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

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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.


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.

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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 pregnancy.

<table>
<thead>
<tr>
<th>Table 1 Experimental groups and their respective diet supplementation</th>
</tr>
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<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>Control pregnant</td>
</tr>
<tr>
<td>Diabetic pregnant</td>
</tr>
<tr>
<td>Diabetic pregnant with GLA</td>
</tr>
<tr>
<td>Diabetic pregnant with ascorbate</td>
</tr>
<tr>
<td>Diabetic pregnant with ascorbyl-GLA</td>
</tr>
<tr>
<td>Diabetic pregnant with GLA and ascorbate</td>
</tr>
</tbody>
</table>

**Figure 1** Experimental design.
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. 

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

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

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 ± 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).
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 ± s.e.m. and n equals the number of animals. A one-way analysis of variance (ANOVA) with Dunnet’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
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 \( (P=0.053, t\text{-test CP vs DP}) \) 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
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 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 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 ± 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
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.
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-D28k, could be involved in the failure to concentrate urinary Ca in diabetes (Hamilton et al. 2000). However, calbindin-D28k 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-D28k mRNA expression was not affected by diabetes at day 20 of pregnancy, nor was it affected by any of the treatments (Simón 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, Hosetttet 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 tubulo-glomerular 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 E2 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>CP (n=5)</th>
<th>DP (n=5)</th>
<th>DG (n=5)</th>
<th>DA (n=8)</th>
<th>DASG (n=8)</th>
<th>DGA (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of epiphysis (mm)</td>
<td>5·8 ± 1·0*</td>
<td>4·1 ± 0·8</td>
<td>7·0 ± 0·4*</td>
<td>6·2 ± 0·5*</td>
<td>7·4 ± 0·3*</td>
<td>5·4 ± 0·4*</td>
</tr>
<tr>
<td>Width of epiphysis (mm)</td>
<td>0·17 ± 0·004*</td>
<td>0·13 ± 0·01</td>
<td>0·14 ± 0·01</td>
<td>0·13 ± 0·01</td>
<td>0·14 ± 0·01</td>
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<td>Microtrophic cells</td>
<td>6·8 ± 0·4</td>
<td>6·4 ± 0·4</td>
<td>6·6 ± 0·5</td>
<td>6·8 ± 0·3</td>
<td>7·6 ± 0·5</td>
<td>6·2 ± 0·4</td>
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<tr>
<td>Hypertrophic cells</td>
<td>8·0 ± 0·5*</td>
<td>6·3 ± 0·5</td>
<td>6·7 ± 0·1</td>
<td>6·5 ± 0·25</td>
<td>6·7 ± 0·4</td>
<td>6·0 ± 0·2</td>
</tr>
</tbody>
</table>

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.

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Table 4 Femoral histology. Data are presented as means ± s.e.m.
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

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

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