Antioxidant status and lipid peroxidation in diabetic pregnancy

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Pregnancy in insulin-dependent diabetes mellitus is associated with a greater incidence of fetal abnormality. Animal studies suggest that increased free-radical production and antioxidant depletion may contribute to this risk. The aim of the present study was, therefore, to assess nutritional antioxidant status and lipid peroxidation in diabetic mothers in comparison with a control group. A 7 d dietary history and a food-frequency questionnaire were performed and venous blood collected for biochemical analyses from thirty-eight diabetic mothers and matched control subjects before 12 weeks gestation. Protein intake was significantly greater in diabetic patients (81·4 (SE 14·8) *v.* 72·7 (SE 15·8) g/d, $P = 0{\cdot}015$), while total sugar intake was less (79·5 (SE 13·2) *v.* 104·8 (SE 28·8) g/d, $P < 0{\cdot}001$). There were no significant differences in the intake of the major antioxidant vitamins (retinol, vitamin C or vitamin E) or β-carotene. However, intakes of a number of other micronutrients (including Se, Zn, Mg, Mn, riboflavin, thiamin, niacin and folate) were greater in diabetic patients. Among the nutritional chain-breaking antioxidants, serum levels of α-tocopherol (21·6 (SE 5·7) *v.* 17·3 (SE 4·7) μmol/l, $P = 0{\cdot}0013$), β-carotene (0·27 (SE 0·18) *v.* 0·14 (SE 0·11) μmol/l, $P = 0{\cdot}003$) and lycopene (0·23 (SE 0·17) *v.* 0·16 (SE 0·13) μmol/l, $P = 0{\cdot}03$) were greater in diabetic patients. There was no evidence of greater lipid peroxidation in diabetic patients, and total antioxidant capacity was similar in the two groups. Overall, these results indicate that nutritional antioxidant status is better in this group of diabetic mothers than in control pregnant non-diabetic subjects attending the same maternity hospital.

Pregnancy: Diabetes mellitus: Antioxidants

Pregnancy outcome for mothers with insulin-dependent diabetes mellitus (IDDM) has greatly improved during the last 40 years, with the perinatal mortality rate falling to approximately forty per thousand compared with 330 per thousand in the 1940s (Hadden, 1990). This improvement is substantially better than that observed in non-diabetic mothers over the same period, in whom perinatal mortality has fallen from approximately 100 per thousand to twenty per thousand. However, there remains a greater incidence of major fetal congenital abnormalities in diabetic pregnancies in comparison with non-diabetic pregnancies (Hadden, 1991). The factors which cause greater fetal morbidity and mortality in diabetic pregnancy are not fully understood, although maternal hyperglycaemia, particularly in the crucial early weeks of fetal development, is partly responsible. However, this cannot be the only significant factor, as there is still a greater risk of fetal abnormality in the presence of near-optimal blood glucose control. Among the other factors which may be important, there has recently been considerable interest in the idea that greater production of O free-radicals in the fetus of the diabetic mother may contribute to the risk of fetal abnormality (Eriksson & Borg, 1991*a*).

It has been suggested over the last few years that oxidative stress in diabetes may be partly responsible for the development of diabetic complications (Baynes, 1991). Oxidative stress occurs when there is an imbalance between free-radical production and the radical-scavenging capacity of antioxidant systems. Free radicals are continually produced in the body as a result of normal metabolic processes and interactions with environmental stimuli. A range of antioxidant defences are present in cells and body fluids which protect cellular components from free-radical-induced damage. There is evidence that both free-radical production and antioxidant defences are disturbed in diabetes (Lyons, 1991).

Potentially important sources of free-radical production in diabetes include autoxidation of glucose and glycated proteins (Wolff *et al.* 1991). There is also considerable evidence that antioxidant defence systems are depleted; activity of antioxidant enzymes is reduced (Tho *et al.* 1988) and glutathione metabolism is disordered (McLennan *et al.* 1991). Among the chain-breaking antioxidants, ascorbate levels are lower and levels of the oxidation product dehydroascorbate may be greater (Sinclair *et al.* 1991; Young *et al.* 1992). The imbalance between free-radical production and antioxidant defences in diabetes is reflected by greater levels of biochemical markers of free-radical activity, including malondialdehyde, conjugated dienes and fluorescent protein products. Diabetic subjects with microangiopathy have the highest levels of such markers (Jennings & Barnett, 1988). More recently it has become clear that the processes of glycation and free-radical production are intimately linked by the propensity of glucose to undergo 'autoxidation' (Wolff *et al.* 1991). This process is particularly likely to occur in the presence of Fe and Cu.

As indicated earlier, there is considerable evidence that free-radical production may be linked to the development of diabetic complications. Congenital malformations in diabetic pregnancy may be regarded in this context as a complication of maternal disease. Exposure of the developing rat embryo to free-radical generating systems *in vitro* will cause fetal abnormalities (Jenkinson *et al.* 1986), and an increased O tension is also associated with abnormal neural fold and crest development (Morris & New, 1979). Furthermore, when rat embryos are cultured *in vitro* in the presence of high glucose concentrations, protection against abnormal embryonic development is conferred by the radical-scavenging enzymes superoxide dismutase (*EC* 1.15.1.1), catalase (*EC* 1.11.1.6) and glutathione peroxidase (*EC* 1.11.1.9) (Eriksson & Borg, 1991*a*). The embryonic mitochondria appear to be an important site of free-radical generation in this model (Eriksson & Borg, 1991*b*). Several chain-breaking antioxidants, including Trolox®, N-acetylcysteine, butylated hydroxytoluene and vitamin E have also been reported to prevent fetal abnormalities in this experimental system (Eriksson, 1994; Eriksson & Siman, 1995; Siman *et al.* 1995).

Little information is available with regard to the early human development of antioxidant defences, but the fetus is likely to be particularly sensitive to free-radical damage early in development when the major organ systems are developing. It is likely that antioxidant status is especially important during this period. The aim of the present study was, therefore, to assess nutritional antioxidant status in pregnant diabetic women in comparison with control subjects.

PATIENTS AND METHODS

Thirty-eight successive pregnant women with IDDM presenting before 12 weeks gestation were recruited at the booking visit at the Royal Maternity Hospital, Belfast. Control subjects matched for age, smoking status, duration of pregnancy and social class (by occupation of husband or partner, as classified in the Northern Ireland Census, Department

of Health and Social Services, 1991) were recruited at the same season of the year as matched diabetic mothers. Smoking status was defined on the basis of current smoking at the estimated time of conception. Patients or control subjects with a history of fetal congenital abnormality were excluded from the study. At the booking visit the study was explained to the patient, venous blood was collected for biochemical analyses, and anthropometric measurements were taken before the patient was seen by the study dietitian. Patient weight was measured using calibrated scales and height using a wall-mounted scale. BMR estimates were determined as suggested by Prentice *et al.* (1996) for the first trimester of pregnancy, by adding an increment of 0·2 MJ to the non-pregnancy BMR predicted from the Schofield equation (Schofield, 1985) using the weight and height measured at booking. All patients and controls gave informed consent and the study was carried out according to the guidelines of the ethics committee of the Queen's University of Belfast.

Dietary assessment

Patients were seen by the dietitian at their first hospital attendance and received instruction on completing a previously validated food-frequency questionnaire (FFQ) (Bolton-Smith *et al.* 1991). This was checked by the dietitian within 2 weeks of booking, at which stage a 7 d dietary history was performed to determine all food and drink consumed in a typical week in early pregnancy (including weekends and food consumed outside the home). The interview lasted between 45 and 60 min. Food portion sizes were recorded in terms of everyday household measures, and a validated food photograph album was used to assist in the estimation of portion sizes (Livingstone *et al.* 1990). The FFQ served as a check-list to compare with foods recorded in the dietary history and was used to record information on smoking status, alcohol consumption, presence or absence of nausea and/or vomiting and use of supplements. There were no significant differences in these variables between the two groups.

Dietary analysis

Foods were coded from *McCance and Widdowson's The Composition of Foods* (Holland *et al.* 1991) and appropriate weights were calculated with the aid of the food photograph album and the book *Food Portion Sizes* (Ministry of Agriculture, Fisheries and Food, 1993). Dietary analysis was then performed using the Microdiet computer software package (version 8, Department of Mathematics and Computer Science, University of Salford, Lancashire). Vitamin supplements were disregarded for the purposes of dietary calculations. The dietary intakes of diabetic and control women were compared with the recommended dietary intakes for pregnant women (Department of Health, 1991).

Biochemical analyses

Venous blood was collected for biochemical analysis and separated within 1 h of collection. Serum and plasma were stored at $-70°$ before analysis. Malondialdehyde, a marker of lipid peroxidation, was measured by HPLC as described by Young & Trimble (1991). Retinol, α-tocopherol, β-carotene and lycopene were measured by HPLC as described by Ito *et al.* (1990), with the assay standardized against the National Institute of Standards and Technology Standard Reference Material 968b. Ascorbate was measured by HPLC as described by Speek *et al.* (1984). Protein thiols were measured by

spectrophotometric assay (Ellman, 1959). Cholesterol was measured using standard enzymic techniques on a Kodak Ektachem analyser (Kodak Clinical Diagnostics, Amersham, Bucks.), and haemoglobin A1c (HbA1c) was measured by immunoassay (Novo Clone HbA1c, Novo Nordisk Pharmaceuticals Ltd, Crawley, Sussex) in the NHS Biochemistry and Haematology Laboratories of the Royal Group of Hospitals, Belfast. The reference range for HbA1c (2·3–4·9 %) was established using non-diabetic healthy adult subjects of both sexes and was the range quoted by the NHS laboratory.

Total antioxidant capacity was measured using the enhanced chemiluminescence assay described by Whitehead *et al.* (1991). In this method light emission occurs when the chemiluminescent substrate luminol is oxidized in a reaction catalysed by horseradish peroxidase in the presence of the enhancer *p*-iodophenol. The steady emission of light is absolutely dependent on constant production of free-radical intermediates derived from *p*-iodophenol, luminol and O. Light emission is therefore sensitive to interference by chain-breaking antioxidants but is restored when all such antioxidants have been consumed in the reaction. The time period of light supression (calculated as the time after the addition of sample or calibrator until the return of 10 % of the initial signal) is directly related to the amount of antioxidant present. The assay was calibrated against calibrators of the water soluble vitamin-E analogue Trolox®. Serum samples were stored for up to 2 months at $-70°$ before assay. Preliminary experiments showed < 5 % deterioration in antioxidant capacity during this time.

The CV (both inter-assay and intra-assay) were < 10 % for HPLC assays and the total antioxidant assay, < 5 % for other assays. All assays, except ascorbate, lycopene, thiols, malondialdehyde and total antioxidant capacity, routinely participated in National External Quality Control Schemes. Where external quality control schemes were not available, internal quality control samples were routinely run in duplicate as part of the normal assay protocol.

Statistical analysis

Data are given as means with their standard errors unless otherwise stated. Patient groups were compared using the unpaired *t* test or the Mann–Whitney U test if data were non-parametrically distributed. Normality of data was tested for individual analyses using the Kolmogorov–Smirnoff test. Correlations were sought using linear regression analysis or Spearman's rank correlation coefficient if data were non-parametrically distributed. Analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 1.1, University CHEST Agreement, UK).

RESULTS

Thirty-eight pregnant diabetic patients and thirty-eight matched control subjects were recruited. Demographic characteristics of patients and controls are given in Table 1. In general, glycaemic control was good in diabetic patients (HbA1c $6·6 \pm 0·25$ %, reference range 2·3–4·9 %). Dietary intakes of macro- and micronutrients are shown in Table 2. Energy intake was similar in the two groups. Among the macronutrients, protein intake was significantly greater in diabetic patients (81·4 (SE 14·8) *v.* 72·7 (SE 15·8) g/d, $P = 0·015$), while total sugar intake was less (79·5 (SE 13·2) *v.* 104·8 (SE 28·8) g/d, $P < 0·001$). There were no significant differences in the intake of the major antioxidant vitamins (retinol, vitamin C or vitamin E) or β-carotene. However, intakes of a number of other

Table 1. *Characteristics of diabetic and control subjects*

(Mean values and standard errors for thirty-eight subjects per group)

	Diabetics		Controls	
	Mean	SE	Mean	SE
Age (years)	31	5·2	30	1·4
No. of smokers (%)	11 (29)		13 (34)	
No. of husbands in manual work (%)	19 (50)		16 (42)	
BMI (kg/m^2)	25·3	0·59	24·9	0·90
EI : BMR*	1·25		1·25	
HbA1c (%)	6·6	0·25	–	

EI, energy intake; HbA1c, haemoglobin A1c.
* BMR for first trimester, as suggested by Prentice *et al.* (1996).

Table 2. *Comparison of results of daily nutrient intakes of pregnant women with diabetes and pregnant controls*

	Diabetics			Controls			
	Mean intake	SEM	Intake (% RNI)	Mean intake	SEM	Intake (% RNI)	Statistical significance of difference (*P*)
Energy (kcal)	1823	41	94	1807	64	93	0·832
Energy (kJ)	7664	174	94	7596	267	93	0·832
Protein (g)	81·4	2·4	160	72·7	2·5	145	0·015*
Carbohydrate (g)	214·5	4·3	98	220·1	7·0	98	0·505
Total sugar (g)	79·5	3·7		104·8	4·8		0·001*
Fat (g)	76·9	2·6	112	76·1	3·7	112	0·869
Total saturated fat (g)	32·3	1·5	160	31·9	1·9	160	0·861
Total mono-unsaturated fat (g)	25·7	0·9	100	24·8	1·2	100	0·536
Total polyunsaturated fat (g)	11·3	0·6	83	12·3	0·8	67	0·305
Cholesterol (mg)	272·8	13·3		241·4	14·1		0·110
Alcohol (g)	0·18	0·90		0·88	0·33		0·048*
Fibre (g)	15·9	0·5	88	12·3	0·4	69	0·001*
Vitamin A (μg)	839	49	120	796	54	114	0·340
β-Carotene (μg)	1929	99		1976	112		0·755
Vitamin C (mg)	84·8	4·9	170	82·6	5·0	165	0·763
Vitamin E (mg)	5·2	0·2		4·9	0·2		0·402
Vitamin E: PUFA	0·49	0·02	123	0·44	0·10	110	0·165
Riboflavin (mg)	2·04	0·11	145	1·73	0·08	124	0·021*
Thiamin (mg)	1·68	0·04	210	1·39	0·05	174	0·001*
Niacin (mg)	17·8	0·4	137	14·7	0·5	114	0·001*
Folate (μg)†	255	7	85	217	7	73	0·001*
Vitamin B_{12} (μg)	4·77	0·26	318	4·22	0·21	281	0·109
Iron (mg)	11·68	0·33	79	9·72	0·33	66	0·001*
Selenium (μg)	55·7	2·0	93	44·3	1·7	74	0·001*
Zinc (mg)	10·5	0·3	150	8·7	0·3	125	0·001*
Copper (mg)	1·04	0·02	87	0·99	0·04	83	0·243
Manganese (mg)	2·54	0·11		1·80	0·08		0·001*
Calcium (mg)	1078	65	154	1018	48	145	0·462
Magnesium (mg)	273	8	101	232	7	86	0·001*

RNI, reference nutrient intake (Department of Health, 1991); PUFA, polyunsaturated fatty acids.
* $P < 0·05$.
† Folic acid supplementation of 13 % of diabetics and 26 % of controls not included.

Table 3. *Serum malondialdehyde and antioxidant concentrations (μmol/l) in pregnant women with diabetes and pregnant controls*

(Mean values with their standard errors for thirty-eight women per group)

	Diabetics		Controls		Statistical significance of difference (*P*)
	Mean	SE	Mean	SE	
MDA	0·78	0·07	0·77	0·06	0·51
TAC	395	27	414	24	0·56
Vitamin C	42·9	4·2	40·3	0·2	0·36
Thiols	398	10	406	9	0·51
Retinol	1·08	0·07	0·99	0·05	0·28
Vitamin E	21·5	0·9	17·3	0·7	0·0013
α-Tocopherol (lipid corrected)*	4·72	0·12	4·06	0·10	0·0084
α-Carotene	0·021	0·010	0·013	0·010	0·12
β-Carotene	0·27	0·18	0·14	0·11	0·003
Lycopene	0·23	0·17	0·16	0·13	0·03

MDA, malondialdehyde; TAC, total antioxidant capacity.
*Expressed as μmol/mmol cholesterol.

micronutrients (including Se, Zn, Mg, Mn, riboflavin, thiamin, niacin and folate) were greater in diabetic patients.

Serum levels of malondialdehyde (a marker of lipid peroxidation), total antioxidant capacity and the major chain-breaking antioxidants are shown in Table 3. There was no evidence of greater lipid peroxidation in diabetic patients, and total antioxidant capacity was similar in the two groups. Among the nutritional chain-breaking antioxidants, levels of α-tocopherol (21·6 (SE 5·7) *v.* 17·3 (SE 4·7) μmol/l, $P = 0{\cdot}0013$), β-carotene (0·27 (SE 0·18) *v.* 0·14 (SE 0·11) μmol/l, $P = 0{\cdot}003$) and lycopene (0·23 (SE 0·17) *v.* 0·16 (SE 0·13) μmol/l, $P = 0{\cdot}03$) were greater in diabetic patients. Further analysis revealed no difference in malondialdehyde or chain-breaking antioxidants when patients and controls were analysed separately as smokers or non-smokers.

In keeping with previous studies, there was a poor correlation between serum levels of antioxidant vitamins and calculated intake, with the exception of vitamin C in the case of control subjects (*r* 0·55). However, no correlation between serum vitamin C and vitamin C intake was found in diabetic patients (*r* 0·10), supporting the previous work of Cunningham *et al.* (1991) who failed to find a correlation between diet and mononuclear leucocyte ascorbate in diabetic subjects.

Following birth, only one pregnancy (diabetic) resulted in significant fetal abnormality (multiple cardiac abnormalities and sacral abnormalities). Serum vitamin C was below threshold or cut-off for adequacy (> 32·0 μmol/l) in this patient (21·0 μmol/l), but all other results were unremarkable.

DISCUSSION

The hypothesis underpinning this study was that impaired antioxidant status in diabetic pregnancy might contribute to an increased risk of fetal abnormality. As outlined earlier, substantial evidence from animal models suggests that increased oxidative stress in the developing embryo is an important cause of abnormality (Eriksson & Borg, 1991*b*), and that this can be prevented by chain-breaking antioxidants, including tocopherol. However,

we have found no evidence of increased oxidative stress or antioxidant depletion in diabetic mothers in early pregnancy; indeed, levels of several important chain-breaking antioxidants (including α-tocopherol, β-carotene and lycopene) were actually found to be higher than in non-diabetic subjects. These results stand in contrast to the majority of previous studies which have reported lower levels of chain-breaking antioxidants, particularly ascorbate, and greater lipid peroxidation in non-pregnant diabetic subjects. However, there are several possible explanations for these findings.

In previous studies of antioxidant status in diabetes, abnormalities have usually been reported in the presence of diabetic complications or poor metabolic control. Indeed, in several studies antioxidant status and markers of lipid peroxidation have been normal in well-controlled diabetic subjects with no evidence of micro- or macroangiopathic complications (Jennings & Barnett, 1988; McLennan *et al.* 1991). Noberasco *et al.* (1991) reported a positive correlation between lipid peroxidation and markers of glycaemic control, with no increase in peroxidation in subjects with near normal HbA1c levels. Faure *et al.* (1993) reported that several antioxidants which were abnormal in poorly controlled diabetic subjects returned to normal following an improvement in diabetic control, and Armstrong *et al.* (1996) reported an improvement in lipid peroxidation and antioxidant status following 3 months of dietary treatment in newly diagnosed non-insulin-dependent diabetes. All of the diabetic mothers in the present study were attending a hospital diabetic clinic before their pregnancy and received detailed dietary advice. At the maternity hospital attended by the mothers, the majority of pregnancies are carefully planned in respect of diabetic control, as is becoming normal practice in diabetic pregnancy (Steele, 1996). In the case of planned pregnancies, mothers are encouraged to pay particular attention to their diet and diabetic control before conception, and this is reflected in the relatively low HbA1c values and greater dietary intake of a range of micronutrients. This is likely to be the main explanation for the absence of increased lipid peroxidation and good antioxidant levels in these subjects.

In general, the dietary intakes of the main antioxidant nutrients in both diabetic mothers and controls were found to be adequate when compared with dietary reference values for pregnancy (Department of Health, 1991). Dietary intakes of vitamin C (85 and 83 mg/d for diabetic and control groups respectively) were somewhat higher than those recorded by Gregory *et al.* (1990), who found a mean intake of 66 mg/d for non-pregnant women aged 25–34 years. β-Carotene intakes were similar to those recorded by Gregory *et al.* (1990). The vitamin E : polyunsaturated fatty acid (PUFA) ratio was found to be greater in both groups than the 0·4 mg α-tocopherol equivalent/g dietary PUFA recommended in the United States, although no such recommendation currently exists in the UK. The intakes of other antioxidant micronutrients were found to be adequate in comparison with the dietary reference values with the exception of Se, where intake was 93 % (diabetic patients) and 74 % (controls) of recommended values. Anderson (1993) examined the impact of pregnancy on diet in a group of 100 Scottish women in early pregnancy and found that pregnant women ate differently in comparison with non-pregnant women, with higher intakes of Na, Zn and vitamin C seen during pregnancy.

The lack of correlation between dietary intake and serum antioxidant levels highlights the problems of assessing dietary intake accurately (Bingham, 1987). Errors in dietary assessment may arise from the assessment methodologies chosen, food tables used, food coding, seasonal variation and estimation of food consumed. The dietary history method, first developed by Burke (1947), attempts to estimate the usual food intakes of individuals over a relatively long period of time, and generally yields good precision when used for a group. Underestimation of food portion size is common and overall energy intake may be

underestimated by as much as 21 % (Acheson *et al.* 1980). In an effort to overcome these problems, we used a food photograph album to assist in the estimation of food portion size (Robson *et al.* 1994). Few studies have measured the validity of the dietary history method in comparison with actual food intake. Livingstone *et al.* (1992) found that mean daily energy intake assessed by the diet history method was in closer agreement with energy expenditure (independently validated using doubly-labelled water) than energy intake calculated from weighed dietary records. In the present study we considered that validation using a 7 d weighed or estimated food record would place too many demands on the subjects and might result in altered food intake. Daily, weekly and seasonal variations in food intake also need to be considered. Due to between- and within-subject variation, accurate quantification of antioxidant nutrient intake would require that the weighing process be performed continually for 5–6 weeks (Nelson *et al.* 1989) which was not possible in the present study.

The energy intake : BMR ratio was estimated to determine whether or not energy intake (and, by inference, food intake) was under-reported. Bias may arise as subjects know they are being interviewed by a dietitian and obese subjects are more likely to underestimate energy intake (Prentice *et al.* 1986). It has been estimated that a value of 1·4 or greater should be seen in a study of less than 100 individuals (Black *et al.* 1991). Our results (a value of 1·25 for both diabetic and control groups) suggest bias towards under-reporting of habitual energy intake, although the nausea and vomiting which some women experienced may have contributed towards this finding. Prentice *et al.* (1996) highlighted the unavailability of predictive equations for BMR during the different stages of pregnancy, and suggested a formula for the estimation of BMR during the first trimester, and we have made use of this calculation.

In conclusion, we have found no evidence of lower antioxidant status in early diabetic pregnancy. Indeed, diabetic mothers had a greater intake of a range of antioxidant micronutrients in comparison with control subjects, probably as a result of paying particular attention to their diet before conception and during early pregnancy. It remains possible that pregnancy in diabetic (and other) mothers with lower antioxidant status is associated with an increased risk of fetal abnormality. As diabetic pregnancies are not always planned, appropriate dietary advice for diabetic women of child-bearing age should continue to be an important goal for all those involved in their management.

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