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## Effect of diabetes mellitus on rat placenta cellularity

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### Abstract

Transcellular placental maternofetal flux of calcium and magnesium is reduced in diabetic pregnancy in the rat which might be due to changes in placental cellularity. In order to investigate this wet and dry weight, DNA and protein content were measured in placentas from untreated diabetic ( $D_o$ ), insulin-treated diabetic ( $D_i$ ) and control rats (C) on day 21 of gestation (term = 23 days). Wet and dry weights (mg; mean  $\pm$  S.E.M.) were  $418 \pm 13$ ,  $474 \pm 19$ ,  $416 \pm 14$  and  $66 \pm 3$ ,  $75 \pm 3$ ,  $67 \pm 3$  in C,  $D_o$  and  $D_i$  groups, respectively. Total DNA and protein content (mg) was  $1.8 \pm 0.2$ ,  $1.7 \pm 0.1$ ,  $1.5 \pm 0.1$  and  $50.4 \pm 2.4$ ,  $54.9 \pm 2.6$ ,  $51.9 \pm 3.3$  in C,  $D_o$  and  $D_i$  groups, respectively. The data suggest that placental cellularity is unaffected by maternal diabetes mellitus in the rat and is unlikely to directly affect maternofetal flux of calcium and magnesium. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords:* Placenta; DNA; Protein; Water; Diabetes

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### 1. Introduction

Infants of diabetic mothers are at risk of developing neonatal hypocalcaemia [1–5] and hypomagnesaemia [4,6] the incidence and severity of which is directly related to the severity of the maternal diabetes mellitus. Furthermore, the bone mineral content

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of these infants is reduced by about 10% when compared to normal infants [7]. Reduced fetal skeletal mineralisation and/or retarded ossification has also been reported in both streptozotocin-induced and genetically diabetic rats [8–10]. The aetiology of these disturbances is not fully understood but is likely to be multifactorial.

We have previously demonstrated reduced unidirectional maternofetal flux of calcium and magnesium across the diabetic rat placenta; the reduced calcium flux can be partly explained by an effect on the expression of placental transport components involved in calcium transfer [11]. Altered placental histology and cellularity in the presence of maternal diabetes mellitus has been reported in both humans [12] and rats [13,14]. Since both calcium and magnesium utilise active transcellular transport pathways [15,16] their transport characteristics might be affected by changes in placental cellularity in the presence of maternal diabetes mellitus.

Thus, the aim of this study was to examine the effect of maternal diabetes mellitus on placental cellularity by measurement of wet and dry weight, DNA and protein content in placentas from untreated diabetic ( $D_0$ ), insulin-treated diabetic ( $D_1$ ) and control rats (C).

## 2. Methods

All work carried out in this study was in accordance with the UK Animals (Scientific Procedures) Act 1986. Reagents and chemicals used were of Analar grade and purchased from Sigma or BDH Chemicals, Poole, UK, unless otherwise stated.

### 2.1. Animals

The cohort of animals used for these experiments were the same as those in which reduced maternofetal flux of calcium and magnesium in the presence of untreated maternal diabetes mellitus was reported [11]. Five days before being allowed to mate, female Sprague–Dawley rats (Charles River, Wilmington, UK) aged 6–8 weeks were rendered diabetic by an intraperitoneal injection of 60 mg/kg streptozotocin dissolved in citrate buffer (pH 6.8). Control rats received an equivalent volume of citrate buffer only. Two days later, diabetes mellitus (glucose concentration  $> 15$  mmol/l) was confirmed by the measurement of plasma glucose concentration from a tail vein sample using a glucose oxidase kit (Sigma). Thereafter, some diabetic rats received a once-daily subcutaneous injection (4–16 units) of a long-acting, heat-treated bovine insulin (Novo Nordisk; supplied by Ladybee, Horam, UK) to maintain their glucose concentration below 10 mmol/l. The remaining diabetic and control rats received once-daily subcutaneous injections of sterile water. Three days later the rats were caged with a male and the day on which a vaginal plug was found was designated day 1 of gestation. Plasma glucose concentration was measured periodically throughout gestation and the insulin dose adjusted accordingly. On day 21 of gestation, the rats were anaesthetised, initially with ether, followed by an intraperitoneal injection of sodium amytal (100 mg/kg). Following laparotomy and hysterotomy, studies on

placental calcium and magnesium flux were performed [11], and at the end of these experiments some of the remaining placentas were harvested, trimmed of membranes, blotted and stored at  $-20^{\circ}\text{C}$ .

### *2.2. Measurement of placental DNA content*

Extraction and measurement of DNA were carried out using the method described by Gendimenico [17]. Two placentas from each rat were briefly thawed, weighed and homogenised separately in 15 ml ice cold 0.5 M  $\text{HClO}_4$ , and the acid-insoluble material containing DNA isolated by centrifugation at  $800 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The supernatant was discarded and the pellet containing DNA was hydrolysed in 0.5 M  $\text{HClO}_4$  for 30 min at  $90^{\circ}\text{C}$ . This solution was centrifuged at  $800 \times g$  for 20 min at  $4^{\circ}\text{C}$  and the DNA-containing supernatant was aspirated and made up to 10 ml with 0.5 M  $\text{HClO}_4$ . Two ml freshly prepared diphenylamine reagent (0.75 g diphenylamine, 50 ml glacial acetic acid, 0.75 ml sulphuric acid, 0.25 ml 1.6% acetaldehyde) was added to 0.1 ml aliquots of unknown samples and standards (prepared using calf thymus DNA) and incubated in a waterbath at  $50^{\circ}\text{C}$  for 3 h. The absorbance of samples and standards was read at 600 nm using a double beam spectrophotometer (Cecil CE 3500, Cambridge, UK). The relationship between DNA content and absorbance at 600 nm is linear [17], allowing the amount of DNA in each unknown sample to be determined from the standard curve. All assays were performed in duplicate and the mean of the DNA content in the two placentas from each animal was taken as the single value for that animal.

### *2.3. Measurement of placental protein content*

Two placentas from each rat were briefly thawed, weighed and homogenised separately in 20 ml of distilled water. The protein in 0.1 ml aliquots of this solution was solubilised by incubation with 0.1 ml 1 M NaOH at  $55^{\circ}\text{C}$  for 5 min, and the solution then neutralised by the addition of 0.1 ml 1 M HCl. The protein content was measured using a commercially available kit (Bio-Rad Protein Assay, Bio-Rad, Hemel Hempstead, UK). This is a dye-binding assay, based on the differential colour change of an acidic solution of Coomassie Brilliant Blue G-250 in response to various concentrations of protein. As recommended by the manufacturer, sample aliquots were incubated with Bio-Rad reagent for 30 min at room temperature, and the absorbance of the solutions measured at 595 nm using a spectrophotometer (CE 5500). Bovine serum albumin was used to prepare a standard curve having previously determined that the use of the Bio-Rad kit produces a curve that obeys Beer's Law. All assays were carried out in duplicate and the mean of the protein content in the two placentas from each animal was taken as the single value for that animal.

### *2.4. Measurement of wet and dry weight*

Three placentas from each animal were briefly thawed, weighed in a crucible and dried to constant weight in an oven at  $80^{\circ}\text{C}$ . The mean of the weights and percentage

water content of the three placentas from each animal was taken as the single value for that animal.

### 2.5. Statistics

All results are presented as means  $\pm$  S.E.M. with  $n$  being the number of animals. Comparisons between groups were carried out by unpaired Student's  $t$ -test or one-way analysis of variance (ANOVA) followed by Tukey's post tests. A  $P$  value less than 0.05 was considered significant.

## 3. Results

Two days after streptozotocin injection, mean plasma glucose concentration in the diabetic rats was  $> 20$  mmol/l. Thereafter, mean plasma glucose concentration in the  $D_1$  group was maintained below 10 mmol/l and compared favourably with the glucose concentration in the C group, which was measured only on days 7 and 21 of gestation. By contrast,  $D_0$  rats remained hyperglycaemic throughout gestation (Fig. 1).

Placental wet weight in the  $D_0$  group was greater than the other two groups, but this was only significant at the 0.1 level (Table 1). However, if the wet weights of the placentas used in the measurement of placental DNA and protein content are included in the analysis (i.e., seven placentas from each animal are used to calculate mean placental wet weight from that animal), then the weight in the untreated diabetic group ( $474 \pm 19$  mg) was significantly greater ( $P < 0.05$ ) than the control group ( $416 \pm 13$  mg) and the insulin-treated diabetic group ( $416 \pm 14$  mg).

The dry weight of the placentas in the  $D_0$  group was significantly greater than the C group, whereas in the  $D_1$  group placental dry weight was almost the same as the C group (Table 1). There was no significant difference between the groups in their percentage water content (Table 1).

Placental protein and DNA content, and the protein/DNA ratio are shown in Table 2. There were no significant differences between the three groups.

## 4. Discussion

In this series of experiments, the placental contents of water, protein and DNA were measured and compared in three groups of rats: a control group, an untreated diabetic group and an insulin-treated diabetic group. Quantification of DNA content was used as a measure of tissue cellularity [18] but it must be recognised that the rat placenta is a complex organ that is partially syncytial in nature. Therefore, measurement of placental DNA gives a measure of nuclear number, rather than cell number, and might bear little relationship to functional capacity. In addition, calculation of the protein/DNA ratio simply gives a measure of the amount of total protein per unit nuclear weight rather than estimating 'cellular' size. Furthermore,

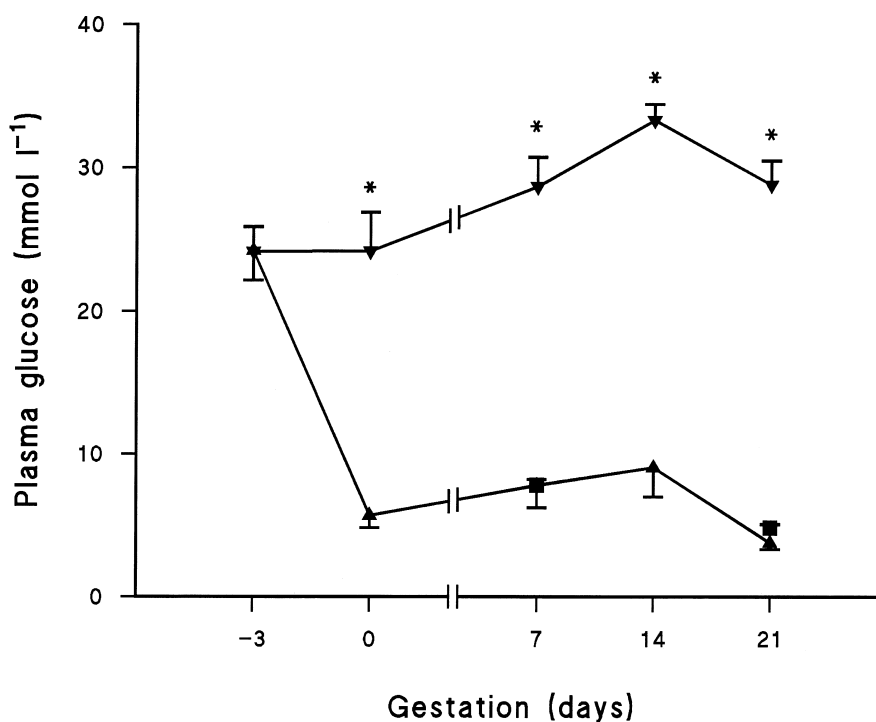


Fig. 1. Maternal plasma glucose concentration in control (C (■)  $n = 15$ ; measured only on days 7 and 21), untreated diabetic (D<sub>0</sub> (▼)  $n = 16$ ), and insulin-treated diabetic (D<sub>1</sub> (▲)  $n = 9$ ) rats before and during gestation. Rats were injected with streptozotocin or vehicle 5 days before being allowed to mate (day 0). The break represents the period during which mating occurred; the day on which a vaginal plug was found was designated day 1 of gestation. Injections of insulin in the D<sub>1</sub> group and sterile water in the C and D<sub>0</sub> groups were commenced 3 days before mating. Data are means  $\pm$  S.E.M. \* $P < 0.001$  versus D<sub>1</sub> by unpaired Student's  $t$ -test (two-tailed) on days 0 and 14, and  $P < 0.001$  versus C and D<sub>1</sub> by ANOVA and Tukey's post tests on days 7 and 21.

measurement of placental total protein content does not differentiate between structural proteins and those involved in placental metabolism or transport functions. Finally, the contribution of fetal blood to placental protein content was not differentiated in these studies — in the human placenta, fetal blood in the placenta

Table 1  
Placental weight (mg) and water content (%)<sup>a</sup>

	C	D <sub>1</sub>	D <sub>0</sub>
Wet weight	412 $\pm$ 21	420 $\pm$ 15	469 $\pm$ 20
Dry weight	66 $\pm$ 3	67 $\pm$ 3	76 $\pm$ 3*
Water	83.9 $\pm$ 0.1	84.0 $\pm$ 0.1	83.8 $\pm$ 0.2

<sup>a</sup> Data are means  $\pm$  S.E.M. in control (C,  $n = 15$ ), insulin-treated diabetic (D<sub>1</sub>,  $n = 9$ ) and untreated diabetic (D<sub>0</sub>,  $n = 15$ ) rats.

Statistical significance between groups by ANOVA and Tukey's post tests, \* $P < 0.05$  versus C.

Table 2  
Placental protein and DNA content (mg) and ratio<sup>a</sup>

	C	D <sub>I</sub>	D <sub>O</sub>
Protein	50.4±2.4	51.9±3.3	54.9±2.6
DNA	1.8±0.2	1.5±0.1	1.7±0.1
Protein/DNA	31.0±2.9	34.0±2.4	34.3±2.1

<sup>a</sup> Data are means±S.E.M. in control (C, *n* = 15), insulin-treated diabetic (D<sub>I</sub>, *n* = 9) and untreated diabetic (D<sub>O</sub>, *n* = 16) rats.

and umbilical cord may account for almost 30% of placental weight [19]. Nevertheless, a comparison of the placental content of DNA and protein in the three groups of rats might reveal gross changes in structure and cellularity.

The percentage water content of the placentas in all three groups was 84% (Table 1), which is in agreement with other studies [20–22]. If only the placentas in which wet and dry weights were measured are considered, then there was no significant difference between the three groups with respect to wet weight (Table 1). However, if the wet weights of the placentas that were used for DNA and protein measurement are also included in the analysis, then it was found that the wet weight in the D<sub>O</sub> group was significantly higher than the other two groups. Although this appears to be a consistent finding in diabetic rat pregnancy [20,23,24], there is disagreement in the literature about the presence of placentomegaly in diabetic human pregnancies which might reflect differences in the control, duration and severity of the maternal diabetes. It is generally believed that placentomegaly in human diabetic pregnancy might support the increased demand of substrates made by the macrosomic fetus. However, this situation is in contrast to rodent diabetic pregnancy, in which fetuses are generally growth-retarded, and, therefore, the functional significance of placentomegaly in the presence of maternal diabetes in this and other species is unclear [25].

The dry weight of the placentas in the D<sub>O</sub> group of animals was significantly greater when compared to the C group (Table 1). This finding is in agreement with previous reports [20,23]. Although the composition of the dry residue was not analysed in these experiments, it is likely that the increase in weight was a result of not only the increased glycogen content found in placentas of diabetic pregnancies [23,24] but also an increase in intracellular solutes required to maintain osmotic equilibrium in the presence of maternal hyperglycaemia.

There were no significant differences between the three groups with respect to the placental content of DNA and protein, and the protein/DNA ratio (Table 2), which is in agreement with a previous report [23]. By contrast, Winick and Noble [26] reported an increase in placental DNA and protein content in placentas of human diabetic pregnancies in proportion to the increase in placental weight suggesting an increased number of cells of normal size. Similarly, Diamant et al. [27] found an increase in placental DNA content in both human and rat diabetic pregnancy, but when the DNA content was normalised to placental wet weight there was no difference between diabetics and controls in humans, whereas in the diabetic rat DNA content was significantly lower. It is likely, therefore, that there is no difference, in placental DNA

and protein content normalised to placental weight between normal and diabetic pregnancies. It follows that placental cellularity, as measured by these parameters, is unaffected by the presence of maternal diabetes mellitus.

In summary, the placentas of untreated diabetic rats were heavier than those of insulin-treated diabetic and control animals and this increase in weight was as a result of an increase in dry weight. The composition of the dry residue was not examined but a major contribution from increased placental glycogen is likely. Placental content of DNA and protein did not differ significantly between the three groups, suggesting unaltered placental cellularity in the presence of maternal diabetes mellitus.

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