γ -Linoleic acid and ascorbic acid ameliorate the effects of experimental diabetes on electrolyte and bone homeostasis in pregnant rats

R Braddock, C M Simán¹, K Hamilton¹, H Devlin², H Garland and C P Sibley¹

School of Biological Sciences, University of Manchester, G.38 Stopford Building, Oxford Road, Manchester M13 9PT, UK

¹Academic Unit of Child Health, University of Manchester, St Mary's Hospital, Hathersage Road, Manchester M13 0JH, UK

²Department of Dental Medicine and Surgery, University Dental Hospital of Manchester, Higher Cambridge Street, Manchester M15 6FH, UK

(H Garland is now deceased)

(Requests for offprints should be addressed to C P Sibley; Email: colin.sibley@man.ac.uk)

Abstract

Experimental diabetes in rats is associated with excessive electrolyte loss in the urine, which is further accentuated by pregnancy, particularly of Ca. Supplementation with essential fatty acids and antioxidants has proven beneficial in treating several types of complications, including nephropathy. The present study investigated the effect of γ -linoleic acid (GLA; 500 mg/kg per day; group DG) and ascorbate (290 mg/kg per day; group DA), alone and in combination (group DGA), as well as ascorbyl-GLA (790 mg/kg per day; group DASG), on urinary electrolyte output and skeletal composition in pregnant streptozotocin-diabetic rats. Urine was collected in metabolism cages before and throughout pregnancy. Diabetic rats (DP) increased their urine volume as compared with control (CP) throughout the experiment, reaching an output of more than 13 times that of the control group by the end of pregnancy (CP 24 ± 4 , DP 316 ± 21 , DG

223 ± 21 , DA 221 ± 14 , DASG 163 ± 17 , DGA 220 ± 19 ml urine/24 h). Concomitant with increased urine volume was a reduction of urinary Na (CP 47 ± 14 , DP 22 ± 5 mmol/l), K (CP 210 ± 34 , DP 31 ± 1 mmol/l) and Mg (CP 14 ± 1 , DP 3.8 ± 0.2 mmol/l) concentration, but not of Ca concentration (CP 5.4 ± 1.5 , DP 6.3 ± 0.6 mmol/l), and hence total Ca loss was relatively most severe. All the treatments reduced urine volume with no effects on electrolyte concentration as compared with DP, with no significant difference between the treatments. A reduced bone size and bone Ca content was partially ameliorated by the diet supplementation. We have concluded that GLA and ascorbate, alone or in combination, prevent urinary electrolyte loss in pregnant rats and do so by reducing urine production.

Journal of Endocrinology (2002) **173**, 273–284

Introduction

Patients with diabetes mellitus are susceptible to a variety of secondary complications, such as nephropathy, retinopathy and neuropathy. Hypercalciuria is also common during the early stage of diabetes and contributes to the alterations in calcium homeostasis in the disease (Raskin *et al.* 1978, Wood *et al.* 1984, Harangi *et al.* 1988, Anawa & Garland 1990). The hypercalciuria is further escalated in pregnancy complicated by diabetes (Birdsey *et al.* 1995). It is likely that the cause of hypercalciuria in diabetes involves abnormal function of the thick ascending limb of the loop of Henle, although the detailed mechanism is not known (Guruprakash *et al.* 1988, Garland *et al.* 1991). Renal dysfunction in diabetes also appears to be related to bone diseases (Hercz *et al.* 1993, Pei *et al.* 1993, Sherrard

et al. 1993), characterised by low bone turnover (Aubia *et al.* 1988) and reduced levels of parathyroid hormone (PTH) (Felsenfeld *et al.* 1991) as well as reduced bone mineral content (BMC) (Levin *et al.* 1976, McNair *et al.* 1978, Hough 1994). The loss of BMC is aggravated in poorly controlled diabetes by renal calcium leak (Gregorio *et al.* 1994). Osteoporosis is present in experimental diabetes of rats (Verhaeghe *et al.* 1990, Jara *et al.* 1995) but the involvement of PTH is controversial, with some (Hough *et al.* 1981, 1982, Shires *et al.* 1981) but not all (Glajchen *et al.* 1988, Locatto *et al.* 1990, Jara *et al.* 1995, Hamilton *et al.* 2000) studies reporting reduced circulating PTH concentrations. Bone mineralisation is also regulated by prostaglandins (Conaway *et al.* 1986) which potentially could be a pathway for intervention in diabetes, and a target for the development of treatments.

Table 1 Experimental groups and their respective diet supplementation

	Group	No.	Diet
Control pregnant	CP	7	CRM, deionized water
Diabetic pregnant	DP	7	CRM, deionized water
Diabetic pregnant with GLA	DG	8	CRM with GLA (500 mg/kg per day), deionized water
Diabetic pregnant with ascorbate	DA	8	CRM, deionized water with ascorbate (290 mg/kg per day)
Diabetic pregnant with ascorbyl-GLA	DASG	9	CRM, deionized water with ascorbyl-GLA (790 mg/kg per day) (corresponding to 500 mg GLA and 290 mg ascorbate)
Diabetic pregnant with GLA and ascorbate	DGA	8	CRM with GLA (500 mg/kg per day), deionized water with ascorbate (290 mg/kg per day)

Essential fatty acids (EFA) have been found to have protective properties in experimental diabetes against nephropathy and neuropathy (Barcelli *et al.* 1990, Tulloch *et al.* 1994, Cameron & Cotter 1996, Garland *et al.* 1997, Cameron *et al.* 1998, Hounsom *et al.* 1998). The mechanisms for the protective effects have not been fully elucidated, but EFAs are known to be regulators of protein kinase functions (Blobe *et al.* 1995), being constituents of diacylglycerols released from phospho-inositol (Nunez 1993), and being precursors of prostaglandins, leukotrienes, thromboxanes and hydroxy fatty acids (Nunez 1993). Diabetes causes a reduced activity of the enzyme Δ -6-desaturase which converts linoleic acid to γ -linoleic acid (GLA) (Horrobin & Carmichael 1992). GLA is then further metabolised to arachidonic acid. Additional intake of GLA would circumvent the metabolic block caused by diabetes.

Antioxidants have proved to be effective in preventing complications of diabetes (Kelly 1998). Ascorbate, the primary antioxidant to be consumed in plasma exposed to oxidative stress (Frei *et al.* 1989, 1990), is reduced in tissue and plasma of patients with diabetes (Som *et al.* 1981, Yew 1983, McLennan *et al.* 1988). As arachidonic acid is a target for free oxygen radicals, ascorbate may prevent its degeneration in diabetes.

This study was designed to examine whether GLA and ascorbate can ameliorate electrolyte loss in urine and bone composition of diabetic rats during pregnancy. The compounds were administered in combination and as ascorbyl-GLA in order to test the hypothesis that there are synergistic benefits of providing the two substances together.

Materials and Methods

From the age of 3 weeks, female Sprague–Dawley rats (Charles River Laboratories, Wilmington, Kent, UK) were maintained under a constant 12-h photoperiod at a temperature of 20–22 °C in standard and metabolism

cages. The rats had free access to food (CRM; Special Diet Services, Whitham, Essex, UK) and deionized water, with or without supplements of GLA and/or ascorbate and ascorbyl-GLA throughout the experiment. Experimental groups and corresponding diets are outlined in Table 1. GLA-enriched food was prepared by dropping GLA (Scotia Pharmaceuticals Ltd, Guildford, Surrey, UK) onto food pellets. Ascorbate (Sigma, Poole, Dorset, UK) and ascorbyl-GLA (Scotia Pharmaceuticals Ltd) were dissolved in deionized water. All doses were corrected to the food and water consumption of the previous day. The doses were chosen based on previously reported effectiveness on neurovascular deficits in diabetic rats (Cameron & Cotter 1996b).

At 8 weeks of age, rats were made diabetic with a single intraperitoneal injection of streptozotocin (STZ; Sigma, Poole, Dorset, UK 60 mg/kg) dissolved in citric acid buffer (pH 4.8). Control animals were injected with buffer only. Only animals demonstrating polydipsia, polyuria and glycosuria (>5.5 mmol/l) within 36 h of injection were included in the study. Blood glucose was measured with the glucose oxidase–peroxidase method (Ames Sera-Pak Reagent Kit, Ames DVN; Miles Ltd, Slough, Bucks, UK) at the termination of the experiment in all pregnant rats.

At 9 weeks of age, the female rats were mated with control males of the same strain. The morning on which a sperm plug was identified was designated day 0 of

Figure 1 Experimental design.

Table 2 Concentration of glucose and electrolytes in terminal plasma samples. Data are presented as means $\pm$ S.E.M.

	CP (n=7)	DP (n=7)	DG (n=8)	DA (n=8)	DASG (n=9)	DGA (n=8)
Concentrations						
Glucose (mmol/l)	6.4 $\pm$ 0.6***	40.1 $\pm$ 1.4	35.4 $\pm$ 3.9	39.4 $\pm$ 2.3	39.1 $\pm$ 2.8	44.0 $\pm$ 1.6
Na (mmol/l)	134 $\pm$ 5	123 $\pm$ 3	125 $\pm$ 3	121 $\pm$ 3	124 $\pm$ 3	123 $\pm$ 3
K (mmol/l)	6.1 $\pm$ 0.4	6.7 $\pm$ 0.6	7.5 $\pm$ 0.8	6.7 $\pm$ 1.0	7.7 $\pm$ 0.9	6.9 $\pm$ 0.3
Mg (mmol/l)	0.98 $\pm$ 0.02	0.92 $\pm$ 0.05	0.79 $\pm$ 0.06	0.50 $\pm$ 0.06*	0.83 $\pm$ 0.05	0.093 $\pm$ 0.05
Ca (mmol/l)	2.24 $\pm$ 0.11*	2.41 $\pm$ 0.14	2.45 $\pm$ 0.17	2.49 $\pm$ 0.13	2.79 $\pm$ 0.13*	2.53 $\pm$ 0.13
Ultrafiltrable Ca (mmol/l)	1.55 $\pm$ 0.19	1.38 $\pm$ 0.33	1.79 $\pm$ 0.09*	1.48 $\pm$ 0.30	1.51 $\pm$ 0.26	1.51 $\pm$ 0.23

Group abbreviations are given in Table 1.

Statistical analysis with ANOVA with Dunnett's post-hoc test. Only significant differences versus the DP group are indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2 Weight (a) and food intake (b) of rats before and 5 days after STZ injection, and at 7, 14 and 21 days of pregnancy. Group abbreviations are given in Table 1. Data are presented as means $\pm$ S.E.M., statistical analysis with ANOVA with Dunnett's post-hoc test. Only significant differences versus the DP group are indicated (* $P < 0.05$).

pregnancy. All rats had an acclimatisation period of 14 days in a metabolic cage (Metabowl; Jencons Scientific Ltd, Hemel Hempstead, Herts, UK), starting at 6 weeks of age, before a 24-h collection of urine started. Urine was then

collected 1 day before the STZ injection, 5 days after the injection, and after mating on gestational days 7, 14 and 21. The experiment was interrupted on gestational day 21 and arterial blood, kidneys and femurs were collected

Figure 3 Water intake (a) and urine production (b) over 24 h of rats before and 5 days after STZ injection, and at 7, 14 and 21 days of pregnancy. Data are presented as means $\pm$ S.E.M. Group abbreviations are given in Table 1. Statistical analysis with ANOVA followed by Dunnett's post-hoc test. Only significant differences versus the DP group are indicated (* $P < 0.05$).

for analyses. The experimental procedure is outlined in Fig. 1.

Urinary samples were analysed for sodium and potassium by flame photometry (Corning EE1 model 450; Scientific and Medical Products Ltd, Manchester, UK) and for calcium and magnesium by atomic absorption spectrophotometry (Perkin-Elmer 3100; Beaconsfield, Bucks, UK) as previously described (Simán *et al.* 2000).

Left femurs were ashed at 750 °C for 48 h and the ash was weighed. The ash was dissolved in 6 M HCl and diluted in 0.3% lanthanum or distilled water for Ca and Mg analysis respectively, by atomic absorption spectrophotometry.

Right femurs were studied histologically after longitudinal sectioning and haematoxylin and eosin staining, with the examiner (R.B.) blinded to the identity of the specimen. The length and width of the growth plate

were measured as well as the ratio of microtrophic to hypertrophic cells according to Marchi *et al.* (1991).

All data are presented as means $\pm$ S.E.M. and n equals the number of animals. A one-way analysis of variance (ANOVA) with Dunnett's post-hoc test was used to compare groups at each time-point measured. All comparisons were made against the DP group.

Results

Injection with STZ resulted in diabetes, as verified by glycosuria, polyuria and polydipsia. Diabetes was confirmed in 9/10 of DP, 7/11 of DG, 11/13 of DA, 8/13 of DGA and 10/12 of DGA rats. By the end of pregnancy, plasma glucose concentration was increased in all the diabetic groups and was not further affected by any of the

Figure 4 Concentration (a) and total urinary output (b) of sodium of rats before and 5 days after STZ injection, and at 7, 14 and 21 days of pregnancy after 24-h urine collection. Data are presented as means $\pm$ S.E.M. Group abbreviations are given in Table 1. Statistical analysis with ANOVA followed by Dunnett's post-hoc test. Only significant differences versus the DP group are indicated (* $P < 0.05$).

treatments (Table 2). The body weight of the rats was not significantly different between the groups at any stage of the experiment (Fig. 2a). Food consumption increased significantly in the DP group as compared with the CP group after induction of diabetes. The DASG group had a reduction in food consumption on gestational days 7 and 14 (Fig. 2b).

Water consumption paralleled urine production in all animals (Fig. 3). Urine production did not change over the course of pregnancy in the CP group. In the DP group, urine production was significantly increased as compared with CP rats within 5 days of STZ injection and increased over the course of pregnancy. The DASG group had significantly decreased urine production before STZ injection as compared with non-supplemented rats. After induction of diabetes, the urine output was lower in animals receiving any of the diet supplementation, most

prominently in the DASG group and was significant in all treatment groups at day 21 of pregnancy (Fig. 3).

Total output of Na, K, Mg and Ca was similar in all groups at the baseline measurement (Figs 4–7). Five days after STZ injection and throughout pregnancy, the urinary concentration of Na, K and Mg was reduced in the DP group as compared with the CP group (Figs 4a, 5a and 6a). However, this was not sufficient to normalise urinary ion output (Figs 4b, 5b and 6b).

For Ca, there was a borderline significant ($P = 0.053$, t -test CP vs DP) reduction of urinary concentration 5 days after STZ injection. The urinary Ca concentration broadly increased in all groups during pregnancy. This increase was slightly greater in the diabetic group, being significant as compared with control at day 14 of pregnancy (Fig. 7a). There was a tendency of the diet supplementation to reduce Ca concentration after STZ injection, but this

Figure 5 Concentration (a) and total urinary output (b) of potassium of rats before and 5 days after STZ injection, and at 7, 14 and 21 days of pregnancy after 24 h urine collection. Data are presented as means $\pm$ S.E.M. Group abbreviations are given in Table 1. Statistical analysis with ANOVA followed by Dunnett's post-hoc test. Only significant differences versus the DP group are indicated (* $P < 0.05$).

never reached statistical significance. The urinary Ca output increased 15 and 20 times over CP rats in the DP group at gestational days 14 and 21 respectively. A significant reduction of Ca output, as compared with DP rats, was noted with all treatments for gestational days 7, 14 and 21 (Fig. 7b).

Terminal plasma concentration of Na and K was not affected by diabetes or diet supplementation. The Mg plasma concentration was not affected by diabetes but was decreased significantly in the DA group. The total Ca concentration, but not ultrafiltrable Ca, was increased in the DP group as compared with CP rats. Total plasma Ca concentration in the DASG group and ultrafiltrable Ca in the DG group was increased as compared with the DP group (Table 2).

Femurs of DP rats were slightly shorter than femurs of CP rats but the wet weight was not significantly different

(Table 3). The ash weight was reduced in DP femurs as compared with the CP group. Diet supplementation increased ash weight, which was significant in the DG and DASG groups. Ca and Mg contents per femur were reduced in DP rats as compared with CP rats, but the concentration per ash weight was not affected. Ca content was returned towards normal in the DG and DASG groups. The Ca concentration was reduced in the DA and DGA group (Table 3).

The length and width of the femoral epiphysis was reduced in the DP rats as compared with CP rats. All diet-supplemented groups had normalised length but not width of the epiphysis (Table 4). The number of hypertrophic cells, but not microtrophic cells, was reduced in DP rat femurs. No significant effect of the treatments on numbers of hypertrophic or microtrophic cells were noted (Table 4 and Fig. 8).

Figure 6 Concentration (a) and total urinary output of magnesium (b) of rats before and 5 days after STZ injection, and at 7, 14 and 21 days of pregnancy after 24 h urine collection. Data are presented as means $\pm$ S.E.M. Group abbreviations are given in Table 1. Statistical analysis with ANOVA followed by Dunnett's post-hoc test. Only significant differences versus the DP group are indicated (* $P < 0.05$).

Discussion

This study has demonstrated that treatment of diabetic rats with GLA and/or ascorbate can reduce ion loss via the urine, an effect which appears to be mediated primarily by a reduction of total urine volume.

Excess urinary excretion in diabetes is caused by glucose-induced osmotic retention of fluid in the renal tubule (Atherton *et al.* 1968). Hence, urine volume is dependent on blood glucose concentration, and the findings in this report could potentially be explained by a reduced severity of the diabetic state in the diet-supplemented animals. There was a nominal, statistically non-significant reduction in the number of rats becoming diabetic after STZ injection in the diet-supplemented groups. The terminal plasma glucose concentration, however, was not affected by any of the supplements. Furthermore, we have found that GLA and ascorbate

given to male rats with the same treatment regimen as in this study do not affect glucose tolerance or pancreatic insulin content (Braddock *et al.* 2002), suggesting that the severity of diabetes was not affected by the diet supplementation. Thus, the treatment effect observed in this study is not related to the severity of diabetes in the rats.

In a previous study, we found no effect on any urinary variables in rats which received a GLA-supplemented diet starting on the day of STZ injection (Simán *et al.* 2000) in contrast to the data reported here. It appears therefore that the effects of GLA and ascorbate seen in this study require that the diet supplementation commences before the induction of diabetes with STZ. In other reports demonstrating a Ca-conserving effect of evening primrose oil (which is rich in GLA) in diabetic rats, the animals were treated for several weeks before induction of diabetes (Tulloch *et al.* 1994, Garland *et al.* 1997). It is notable, in

Figure 7 Concentration (a) and total urinary output of calcium (b) of rats before and 5 days after STZ injection, and at 7, 14 and 21 days of pregnancy after 24-h urine collection. Data are presented as means $\pm$ S.E.M. Group abbreviations are given in Table 1. Statistical analysis with ANOVA followed by Dunnett's post-hoc test. Only significant differences versus the DP group are indicated (* $P < 0.05$).

Table 3 Analysis of femurs at gestational day 21. Data are presented as means $\pm$ S.E.M.

	CP (n=7)	DP (n=7)	DG (n=8)	DA (n=8)	DASG (n=9)	DGA (n=8)
Length (mm)	33.7 $\pm$ 0.4*	32.2 $\pm$ 0.3	32.3 $\pm$ 1.1	33.6 $\pm$ 0.2	32.9 $\pm$ 0.3	32.7 $\pm$ 0.2
Weight (g)	0.81 $\pm$ 0.33	0.79 $\pm$ 0.21	0.85 $\pm$ 0.30	0.83 $\pm$ 0.22	0.83 $\pm$ 0.20	0.80 $\pm$ 0.27
Ash weight (mg)	360 $\pm$ 4*	307 $\pm$ 4	364 $\pm$ 2*	344 $\pm$ 1	359 $\pm$ 3*	341 $\pm$ 2
Ca content (mmol)	3.00 $\pm$ 0.03*	2.60 $\pm$ 0.08	3.01 $\pm$ 0.06*	2.80 $\pm$ 0.09	2.98 $\pm$ 0.08*	2.77 $\pm$ 0.11
Ca/ash weight (mmol/g)	8.34 $\pm$ 0.05	8.47 $\pm$ 0.04	8.28 $\pm$ 0.05	8.14 $\pm$ 0.04*	8.31 $\pm$ 0.04	8.11 $\pm$ 0.05*
Mg content (μmol/bone)	96 $\pm$ 4*	78 $\pm$ 4	89 $\pm$ 2	89 $\pm$ 7	86 $\pm$ 3	89 $\pm$ 5
Mg/ash weight (mmol/g)	0.26 $\pm$ 0.01	0.25 $\pm$ 0.01	0.24 $\pm$ 0.01	0.26 $\pm$ 0.02	0.24 $\pm$ 0.00	0.26 $\pm$ 0.01

Group abbreviations are given in Table 1. Statistical analysis with ANOVA with Dunnett's post-hoc test. Only significant differences versus the DP group are indicated. * $P < 0.05$.

this context, that insulin itself only reverses diabetic hypercalciuria in rats if delivered shortly after induction of diabetes (Hoskins & Scott 1983, Anawa & Garland 1990).

Thus, it appears that GLA has a slow onset in its therapeutic effect, while diabetes causes a rapid and permanent damage to the kidney in rats.

Table 4 Femoral histology. Data are presented as means $\pm$ S.E.M.

	CP (n=5)	DP (n=5)	DG (n=5)	DA (n=8)	DASG (n=8)	DGA (n=7)
Length of epiphysis (mm)	5.8 $\pm$ 1.0*	4.1 $\pm$ 0.8	7.3 $\pm$ 0.4*	6.2 $\pm$ 0.5*	7.6 $\pm$ 0.3*	5.4 $\pm$ 0.4*
Width of epiphysis (mm)	0.17 $\pm$ 0.004*	0.13 $\pm$ 0.01	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01	0.14 $\pm$ 0.01	0.13 $\pm$ 0.01
Microtrophic cells	6.8 $\pm$ 0.4	6.4 $\pm$ 0.4	6.6 $\pm$ 0.5	6.8 $\pm$ 0.3	7.76 $\pm$ 0.5	6.2 $\pm$ 0.4
Hypertrophic cells	8.0 $\pm$ 0.5*	6.3 $\pm$ 0.5	6.7 $\pm$ 0.1	6.5 $\pm$ 0.25	6.7 $\pm$ 0.4	6.0 $\pm$ 0.2

Group abbreviations are given in Table 1.

Statistical analysis with ANOVA with Dunnett's post-hoc test. Only significant differences versus the DP group are indicated. * $P < 0.05$.

The protective effects of the different diets used in this study were not significantly different between the treated groups. Hence, no additional or synergistic effect was noted when the two compounds were administered together or as ascorbyl-GLA. This could be explained by both compounds acting on the same mechanism, and maximal response already being achieved with the doses used in this experiment.

The inability of non-pregnant diabetic rats to concentrate Ca has been ascribed to a defect of reabsorption in the thick ascending limb of the loop of Henle and is independent of acute changes in glucose concentration (Garland *et al.* 1991). The current study of pregnant rats was concomitant with this, as diabetes caused little change in urinary Ca concentration while urinary Na, K and Mg concentrations were reduced. This explains the exacerbation of Ca output in diabetic pregnancy as compared with the other electrolytes. It has been proposed that the rate-limiting Ca-transporting protein calbindin- D_{28k} could be involved in the failure to concentrate urinary Ca in diabetes (Hamilton *et al.* 2000). However, calbindin- D_{28k} mRNA expression is already maximal during late pregnancy to compensate for the increased Ca output (Hamilton *et al.* 2000). This view was strengthened in the current study as the renal calbindin- D_{28k} mRNA expression was not affected by diabetes at day 20 of pregnancy, nor was it affected by any of the treatments (Simán CM, unpublished observation). In short, none of the treatments used here affected the concentration of any of the electrolytes measured. This suggests that the amelioration of the diabetic effects by the GLA and ascorbate treatments acted on a mechanism not involving tubular concentration of electrolytes.

The most notable observation in this study was a reduction in urinary volume in diabetic rats supplemented with GLA and ascorbate, alone or in combination. This effect was present before induction of diabetes in some groups. Hence the treatment effect on urinary ion loss can be ascribed to changes in urinary volumes. This is in contrast to the report by Tulloch *et al.* (1994) who found an almost complete normalisation of Ca output without any effects on urine volume in male rats treated with evening primrose oil. Another study of diabetic pregnant rats fed primrose oil, however, did find reduced urinary

volumes concomitant with reduced Ca output (Garland *et al.* 1997). In this study, the reduced electrolyte output without concentration changes are clearly pinpointing urine production as the cause for the treatment effects.

Glomerular filtration rate (GFR) is increased by both diabetes (Mogensen & Andersen 1975, Østerby & Gundersen 1975, Carney *et al.* 1979, Hosetter *et al.* 1981, Wiseman *et al.* 1985) and pregnancy (Davison & Hytten 1975, Dunlop 1981, Dafnis & Sabatini 1992). It is affected by the pressure in the afferent and efferent glomerular arteriole, and glucose increases GFR through a tubuloglomerular feedback mechanism (Ditzel & Brochner-Mortensen 1983). Endogenously produced prostaglandins are involved in the autoregulatory process determining the glomerular filtration pressure (Arima *et al.* 1994). Diabetes is known to increase prostaglandin production in the kidney (Kreisberg & Patel 1983, Schambelan *et al.* 1985, Craven *et al.* 1987, DeRubertis & Craven 1993) and it appears that protein kinase C activity in glomerular mesangial cells mediates the effects of high glucose concentration on prostaglandin production (Williams & Schrier 1993). In the renal cortex of STZ-diabetic rats, the concentrations of arachidonic acid are reduced concomitant with increased prostaglandin E_2 synthesis (Ramsammy *et al.* 1993). Modulation of substrate supply for prostaglandins, by EFA supplementation, attenuates abnormal haemodynamics caused by diabetes (Tomlison *et al.* 1989, Cameron *et al.* 1993, Stevens *et al.* 1993), possibly involving regulation of cyclo-oxygenase expression (Fang *et al.* 1997). We therefore speculate that the reduction of urine production noted in this study after GLA and/or ascorbate supplementation is due to alterations of the prostaglandin metabolism that regulates glomerular filtration.

The present finding of reduced bone size and thereby reduced Ca content, but with maintained Ca concentration, corroborates previous reports of diabetes affecting bone development (Verhaeghe *et al.* 1986, Tein *et al.* 1998). The higher ratios of hypertrophic cells to proliferative cells in the growth plate found in the diabetic rat femurs in this study may be explained by a reduced local synthesis of mitogenic growth factors, such as insulin-like growth factor-I (Lazowski *et al.* 1994). Insulin and insulin-like growth factor-I are known to initiate chondrocyte proliferation and maturation (Bohme *et al.*

Figure 8 Longitudinal section of the femur from a CP rat (a) and a DP rat (b). Sections (5 μ m) of the femur were stained with haematoxylin–eosin. A smaller zone of hypertrophic cells is notable in the femurs of the diabetic rat.

1992). Supplementation of the diet with essential fatty acids and antioxidants had only minor ameliorative effects on femoral maturation. It is possible that this effect is mediated through modulation of prostaglandin signalling, as it is known to be involved in bone metabolism (Kawaguchi *et al.* 1995).

The excessive loss of electrolytes in pregnant animals could potentially be harmful for the offspring. Skeletal malformations and reduced Ca content are commonly seen in offspring of diabetic rats (Eriksson *et al.* 2000). Recent evidence, however, suggests that the abnormal development is intrinsic to the fetus itself and not related to Ca

supply (Verhaeghe *et al.* 1999), and that the observed down-regulation of the placental Ca-transporting protein calbindin-D_{9K} is a secondary adjustment to reduced fetal Ca demand (Braddock *et al.* 2002).

In summary, this study has demonstrated a partial protective effect of GLA and ascorbate on urine and electrolyte loss in pregnant diabetic rats, as well as a partial restoration of skeletal growth. The treatment effect on the kidney relates to urine volume and not tubular ion reabsorption.

Acknowledgements

We are grateful to Ms C Davidge for advice and assistance. This study was supported by Scotia Pharmaceuticals Ltd and the Thai government.

References

- Anawa A & Garland H 1990 Renal calcium and magnesium handling in experimental diabetes mellitus. *Acta Endocrinologica* **122** 479–486.
- Arima S, Ren Y, Juncos LA, Carretero OA & Ito S 1994 Glomerular prostaglandins modulate vascular reactivity of the downstream efferent arterioles. *Kidney International* **45** 650–658.
- Atherton JC, Hui MA & Thomas S 1968 Effects of water diuresis and osmotic (mannitol) diuresis on urinary solute excretion by the conscious rat. *Journal of Physiology* **197** 395–410.
- Aubia J, Serrano S, Marioso L, Hojman L, Diez A, Lloveras J & Masramon J 1988 Osteodystrophy of diabetes in chronic dialysis: a histomorphometric study. *Calcified Tissue International* **42** 297–301.
- Barcelli UO, Weiss M, Beach D, Motz A & Thompson B 1990 High linoleic acid diets ameliorate diabetic nephropathy in rats. *American Journal of Kidney Diseases* **16** 244–251.
- Birdsey TJ, Husain SM, Garland HO & Sibley CP 1995 The effect of diabetes on urinary excretion in pregnant rats and their offspring. *Journal of Endocrinology* **145** 11–18.
- Blobe GC, Khan WA & Hannum YA 1995 Protein kinase C: cellular target of the second messenger arachidonic acid? *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **52** 129–135.
- Bohme C, Conscience-Egli M, Tschan T, Winterhalter KH & Bruckner P 1992 Induction of proliferation of hypertrophy of chondrocytes in serum free culture: the role of insulin-like growth factor-I, insulin or thyroxine. *Journal of Cell Biology* **116** 1035–1042.
- Braddock R, Simán CM, Hamilton K, Garland HO & Sibley CP 2002 Gamma-linoleic acid and ascorbate improves skeletal ossification in offspring of diabetic rats. *Pediatric Research*, In Press.
- Cameron NE & Cotter MA 1996 Comparison of the effects of ascorbyl γ -linoleic acid in the correction of neurovascular deficits in diabetic rats. *Diabetologia* **39** 1047–1054.
- Cameron NE, Cotter MA, Dines KC, Robertson S & Cox D 1993 The effects of evening primrose oil on nerve function and capillarization in streptozotocin-diabetic rats: modulation by the cyclo-oxygenase inhibitor flurbiprofen. *British Journal of Pharmacology* **109** 972–979.
- Cameron NE, Cotter MA, Horrobin DH & Tritschler HJ 1998 Effects of α -lipoic acid in neurovascular function in diabetic rats: interaction with essential acids. *Diabetologia* **41** 390–399.
- Carney SL, Wong NLM & Dirks JH 1979 Acute effects of streptozotocin diabetes on renal function. *Journal of Laboratory and Clinical Medicine* **93** 950–961.
- Conaway HH, Diez LF & Raisz LG 1986 Effects of prostacyclin and prostaglandin E₁ (PGE₁) on bone resorption in the presence and absence of parathyroid hormone. *Calcified Tissue International* **38** 130–134.
- Craven PA, Caines MA & DeRubertis FR 1987 Sequential alterations in glomerular prostaglandin and thromboxane synthesis in diabetic rats: relationship to the hyperfiltration of early diabetes. *Metabolism* **36** 95–103.
- Dafnis E & Sabatini S 1992 The effect of pregnancy on renal function: physiology and pathophysiology. *American Journal of the Medical Sciences* **303** 184–205.
- Davison JM & Hytten FE 1975 The effect of pregnancy on renal handling of glucose. *British Journal of Obstetrics and Gynaecology* **82** 374–381.
- DeRubertis FR & Craven PA 1993 Eicosanoids in the pathogenesis of the functional and structural alterations of the kidney in diabetes. *American Journal of Kidney Diseases* **22** 727–735.
- Ditzel J & Brochner-Mortensen J 1983 Tubular reabsorption rates as related to elevated glomerular filtration in diabetic children. *Diabetes* **32** 28–33.
- Dunlop W 1981 Serial changes in renal haemodynamics during normal human pregnancy. *British Journal of Obstetrics and Gynaecology* **88** 1–9.
- Eriksson UJ, Borg LAH, Cederberg J, Nordstrand H, Siman CM, Wentzel C & Wentzel P 2000 Pathogenesis of diabetes-induced congenital malformations. *Uppsala Journal of Medical Sciences* **105** 53–84.
- Fang C, Jiang Z & Tomlinson DR 1997 Expression of constitutive cyclo-oxygenase (COX-1) in rats with streptozotocin-induced diabetes; effects of treatment with evening primrose oil or an aldose reductase inhibitor on COX-1 mRNA levels. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **56** 157–163.
- Felsenfeld AJ, Rodriguez M, Dunley R & Llach F 1991 A comparison of parathyroid gland function in hemodialysis patients with different forms of renal osteodystrophy. *Nephrology, Dialysis, Transplantation* **6** 244–251.
- Frei B, England L & Ames BN 1989 Ascorbate is an outstanding antioxidant in human blood plasma. *PNAS* **86** 6377–6381.
- Frei B, Stocker R, England L & Ames BN 1990 Ascorbate: the most effective antioxidant in human blood plasma. *Advances in Experimental Medicine and Biology* **264** 155–163.
- Garland H, Harris P & Morgan T 1991 Calcium transport in the proximal convoluted tubule and loop of Henle of rats made diabetic with streptozotocin. *Journal of Endocrinology* **131** 373–380.
- Garland HO, Forshaw AG & Sibley CP 1997 Dietary essential fatty acid supplementation, urinary calcium excretion and reproductive performance in the diabetic pregnant rat. *Journal of Endocrinology* **153** 357–363.
- Glajchen N, Epstein S, Ismail F, Thomas S, Fallon M & Chakrabarti S 1988 Bone mineral metabolism in experimental diabetes mellitus: osteocalcin as a measure of bone remodeling. *Endocrinology* **123** 290–295.
- Gregorio F, Cristallini S, Santeusano F, Filipponi P & Fumelli P 1994 Osteopenia associated with non-insulin-dependent diabetes mellitus: what are the causes? *Diabetes Research and Clinical Practice* **23** 43–54.
- Guruprakash G, Krothapalli R, Rouse D, Babino H & Suki W 1988 The mechanism of hypercalciuria in streptozotocin-induced diabetic rats. *Metabolism* **37** 306–311.
- Hamilton K, Tein M, Glazier J, Mawer EB, Berry JL, Balment RJ, Boyd RD, Garland HO & Sibley CP 2000 Altered calbindin mRNA expression and calcium regulating hormones in rat diabetic pregnancy. *Journal of Endocrinology* **164** 67–76.
- Harangi F, Soltesz G & Mehes K 1988 Hypercalciuria in children with diabetes mellitus. *Helvetica Paediatrica Acta* **43** 267–271.
- Hercz G, Pei Y, Greenwood C, Manuel A, Saiphoo C, Goodman WG, Segre GV, Fenton S & Sherrard DJ 1993 Aplastic osteodystrophy without aluminum: the role of 'suppressed' parathyroid function. *Kidney International* **44** 860–866.
- Horrobin DF & Carmichael HA 1992 Essential fatty acids in relation to diabetes. In *Treatment of Diabetic Neuropathy: A New Approach*, pp 21–39. Ed. DF Horrobin. Edinburgh: Churchill Livingstone.

- Hosetter TH, Troy JL & Brenner BM 1981 Glomerular hemodynamics in experimental diabetes mellitus. *Kidney International* **19** 410–415.
- Hoskins B & Scott J 1983 Effects of insulin on urinary calcium excretion by diabetic rats in the absence of glucose control. *Acta Diabetologia* **21** 263–273.
- Hough FS 1994 Alterations of bone and mineral metabolism in diabetes mellitus. Part II. Clinical studies in 206 patients with type I diabetes mellitus. *South African Medical Journal* **72** 120–126.
- Hough S, Avioli LV, Bergfeld MA, Fallon MD, Slatopolsky E & Teitelbaum SL 1981 Correction of abnormal bone and mineral metabolism in chronic streptozotocin-induced diabetes mellitus in the rat by insulin therapy. *Endocrinology* **198** 2228–2234.
- Hough S, Russell JE, Teitelbaum SL & Avioli LV 1982 Calcium homeostasis in chronic streptozotocin-induced diabetes mellitus in the rat. *American Journal of Physiology* **242** E451–E456.
- Hounsom L, Horrobin DF, Tritschler H, Corder R & Tomlinson DR 1998 A lipoic acid–gamma linoleic acid conjugate is effective against multiple indices of experimental diabetic neuropathy. *Diabetologia* **41** 839–843.
- Jara A, Bover J & Felsenfeld AJ 1995 Development of secondary hyperparathyroidism and bone disease in diabetic rats with renal failure. *Kidney International* **48** 1746–1751.
- Kawaguchi H, Pilbeam CC, Harrison JR & Raisz LG 1995 The role of prostaglandins in the regulation of bone metabolism. *Clinical Orthopaedics and Related Research* **313** 36–46.
- Kelly FJ 1998 Use of antioxidants in the prevention and treatment of disease. *Journal of International Federation of Clinical Chemistry* **10** 21–23.
- Kreisberg JI & Patel PY 1983 The effects of insulin, glucose and diabetes on prostaglandin production by rat kidney and cultured glomerular mesangial cells. *Prostaglandins, Leukotrienes, and Medicine* **11** 431–442.
- Lazowski DA, Fraker LJ, Hudsman A, Sier B, Modrowski D & Han VK 1994 Regional variations of insulin-like growth factor-1 gene expression in mature rat bone and cartilage. *Bone* **15** 563–576.
- Levin ME, Boisseau VC & Avioli LV 1976 Effects of diabetes mellitus on bone mass in juvenile and adult onset diabetes. *New England Journal of Medicine* **294** 241–245.
- Locatto ME, Fernandez MC, Abranzon H, Caferra D & Puche RC 1990 Calcium metabolism of rats with varying degrees of insulinopenia. *Bone and Mineral* **8** 119–130.
- McLennan S, Yue DK, Fisher E, Capogreco C, Heffernan S, Ross GR & Turtle JR 1988 Deficiency of ascorbic acid in experimental diabetes. Relationship with collagen and polyol pathway abnormalities. *Diabetes* **37** 359–361.
- McNair P, Madsbad S, Christiansen C, Faber OK, Transbol I & Binder C 1978 Osteopenia of insulin treated diabetes mellitus: its relation to age of onset, sex and duration of disease. *Diabetologia* **15** 87–93.
- Marchi F, Luder HU & Leblond CP 1991 Changes in cells' secretory organelles and extracellular matrix during endochondral ossification in the mandibular condyle of the growing rat. *American Journal of Anatomy* **190** 41–73.
- Mogensen CE & Andersen MJF 1975 Increased kidney size and glomerular filtration in untreated juvenile diabetes: normalization by insulin-treatment. *Diabetologia* **11** 221–224.
- Nunez EA 1993 Fatty acids and cell signalling. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **48** 1–4.
- Osterby R & Gundersen HJG 1975 Glomerular size and structure in diabetes mellitus: I. Early abnormalities. *Diabetologia* **11** 225–229.
- Pei Y, Hercz G, Greenwood C, Segre G, Manuel A, Saiphoo C, Fenton S & Sherrard D 1993 Renal osteodystrophy in diabetic patients. *Kidney International* **44** 159–164.
- Ramsammy LS, Hayenes B, Josepovitz C & Kaloyanides GJ 1993 Mechanism of decreased arachidonic acid in the renal cortex of rats with diabetes mellitus. *Lipids* **28** 433–439.
- Raskin P, Stevenson M, Barilla D & Pak C 1978 The hypercalciuria of diabetes mellitus; its amelioration with insulin. *Clinical Endocrinology* **9** 329–335.
- Schambelan M, Blake S, Sraer J, Bens M, Nivez MP & Wahbe F 1985 Increased prostaglandin production by glomeruli isolated from rats with streptozotocin-induced diabetes mellitus. *Journal of Clinical Investigation* **75** 404–412.
- Sherrard DJ, Hercz G, Pei Y, Maloney NA, Greenwood C, Manuel A, Saiphoo C, Fenton SS & Serge GV 1993 The spectrum of bone disease in end-stage renal failure – an evolving disorder. *Kidney International* **43** 436–442.
- Shires R, Teitelbaum SL, Bergfeld MA, Fallon MD, Slatopolsky E & Avioli LV 1981 The effect of streptozotocin-induced diabetes mellitus on bone and mineral homeostasis in the rat. *Journal of Laboratory and Clinical Medicine* **97** 231–240.
- Simán CM, Garland HO, Pikongarm R & Sibley CP 2000 Failure of short-term γ -linoleic acid treatment to reduce urinary calcium loss of diabetic rats. *Urology Research* **28** 132–135.
- Som S, Basu S, Mukherjee D, Deb S, Choudhury PR, Mukherjee S, Chatterjee SN & Chatterjee IB 1981 Ascorbic acid metabolism in diabetes mellitus. *Metabolism* **30** 572–577.
- Stevens EJ, Carrington AL & Tomlinson DR 1993 Prostacyclin release in experimental diabetes: effect of evening primrose oil. *Prostaglandins, Leukotrienes, and Essential Fatty acids* **49** 699–706.
- Tein MS, Breen SA, Loveday BE, Devlin H, Balment RJ, Boyd RD, Sibley CP & Garland HO 1998 Bone mineral density and composition in rat pregnancy: effects of streptozotocin-induced diabetes mellitus and insulin replacement. *Experimental Physiology* **83** 165–174.
- Tomlinson DR, Robinson JP, Compton AM & Keen P 1989 Essential fatty acid treatment – effects on nerve conduction, polyol pathway and axonal transport in streptozotocin diabetic rats. *Diabetologia* **32** 655–659.
- Tulloch I, Smellie WS & Buck AC 1994 Evening primrose oil reduces urinary calcium excretion in both normal and hypertensive rats. *Urology Research* **22** 227–230.
- Verhaeghe J, Bouillon R, Nyomba B, Lissens W & Asche FV 1986 Vitamin D and bone mineral homeostasis during pregnancy in the diabetic BB rat. *Endocrinology* **118** 1019–1025.
- Verhaeghe J, Van Herck E, Visser WJ, Suiker AMH, Thomasset M, Einhorn TA, Faierman E & Bouillon R 1990 Bone and mineral metabolism in BB rats with long-term diabetes: decreased bone turnover and osteoporosis. *Diabetes* **39** 477–482.
- Verhaeghe J, van Bree R, van Herck E, Rummens K, Vercruysse L, Bouillon R & Pijnenborg R 1999 Pathogenesis of fetal hypomineralization in diabetic rats: evidence for delayed bone maturation. *Pediatric Research* **45** 209–217.
- Williams B & Schrier RW 1993 Glucose-induced protein kinase C activity regulates arachidonic acid release and eicosanoid production by cultured glomerular mesangial cells. *Journal of Clinical Investigation* **92** 2889–2896.
- Wiseman MJ, Saunders AJ, Keen H & Viberti G 1985 Effects of blood glucose control on increased glomerular filtration rate and kidney size in insulin-dependent diabetes. *New England Journal of Medicine* **312** 617–620.
- Wood R, Allen L & Bronner F 1984 Regulation of calcium metabolism in streptozotocin-induced diabetes. *American Journal of Physiology* **247** R120–R123.
- Yew MS 1983 Effect of streptozotocin diabetes on tissue ascorbic acid and dehydroascorbic acid. *Hormone and Metabolic Research* **15** 158

Received in final form 27 December 2001
Accepted 11 January 2002