Abstract

Background. Intravenous vitamin C supplementation to haemodialysis patients might ameliorate responsiveness to recombinant human erythropoietin (rHuEpo). This study was performed to analyse the relation between vitamin C plasma concentration and response to rHuEpo.

Methods. In a cross-sectional, single-centre observational study including all haemodialysis patients, pre-dialysis plasma vitamin C concentrations were measured by high-performance liquid chromatography and response to rHuEpo (haemoglobin concentration/international units rHuEpo/kg/week) was recorded together with baseline laboratory data.

Results. Univariate analysis yielded a significant correlation between vitamin C plasma levels and response to rHuEpo \((n=130, r=0.25, P=0.004)\), which still persisted after adjustment for transferrin saturation, C-reactive protein, malondialdehyde, parathyroid hormone, route of rHuEpo administration, residual renal function and diabetes mellitus (adjusted \(r=0.23, P=0.014\)). Analysis per quartiles of vitamin C plasma level revealed a significantly lower response to rHuEpo with decreasing vitamin C values \((P=0.026)\).

Conclusions. In unselected haemodialysis patients, vitamin C plasma levels account, at least partially, for the response to rHuEpo. Larger-sized interventional studies are needed to find out whether vitamin C plasma levels may or may not appropriately reflect the potential beneficial effect of vitamin C supplements on rHuEpo responsiveness.

Keywords: anaemia; chronic kidney disease; erythropoietin; vitamin C

Introduction

Current guidelines for the management of anaemia in chronic kidney disease (CKD) patients value vitamin C deficiency ‘worth exploring’ only after the exclusion of several other more common conditions associated with a poor response to recombinant human erythropoietin (rHuEpo) [1]. Vitamin C potentially increases intestinal iron absorption and iron mobilization from inert tissue stores in a poorly understood fashion and may improve iron utilization in the erythron [1]. A recent workshop on the role of adjuvant therapies to rHuEpo recommended that when subclinical vitamin C-deficiency is suspected, CKD patients should receive 1–1.5 g oral vitamin C per week or 300 mg intravenous ascorbic acid three times a week after each haemodialysis session [2]. However, evidence for such recommendations is scarce and it remains unclear when to suspect vitamin C deficiency.

In a small-scale pioneer study, seven dialysis patients with normal iron status (i.e. ferritin >100 \(\mu g/l\), transferrin saturation >25%) and moderately dosed rHuEpo (rHuEpo <100 international units/kg/week) did not profit from a 3 month course of 1 g vitamin C/week intravenously [3]. In contrast, haemoglobin concentrations rose and transferrin saturation increased significantly in four iron-overloaded patients (ferritin >500 \(\mu g/l\)) after ~3 weeks of vitamin C supplementation [3]. Another study on 12 iron-overloaded dialysis patients found a decrease in mean rHuEpo requirements in association with a rising transferrin saturation after 8 weeks of 300 mg vitamin C administered intravenously after each haemodialysis.
session [4]. Only a subset of iron-overloaded dialysis patients may respond: Tarng et al. [5] obtained positive predictive values regarding a response to vitamin C for a transferrin saturation <25% and an erythrocyte zinc protoporphyrin concentration >105 µmol/mol haem (indicating insufficient iron incorporation into haem). Anaemia, however, worsened immediately after discontinuation of vitamin C administration [3,5]; plasma vitamin C levels were considered normal at the beginning of the trial [3], did not differ between responders and non-responders at baseline and rose in a similar manner in both groups during supplementation [5]. Hence, caution is warranted when interpreting the above findings as a result of a possible correction of a vitamin C-deficiency state. The best evidence documenting a beneficial effect of vitamin C on erythropoiesis stems from a prospective, randomized cross-over trial on 27 anaemic dialysis patients [6] with no obvious cause for hyporesponsiveness to rHuEpo other than functional iron-deficiency. Anaemia improved in all study participants during 3 months of vitamin C supplementation (500 mg three times a week intravenously following haemodialysis) and was associated with a rise in transferrin saturation and a fall in serum ferritin levels. Haemoglobin concentration and transferrin saturation declined after cessation of vitamin C therapy. These results strongly support the idea that intravenous vitamin C mobilizes previously not accessible iron from body stores to transferrin. Accordingly, Sezer et al. [7] reported no response to vitamin C supplementation in dialysis patients with a baseline value of hypochromic red blood cells <10%, while responders yielded a mean value of 26%.

In a recent cross-over study, as much as two-thirds of unselected, anaemic dialysis patients responded to 6 months of 500 mg intravenous vitamin C three times a week [8]. The authors, however, did not account for the response rate during the placebo phase of the trial and plasma oxalate levels were not assessed [9]. Vitamin C is metabolized to oxalate; hence, secondary oxalosis may follow prolonged vitamin C supplementation in CKD patients.

To date, parameters indicating absolute or functional iron deficiency (transferrin saturation, erythrocyte zinc protoporphyrin and percentage of hypochromic erythrocytes) may support the decision to prescribe vitamin C in order to overcome hyporesponsiveness to rHuEpo. The value of vitamin C plasma levels, however, in guiding this decision remains unclear. We, therefore, performed a cross-sectional, single-centre study analysing for a potential relation between the plasma level of vitamin C and the response to rHuEpo.

**Subjects and methods**

**Study design and study population**

The study was designed as a cross-sectional analysis including all consecutive patients under haemodialysis at a tertiary care university clinic in January 2002. We analysed for a potential relation between plasma levels of vitamin C and response to rHuEpo (dependent variable). Response to rHuEpo was defined as the ratio of the concentration of haemoglobin (g/dl) on sampling day and the dose of rHuEpo (IU/kg/week). Dose of rHuEpo and iron sucrose, respectively, were defined as the median weekly dose per kg dry body weight (end of dialysis weight) during the 4 weeks preceding sampling. The study complied with the Declaration of Helsinki and written informed consent was obtained from each participant. Blood samples were drawn through dialysis access before commencement of dialysis. We included 130 patients in the present study. Oral multivitamin supplementation (Dreisavit®; GRY-Pharma, Kirchzarten, Germany; containing 100 mg vitamin C, 0.16 mg folic acid, 0.03 mg biotin, 8 mg thiamine, 8 mg riboflavin, 10 mg pyridoxine, 50 mg nicotinamide and 10.9 mg calcium pantothenate) was routinely prescribed to all patients. Compliance, however, was not confirmed. Patients were advised not to take any vitamin supplements on the day of blood sampling. The median age was 60 years and the range from the 25th to the 75th percentile (interquartile range (IQR)) was 48–71 years. Eighty-six (66%) patients were male, 26 (20%) had diabetes mellitus with a median HbA1C of 7.6% (IQR: 6.9–8.1%). Patients were on haemodialysis with high-flux membranes ( polysulfone and cellulose triacetate) for a median of 20 months (IQR: 10–36 months), 123 (95%) received intravenous or subcutaneous rHuEpo therapy [118 (96%) rHuEpo-alpha; 5 (4%) rHuEpo-beta] and 110 (85%) patients were dialysed via fistulae, 3 (2%) via synthetic grafts and 17 (13%) via central catheters. Demographic data, clinical characteristics and laboratory findings are presented in Table 1.

**Laboratory evaluation**

Standard methods were applied for the estimation of routine parameters. For vitamin C analyses, blood was collected into chilled tubes containing Li-heparin as an anticoagulant. Subsequently, the blood was transported in an ice bath to the laboratory and was centrifuged at 4°C. Vitamin C was analysed with commercial high-performance liquid chromatography (HPLC) kits from Immundiagnostik (Bensheim, Germany) containing the calibrator, mobile phase, a precipitation agent and two control samples. The formula-
calculation of the concentration by Chromleon software (Dionex, Sunny Vale, CA, USA). Interassay coefficients of variation were 13% and 8.6% at 23.5 and 76.9 \( \text{m} \text{mol/l} \), respectively. A method comparison to another commercial vitamin C kit (Chromsystems, Munich, Germany) yielded the Pasing–Pablok regression line:

\[
\text{Chromsystems} = 0.95 \times \text{Imundiagnostik} + 1.9
\]

\((r = 0.95, n = 11)\). Malondialdehyde was estimated by HPLC according to Fukunaga et al. [10].

Statistical methods

Continuous data are given as the median and the IQR (range from the 25th to the 75th percentile). Discrete data are presented as counts and percentages. For univariate comparison of continuous variables, the Wilcoxon rank sum and Kruskal–Wallis tests were used, as appropriate. Spearman’s rank correlation coefficient was calculated for univariate correlations of continuous variables. Multivariate partial correlation was then applied to assess the association between vitamin C plasma levels and rHuEpo response, adjusting for possible confounding variables. As data were skewed to the right, we used log transformation to achieve a distribution resembling a normal distribution. Baseline variables were selected for the model if they (i) had either a clinically or biologically plausible relation with rHuEpo response or (ii) appeared to be imbalanced with respect to rHuEpo response, indicated by a \( P \)-value of < 0.20. We used a hierarchical modelling strategy assessing the effects of clinical and laboratory covariables separately and jointly. A two-sided \( P \)-value of < 0.05 was considered as statistically significant. Calculations were performed with Stata, release 8 (Stata, College Station, TX, USA) and SPSS for Windows® (version 10.0; SPSS Inc., Chicago, IL, USA).

Results

Vitamin C plasma levels were significantly correlated with rHuEpo response in univariate analysis \((r = 0.25, P = 0.004)\), indicating that vitamin C levels account for \(~5\%\) of the variation of the rHuEpo response. To identify possible confounding factors, which may influence this association, all variables listed in Table 1 were analysed with respect to imbalances of rHuEpo response and vitamin C plasma levels. Transferrin saturation \((r = 0.26, P = 0.003)\), iron substitution \((r = -0.38, P < 0.001)\) and route of rHuEpo administration \((P < 0.001)\) were significantly correlated with rHuEpo response. Residual kidney urea clearance \((r = 0.13, P = 0.14)\), ferritin \((r = 0.16, P = 0.07)\) and albumin \((r = 0.15, P = 0.09)\) showed a trend towards an association with rHuEpo response. Parathyroid hormone \((r = 0.18, P = 0.044)\) and plasma aluminium \((r = 0.23, P = 0.018)\) were significantly correlated with vitamin C plasma levels. Albumin \((r = 0.16, P = 0.079)\), C-reactive protein \((r = -0.14, P = 0.11)\), malondialdehyde \((r = 0.12, P = 0.18)\) and transferrin saturation \((r = 0.14, P = 0.11)\) were also correlated with vitamin C plasma levels. 

Table 1. Demographic data and clinical characteristics of 130 haemodialysis patients

<table>
<thead>
<tr>
<th></th>
<th>n (%)/median</th>
<th>IQR</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60</td>
<td>48–71</td>
<td>23–93</td>
</tr>
<tr>
<td>Male sex</td>
<td>86 (66%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69</td>
<td>60–79</td>
<td>34–108</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>26 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>30 (23%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of haemodialysis (months)</td>
<td>20</td>
<td>10–36</td>
<td>1–274</td>
</tr>
<tr>
<td>Mode of dialysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemodialysis</td>
<td>34 (26%)</td>
<td></td>
<td></td>
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<tr>
<td>Haemodiafiltration</td>
<td>96 (74%)</td>
<td></td>
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<tr>
<td>Kt/V</td>
<td>1.39</td>
<td>1.25–1.58</td>
<td>0.89–2.55</td>
</tr>
<tr>
<td>Residual kidney urea clearance (ml/min)</td>
<td>0</td>
<td>0–1.1</td>
<td>0–10.5</td>
</tr>
<tr>
<td>Parathyroid hormone (ng/l)</td>
<td>147</td>
<td>63–247</td>
<td>2–2310</td>
</tr>
<tr>
<td>Aluminium (µg/l)</td>
<td>12.3</td>
<td>5.7–20.5</td>
<td>1.3–208.8</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>37.5</td>
<td>35.7–39.4</td>
<td>23.6–43.6</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>9.3</td>
<td>5.5–15.6</td>
<td>&lt;5.0–183</td>
</tr>
<tr>
<td>Malondialdehyde (µmol/l)</td>
<td>1.3</td>
<td>1.0–1.6</td>
<td>0.5–2.6</td>
</tr>
<tr>
<td>Vitamin C plasma level (µmol/l)</td>
<td>45.1</td>
<td>24.3–76.9</td>
<td>1.0–459</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>12.1</td>
<td>11.1–12.8</td>
<td>7.8–14.4</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>20.1</td>
<td>14.5–24.9</td>
<td>6.0–78.8</td>
</tr>
<tr>
<td>Ferritin (µg/l)</td>
<td>299</td>
<td>146–469</td>
<td>8–991</td>
</tr>
<tr>
<td>Iron sucrose (mg/week/kg)</td>
<td>0.77</td>
<td>0.42–1.24</td>
<td>0–2.35</td>
</tr>
<tr>
<td>Dose of rHuEpo (IU/week/kg)</td>
<td>176</td>
<td>96–309</td>
<td>0–871</td>
</tr>
<tr>
<td>Route of rHuEpo administration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No rHuEpo</td>
<td>7 (5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td>101 (78%)</td>
<td></td>
<td></td>
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<tr>
<td>Subcutaneous</td>
<td>22 (17%)</td>
<td></td>
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</table>

Continuous data are given as the median, the IQR and the range.

*The study was performed before administration of rHuEpo-alpha was restricted to the intravenous route only because of the emergence of pure red blood cell aplasia in association with long-term use of erythropoietic agents.
$P = 0.11$) showed a trend towards a correlation with vitamin C. Patients with lower than normal serum albumin levels ($< 34 \text{ g/l}$) showed a significantly lower response to rHuEpo ($P = 0.034$, data not shown). The remaining variables showed no significant correlation with rHuEpo response or vitamin C levels (all $P > 0.2$).

We then calculated partial correlation coefficients for vitamin C plasma levels and rHuEpo response, adjusting for possible confounding effects. The correlation between vitamin C and rHuEpo was widely independent of other investigated parameters ($r = 0.23$, $P = 0.014$), adjusting for transferrin saturation, C-reactive protein, malondialdehyde, parathyroid hormone, route of rHuEpo administration, residual kidney urea clearance and diabetes mellitus. Further adjustment for albumin (instead of C-reactive protein), ferritin (instead of transferrin saturation), $Kt/V$ and aluminium did not cause a significant effect modification ($r = 0.25$, $P = 0.013$).

We further analysed rHuEpo response per quartiles of vitamin C plasma levels and, by overall comparison, found a significantly lower response to rHuEpo with decreasing plasma levels of vitamin C ($P = 0.026$). Figure 1 shows a box plot of this analysis.

**Discussion**

Univariate analysis yielded a significant correlation between vitamin C plasma levels and response to rHuEpo, which still persisted after correction for potential confounding variables. All of our patients were screened routinely to immediately exclude overt, correctable conditions associated with hypo responsiveness to rHuEpo, such as occult blood loss, haemolysis and folate or vitamin B12 deficiency. High levels of intact parathyroid hormone may limit the efficacy of rHuEpo [1]. In our sample, the degree of hyperparathyroidism showed no relevant relation to rHuEpo response, probably due to effective therapy yielding a median parathyroid hormone level of 147 ng/l (IQR: 63–247 ng/l). Dialysis dose was adequate, as evidenced by a median $Kt/V$ of 1.39 (IQR: 1.25–1.58), and the median serum albumin level of our sample lay within the normal range (median: 37.5 g/l; IQR: 35.7–39.4 g/l), indicating good nutritional status. Iron deficiency strongly restricts rHuEpo responsiveness and continuous administration of rHuEpo greatly increases iron demands [1]. Accordingly, we found a significant univariate correlation between transferrin saturation and rHuEpo response and a negative association between administered iron and rHuEpo response. Patients of our sample received 638 mg (IQR: 348–1027 mg) of intravenous iron sucrose per quarter. This value slightly exceeds the estimate of current guidelines (400–500 mg [11]), most probably due to the high normal median haemoglobin of our patients (Table 1). Nevertheless, our baseline data validate our patient sample as an average haemodialysis population.

Our study was a priori targeted to confirm a potential relationship between vitamin C plasma levels and rHuEpo response in haemodialysis patients. Vitamin C levels accounted for $\sim 5\%$ of the variation of the rHuEpo response, suggesting only a small but
significant impact of vitamin C on the efficacy of exogenous rHuEpo. Furthermore, rHuEpo response declined with decreasing vitamin C plasma levels (Figure 1). Importantly, indicators of iron status (ferritin, transferrin saturation and the amount of iron substitution), inflammation (C-reactive protein) and oxidative stress (malondialdehyde) did not cause a significant effect modification. It has been hypothesized that intravenous vitamin C supplementation might ameliorate rHuEpo responsiveness by increasing intestinal iron absorption and iron mobilization from inert tissue stores and/or by improving iron utilization in the erythron. Most intervention studies found a decreasing ferritin concentration in response to vitamin C [5, 6] and a rise of transferrin saturation [3–8]. In our study, transferrin saturation did not modify the relation between vitamin C and rHuEpo response. This finding, however, does not exclude the possibility that a potential impact of vitamin C on rHuEpo response depends on iron metabolism.

Vitamin C constitutes one of the most important water-soluble antioxidants in plasma and serves as the primary intracellular antioxidant in concert with glutathione [12,13]. Oxidative denaturation of haemoglobin and erythrocyte membrane lipid peroxidation renders uraemic red blood cells more sensitive to oxidative, osmotic and mechanical stresses [14,15]. It may be argued that intravenous vitamin C supplementation antagonizes oxidative damage and increases red blood cell survival, as has been shown for exogenously administered glutathione [16]. Vitamin E supplementation to haemodialysis patients resulted in reduced rHuEpo dosage requirements [17]. Haemodialysis patients with stable haemoglobin levels not receiving rHuEpo therapy exhibited a higher hydroxyl radical scavenging activity when compared with those in need of rHuEpo [18]: it may be argued that rHuEpo- or anaemia-associated increments of oxidant burden may be counterbalanced by a higher consumption of antioxidants like vitamin C. In a cross-sectional study on 73 dialysis patients, the dose of rHuEpo as well as the ratio of rHuEpo/haemoglobin was found to correlate positively with serum concentrations of 8-hydroxy-2′-deoxyguanosine, an oxidation product of DNA [19]. Due to the cross-sectional, observational nature of our study, however, no cause–effect relation between the vitamin C plasma level and rHuEpo response should be inferred.

Serum albumin inhibits peroxidation of erythrocyte membrane lipids and persistent hypoalbuminuria decreases serum antioxidant activity [20]. We found a significantly lower response to rHuEpo in patients with lower than normal serum albumin levels, in analogy to other investigators, lending credibility to our data set [21]. Malondialdehyde constitutes a key metabolite of lipid peroxidation and serves as a reference indicator of oxidative stress. In our sample, neither malondialdehyde nor albumin levels affected the relationship between vitamin C plasma levels and rHuEpo response. Vitamin C might also influence renal synthesis of erythropoietin: in rat isolated kidneys, production of erythropoietin was enhanced in the presence of an antioxidative cocktail comprising vitamins A, E and C [22] and pro-oxidants reduced erythropoietin synthesis in human hepatoma cells [23]. In contrast to vitamin A, however, vitamin C alone did not increase erythropoietin production by human hepatoma cells [22].

Our study is limited by its observational nature and by its relatively small number of cases. Previous intervention trials [3–8] on the effect of vitamin C on rHuEpo responsiveness in CKD patients were even smaller in case number, vitamin C plasma levels were not analysed [4,6–8] and, if measured, correlation analyses were not performed [3,5]. Importantly, responders to vitamin C supplementation had normal vitamin C plasma levels [3] or did not differ from non-responders with regards to colorimetrically estimated vitamin C [5]. Plasma concentrations of vitamin C may more adequately reflect dietary intake than tissue and body pool content [23], but, on the other hand, plasma levels of the low range have been observed to correlate with intracellular content [12]. Determination of vitamin C concentrations by HPLC, as performed in this study, is superior to colorimetric estimations [24]. A strong and highly significant correlation between plasma vitamin C concentration and the weekly rHuEpo dose was reported in one of the first studies analysing vitamin C by using HPLC [25]. Data, however, were not adjusted to possible confounding variables, the number of cases was small and the correlation of both parameters was not a predefined endpoint.

In conclusion, we performed a cross-sectional, single-centre observational study on the relation of vitamin C plasma concentration and response to rHuEpo in haemodialysis patients. Only 5% of the variation of rHuEpo response could be accounted for by plasma vitamin C levels, suggesting only a minor impact on rHuEpo responsiveness. Still, response to rHuEpo declined significantly with decreasing vitamin C plasma levels. Larger-sized interventional trials are needed to evaluate whether plasma vitamin C levels may or may not appropriately reflect the potential beneficial effect of vitamin C or other antioxidant supplementations on rHuEpo responsiveness.

Conflict of interest statement. None declared.

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Received for publication: 24.09.03
Accepted in revised form: 12.03.04