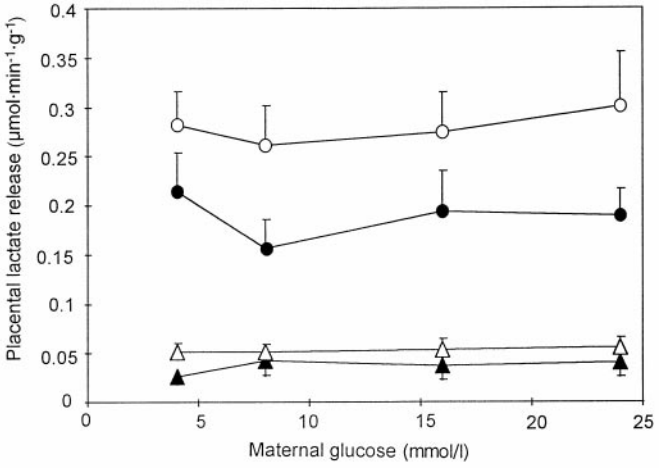


**Fig. 4.** Lactate release into the fetal and maternal effluents by human in vitro perfused placentae (μmol · min$^{-1}$ · g$^{-1}$) at maternal D-glucose concentrations of 4, 8, 16 and 24 mmol/l. Placentae from GDM pregnancies (closed circles) had significantly reduced lactate release into the maternal effluent compared with non-diabetic controls (open circles; ANOVA $p = 0.0107$). Placentae from GDM pregnancies (closed triangles) also had significantly reduced lactate release into the fetal circulation compared with non-diabetic controls (open triangles; ANOVA $p = 0.0353$)

$p = 0.0005$). Mean net D-glucose transfer to the fetal effluent ($r^2 = 0.52$, $p < 0.0001$) and direct materno-fetal D-glucose transfer ($r^2 = 0.42$) were both proportional to the maternal glucose concentration. Both net D-glucose transfer to the fetal circulation ($p = 0.0327$) and direct materno-fetal D-glucose transfer ($p = 0.0147$) were significantly reduced in GDM patients (Fig. 2). Placental glucose utilisation was also proportional to maternal glucose concentration ($r^2 = 0.45$, $p < 0.0001$). Placental glucose utilisation was significantly reduced in GDM compared with control placentae (Fig. 3, $p = 0.0002$).

The release of lactate from the placental lobule into the fetal and maternal effluents was independent of maternal glucose concentration. For both GDM and control placentae the release of lactate into the maternal effluent was significantly higher than into the fetal effluent ($p = 0.0001$). Release of lactate was

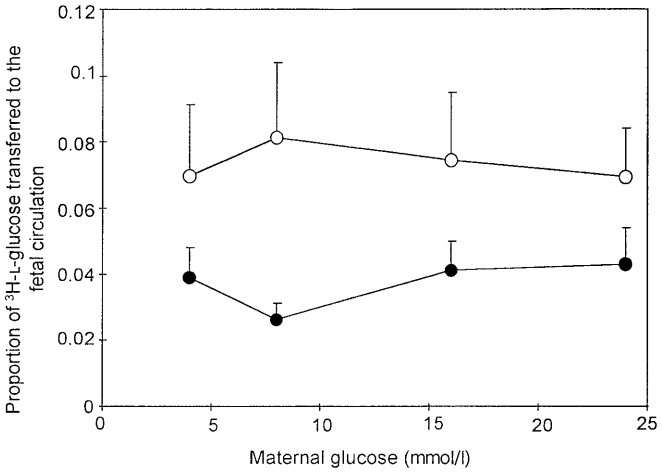


**Fig. 5.** The ratio of L-$^3$H-glucose infused into the maternal perfusate that is detected in the fetal circulation; this is a measure of non-facilitated diffusion. Placentae from GDM pregnancies (closed circles) had significantly reduced L-$^3$H-glucose transfer rate compared with the non-diabetic controls (open circles; ANOVA $p = 0.0028$)

significantly reduced in GDM placenta for both the maternal ($p = 0.0107$) and fetal ($p = 0.0353$) effluents (Fig. 4).

The transfer of $^3$H-L-glucose from the maternal perfusate into the fetal circulation was also significantly reduced (Fig. 5, $p = 0.0028$) compared with controls. $^3$H-L-glucose transfer was independent of maternal glucose concentration for both groups.

Fractionation of the fetal and maternal effluents by HPLC showed peaks associated with $^{14}$C-D-glucose but lacking peaks associated with $^{14}$C-L-lactate (data not shown).

## Discussion

Results of this study suggest that the function of the placenta in gestational diabetes mellitus is altered compared with normal pregnancy. Uptake of D-glucose from the maternal environment, transfer to the fetal circulation, placental D-glucose utilisation and placental production of lactate were all reduced during in vitro bilateral perfusion of human placentae from pregnant patients with gestational diabetes compared with the control group. In addition, the transfer of $^3$H-L-glucose from the maternal perfusate to the fetal circulation was significantly reduced in the GDM compared with control placentae.

Glucose transport into the syncytiotrophoblast from the maternal circulation and from the syncytiotrophoblast to the fetal circulation is mainly by way of the glucose transporter GLUT1 [22–24]. GLUT1 transfers glucose in direct proportion to the glucose concentration gradient within physiological ranges [25]. It has been assumed that the human placenta has a limited role in influencing glucose transport to the fetus; however, there is now evidence that both insulin and glucose can modulate GLUT1 expression (and therefore glucose transport) at the level of the placental trophoblast [26, 27, 35]. Studies using trophoblast cell cultures found that a hyperglycaemic medium resulted in a reduction in GLUT1 protein expression [35]. The reduced D-glucose transport in our studies possibly results from a similar reduction in GLUT1 protein expression due to the diabetic environment experienced in utero . Recent studies using the microvillous (maternal facing) and basal (fetal facing) membrane of the syncytiotrophoblast have suggested that GLUT1 protein concentrations and glucose uptake activity of the basal membrane is increased in diabetes whereas the microvillous membrane is notaffected [28, 29]. Our study, however, which involved perfusion of the intact vascular bed, found reduced D-glucose uptake with GDM. The differences between our study and the previous studies may reflect differences in the two experimental models.

The results of this study also suggest that glucose metabolism of GDM placentae is reduced compared with placentae from control pregnancies. Placental glucose utilisation was calculated as a derivative of the uptake and transfer of glucose and shows that glucose utilisation was altered without showing the fate of the glucose. The release of lactate into the maternal and fetal circulations appears to be reduced in GDM placentae. Further studies need to be done to determine whether the levels of other metabolic products of D-glucose are altered with GDM. The release of lactate by the placenta was not affected by maternal glucose concentration. This is consistent with previous perfusion studies which showed that the rate of lactate production was not affected by maternal glucose concentrations ranging from 5.5 to 53 mmol/l [12]. The HPLC results suggest that during the time of the experiment (1.5 h), the pool of glucose used for lactate production is independent of the glucose being transported from the maternal perfusate to the fetal effluent. This is consistent with previous research suggesting the lactate released may have been synthesised, at least in part, before perfusion [16, 18].

In our studies, the $^3$H-L-glucose transfer is being taken as a measure of non-facilitated diffusion. The reduced non-facilitated diffusion seen in the GDM placentae is possibly due to several factors. One factor could be reduced surface area for transfer. A characteristic feature of placentae from diabetic pregnancies is the enlargement of the villous surface by 30 to 50% [36], which would suggest that surface area available for transfer is not reduced. Other characteristics of GDM placentae possibly cause reduced non-facilitated diffusion. For example, placentae from GDM pregnancies have been shown to have increased cytotrophoblastic cell numbers, thickening

of the basement membrane and reduced vascularisation of the villi [20, 21], as well as reduced syncytiotrophoblast membrane fluidity and increased cholesterol content [37]. One study found a 35 to 45 % reduction in uteroplacental blood flow in diabetic pregnancies and the authors suggested this was due to a reduced intravillous space [38]. An altered intravillous space could change flow dynamics affecting opportunity for diffusion. Studies describing the morphological and histological changes are inconsistent [36]. Further analysis of the cause of reduced in vitro placental $^{3}$H-L-glucose transfer during GDM need to be done. Reduced effective surface area available for exchange may account for some of the reduced transport of D-glucose in the GDM group; however, as non-facilitated diffusion is only responsible for a small proportion of D-glucose transfer, changes in facilitated diffusion are probably more important.

The effects of diabetes on placental function are not well understood. Nevertheless, it is highly likely that metabolic changes occurring in diabetes (e.g. increased glucose concentrations, amino acids and fats, and hyperinsulinaemia) could affect a number of nutrient transport and metabolic pathways, including those of glucose handling. Fetal hyperinsulinaemia has been documented in diabetic pregnancy by a number of analytical techniques, including measurement of insulin and C-peptide in umbilical plasma and amniotic fluid and by cordocentesis [30–32]. Fetal hyperinsulinaemia has also been associated with neonatal macrosomia in both diabetic and non-diabetic pregnancy [32, 33], while maternal glucose concentrations have been shown to be poorly correlated with fetal hyperinsulinaemia and the development of macrosomia [7, 8, 34]. All of the GDM patients in this study were diet-treated, thereby removing exogenous maternal insulin as a possible factor in the observed difference between GDM and control placentae. It is not known if the placentae from insulin-treated GDM patients are different from diet-treated GDM patients, and this is the subject of ongoing studies by our group.

The finding in this study that in GDM glucose transport from the maternal environment to the fetal circulation was reduced, suggests that both the placenta and fetus would be protected from hyperglycaemic peaks that may occur during diabetes. Thus, this mechanism possibly explains why maternal glucose control is poorly correlated with the development of neonatal macrosomia as the placenta is able to protect the fetus from extremes in maternal glucose concentrations during diabetes. We hypothesise that macrosomia occurs when the placenta is incapable of fulfilling its protective role.

We suggest that exposure to the altered in utero milieu or the response of the fetus to this milieu leads to a functionally altered placenta, changing both placental glucose transport and utilisation. This mechanism may protect the placenta and the fetus from these adverse biochemical conditions.

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