



Original Contribution

Acute prooxidant effects of vitamin C in EDTA chelation therapy and long-term antioxidant benefits of therapy[☆]

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Abstract

Chelation therapy is thought to not only remove contaminating metals but also to decrease free radical production. EDTA chelation therapy, containing high doses of vitamin C as an antioxidant, is often used in the treatment of diseases such as diabetes and cardiovascular diseases but the effectiveness of this treatment may be variable and its efficacy has not been demonstrated conclusively. The objective of this work was to determine if the vitamin C added to standard chelation therapy cocktails was prooxidant. We administered a standard EDTA cocktail solution with or without 5 g of sodium ascorbate. One hour following the standard chelation therapy, there were highly significant prooxidant effects on lipids, proteins, and DNA associated with decreased activities of RBC glutathione peroxidase and superoxide dismutase while in the absence of sodium ascorbate, there were no acute signs of oxidative damage. After 16 sessions of standard chelation therapy, the acute prooxidant effects of vitamin C remained, but, even in the absence of nutrient supplements, there were beneficial long-term antioxidant effects of chelation therapy and plasma peroxide levels decreased. In conclusion, multiple sessions of EDTA chelation therapy protect lipids against oxidative damage. However, standard high amounts of vitamin C added to EDTA chelation solutions also display short term prooxidant effects. The added benefits of lower levels of vitamin C in chelation therapy need to be documented.

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Introduction

EDTA chelation therapy is an often used treatment aimed at reducing calcium deposits, removing heavy metals, controlling lipid peroxidation resulting from free radical

pathology, and reducing platelet aggregation in the clinical management of atherosclerosis and related disorders [1] but its effectiveness may be variable and its efficacy has not been demonstrated conclusively [2]. Chelation therapy is thought to not only to remove contaminating metals but also to decrease free radical production [3]. In addition to high sugar and high fat diets [4,5], abnormal amounts of free radicals may be produced by ionizing radiation, heavy metals, poisons, sunlight, and excessive exposure to hyperbaric oxygen and iron overload [1]. Oxidative stress is postulated as one of the major contributors to long-term diabetic complications, including cardiovascular diseases, and the control of oxidative complications may lead to control of diabetes [6–8], inflammation [7] and cardiovascular diseases [6,7]. In addition, modification of cardiac risk

Abbreviations: DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; NAC, N-acetylcysteine; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; ROS, reactive oxygen species.

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factors and antioxidants have been shown to improve endothelial function [9].

The metal and trace metal losses due to EDTA chelation therapy under similar conditions have been documented [10,11]. However, while there is strong evidence that EDTA prevents metal-induced free radical production, the direct in vivo effects of EDTA chelation therapy on oxidative stress markers have not been documented. Moreover, in chelation therapy, high doses of vitamin C are often used as an adjuvant of the treatment [12]. Vitamin C is a known antioxidant but at the high levels (5 g, intravenous) often used during chelation therapy its effects have not been ascertained. In this setting, its use may be beneficial as an antioxidant or deleterious as high doses of ascorbate can have prooxidant effects, especially in the presence of elevated amounts of transition metals [13,14]. The initial reports of prooxidant effects of vitamin C in vivo were in 1998 [15,16]. Earlier studies of vitamin C at lower levels, 72 mg/day [17], reported only antioxidant effects but at higher amounts of vitamin C, 500 mg/day, there were possible prooxidant effects.

Therefore, the objectives of this study were to determine the effects of EDTA chelation therapy on antioxidant status and to address the question of possible prooxidant effects of high doses of vitamin C used during chelation therapy. We measured the frequently used markers for lipids, proteins, and DNA oxidative damage and antioxidant enzyme activities before and after one session and after multiple sessions in patients receiving intravenous EDTA cocktail in the presence or absence of sodium ascorbate.

Materials and methods

Patients

Sixteen patients, 8 female and 8 male, ranging in age from 25 to 74 years participated in the study. The health of the patients varied from those with no obvious health problems to those suffering from diabetes, cardiovascular diseases, hypothyroidism, or heavy metal exposure. The study was approved by the Clinic Human Studies Review Board, Wisconsin Dells, Wisconsin. Patients signed an informed consent form and were not reimbursed for their participation. Data are available to the patients upon request. Patients did not take nutrient supplements 2 weeks prior to and during the study.

Chelation

An intravenous infusion of EDTA mixed in sterile water with 5 g of sodium ascorbate, 2500 units of heparin, 3 ml 2% procaine, 100 mg pyridoxine HCl, 4 meq KCl, 1 ml 8.4% sodium bicarbonate, 1,000 µg hydroxycobalamin, 1 ml vitamin B complex, and 7 ml magnesium sulfate equivalent to 686 mg of elemental magnesium was given

in an arm vein over a 2.5- to 3-h period [12]. The dose of EDTA was 3 g since all patients had good kidney function. Five of the patients (3 female and 2 male) also received the same chelation therapy minus vitamin C.

Blood sampling

Blood samples were collected from the antecubital vein before and immediately after chelation treatment and before and after 5 weeks of treatment. Blood was collected in heparinized tubes protected from light and centrifuged at room temperature for 10 min at 3000g. Plasma and erythrocyte pellets were immediately isolated, aliquoted, and stored at -80°C until measurements within 6 months.

For glutathione measurements, within 20 min of venipuncture, 400 µl whole blood was added to a 3600-µl aqueous solution of metaphosphoric acid (6% w/v). The mixture was centrifuged for 10 min at 4°C . The acidic protein-free supernatant fractions were stored at -80°C until analyses.

For the comet assay and DNA damage determinations [18], 500 µl of blood was stabilized with 500 µl of a 20/80 (v/v) mixture of dimethyl sulfoxide (DMSO) and RPMI 1640 cell culture medium. Aliquots of this mixture were progressively frozen to -80°C by use of cryopreservation vessels (Bicell, Fisher Bioblock Scientific, Lyon, France) in a -80°C freezer overnight. After one night, samples were transferred from the cryopreservation vessels to storage at -80°C until analyses within 4 months.

Biological parameters

Erythrocyte antioxidant metalloenzymes

Erythrocyte Cu–Zn SOD activity was measured after hemoglobin precipitation by monitoring the autooxidation of pyrogallol by the method of Marklund and Marklund [19]. Erythrocyte GSH-Px activity was evaluated by the modified method of Gunzler et al. [20] using *tert*-butyl hydroperoxide (Sigma Chemical Co., Via Coger, Paris, France) as substrate instead of hydrogen peroxide. Results are expressed as micromoles of NADPH (Boehringer-Mannheim, Germany) oxidized per minute per gram of hemoglobin.

Disulfide formation

Total glutathione (GSht) was determined according to the method of Akerboom and Sies [21] using enzymatic cycling of GSH by means of NADPH and glutathione reductase coupled with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). To assay oxidized glutathione (GSSG), GSH was masked by adding 10 µl of 2-vinylpyridine to 500 µl of deproteinized extract adjusted to pH 6 with triethanolamine.

Plasma thiol groups were assayed as described by Faure and Lafond [22]. The calibration was obtained from a stock solution of 100 mM *N*-acetylcysteine (NAC) in the range of 0.125 to 1 mM. Standards and plasma samples were placed

in presence of phosphate buffer 0.05 M, EDTA 1 mM, pH 8, and DTNB, 2.5 mM, and absorbance was measured at 412 nm.

Lipid oxidation

Plasma malondialdehyde (MDA) concentrations were assessed using HPLC as described by Richard et al. [23].

DNA damage

DNA damage was evaluated by the comet assay (single-cell gel electrophoresis) on total blood following the method of Singh et al. [24] modified by Hininger et al. [18]. Results were expressed as tail moment (TEM). Three samples per subject were assayed utilizing 50 cells/sample. The mean of these three determinations was calculated for each subject.

Statistical analyses of the data were performed by analysis of variance. Individual mean comparisons were identified with Duncan's multiple range test (SAS Institute, Cary, NC). Values are means \pm SD.

Results

Within 1 h following the session of standard chelation therapy, there were significant prooxidant effects on all of the variables tested for lipid and disulfide formation and DNA oxidative damage (Table 1). For example, plasma MDA increased 29%, DNA damage, based on the comet assay, increased 38%, and total thiol groups decreased 18%. Moreover, in the RBCs, GPx and SOD antioxidant activities decreased significantly. Similar acute prooxidant effects of chelation therapy were observed after 16 chelation sessions during a 5-week period (data not shown). The acute prooxidant effects of chelation therapy were transitory and were not detected after 1 week (data not shown).

The specific acute effects of vitamin C were determined by comparison of the effects of the chelation cocktail with and without vitamin C in three female and two male patients (Table 2). When the chelation solution contained vitamin C, oxidative stress markers increased and antioxidant enzyme activities decreased while, for all of the variables tested

Table 1
Effects of standard EDTA chelation therapy on oxidative stress markers and antioxidant enzyme activities after one session

Parameters	Before chelation	After chelation
Plasma MDA, $\mu\text{mol/L}$	2.43 \pm 0.32	3.13 \pm 0.30**
Plasma SH, $\mu\text{mol/g prot}$	6.07 \pm 0.75	5.01 \pm 0.94**
Blood GSht, $\mu\text{mol/L}$	855 \pm 181	780 \pm 200**
Blood GSSG, $\mu\text{mol/L}$	24 \pm 16	27 \pm 16**
RBC GPX, U/g Hb	54.4 \pm 8.56	52.0 \pm 8.28**
RBC SOD, U/mg Hb	1.32 \pm 0.15	1.30 \pm 0.17*
Comet, TEM ^a	1.51 \pm 0.45	2.08 \pm 0.55**

Values (means \pm SD) are for 16 patients before and after chelation therapy.

^a Arbitrary units.

* $P < 0.05$.

** $P < 0.005$ for values in the same row.

Table 2

Effects of removal of vitamin C from standard EDTA chelation cocktail on oxidative stress markers and antioxidant enzyme activities after one session

		Standard cocktail	Standard—no vitamin C
Plasma MDA, $\mu\text{mol/L}$	Before	2.46 \pm 0.41	2.46 \pm 0.37
	After	3.26 \pm 0.40*	2.56 \pm 0.29
Plasma SH, $\mu\text{mol/g prot}$	Before	6.70 \pm 0.23	6.08 \pm 0.49
	After	4.16 \pm 0.57*	6.20 \pm 0.23
Blood GSht, $\mu\text{mol/L}$	Before	816 \pm 109	944 \pm 173
	After	644 \pm 192	831 \pm 184
Blood GSSG, $\mu\text{mol/L}$	Before	21.0 \pm 8.4	33.6 \pm 21.8
	After	26.8 \pm 13.7	28.3 \pm 19.1
RBC GPX, U/g Hb	Before	50.0 \pm 7.0	58.7 \pm 8.9
	After	44.8 \pm 4.1*	58.9 \pm 8.2
RBC SOD, U/mg Hb	Before	1.24 \pm 0.15	1.30 \pm 0.16
	After	1.12 \pm 0.22**	1.27 \pm 0.20
Comet, TEM ^a	Before	1.65 \pm 0.53	1.09 \pm 0.17
	After	2.10 \pm 0.66*	1.00 \pm 0.12

Values (means \pm SD) are for 5 patients before and immediately after chelation therapy.

^a Arbitrary units.

* $P < 0.05$.

** $P < 0.005$ for values before and after chelation therapy session.

when vitamin C was omitted, there were no statistical deleterious acute prooxidant effects during the time period studied. All subjects responded similarly and there were no prooxidant effects in any of the subjects immediately following chelation therapy.

After sixteen sessions of standard chelation therapy (5 weeks), there was a significant decrease of plasma MDA ($P < 0.01$) (Table 3) in blood samples drawn prior to the chelation session. Subjects were not taking any form of nutrient supplementation. The acute prooxidant effects of chelation therapy were still present (data not shown).

Discussion

Oxidative stress plays an important role in the development of diabetes [6–8] and cardiovascular diseases [6,25]. Since EDTA chelation therapy is proposed to not only

Table 3
Effects of standard EDTA chelation therapy on oxidative stress markers and antioxidant enzyme activities after 5 weeks (16 sessions)

Parameters	Before chelation	After 16 sessions
Plasma MDA, $\mu\text{mol/L}$	2.50 \pm 0.31	2.35 \pm 0.32**
Plasma SH, $\mu\text{mol/g prot}$	6.08 \pm 0.79	6.06 \pm 0.74
Blood GSht, $\mu\text{mol/L}$	874 \pm 186	834 \pm 180
Blood GSSG, $\mu\text{mol/L}$	25 \pm 15	23 \pm 16
RBC GPX, U/g Hb	54.1 \pm 8.3	54.7 \pm 8.9
RBC SOD, U/mg Hb	1.29 \pm 0.15	1.34 \pm 0.17
Comet, TEM ^a	1.51 \pm 0.45	1.58 \pm 0.55

Values (means \pm SD) are for 16 patients before and after 16 sessions of chelation therapy. Blood samples were taken at least one week following the last chelation session.

^a Arbitrary units.

** $P < 0.01$.

remove metals but also have antioxidant properties [3], it has been used in the treatment of these diseases. The standard EDTA cocktail contains high doses of sodium ascorbate (5 g). Many health benefits have been attributed to vitamin C, such as antioxidant, antiatherogenic, anticarcinogenic and immunomodulator; however, the health benefits of vitamin C are the subject of debate and the potential danger of megadoses of ascorbic acid has been reviewed [26]. The mode of administration of vitamin C also influences its effects since oral doses lead to plasma concentrations that are tightly controlled while intravenous administration of vitamin C produces high plasma and urine concentrations that might have additional effects including antitumor effects [27].

Intravenous infusion of vitamin C has been reported to lead to beneficial antioxidant effects on coronary blood flow postulated to be due to reversal of the inactivation of nitric oxide [28] and improved flow-mediated vasodilation in patients with variant angina [29]. Infused vitamin C has also been shown to inhibit endothelial cell apoptosis in patients with congestive heart failure [30].

However, ascorbic acid may also mobilize iron from iron-binding proteins *in vivo* which in turn could catalyze an oxidative process [13]. Paradoxically, in the presence of transition metals, ascorbate can promote the generation of the same reactive oxygen species it is known to destroy. This prooxidant activity derives from the ability of ascorbate to reduce Fe^{3+} or Cu^{2+} to Fe^{2+} or Cu^{+} , respectively, to reduce O_2 to $\text{O}_2^{\cdot-}$ and to stimulate the Fenton reaction [14]. Damage to nucleic acids and proteins results from the binding of either Fe^{2+} or Cu^{+} to metal binding sites on these macromolecules followed by reaction of the metal complexes with H_2O_2 . This leads to the production of active oxygen species that attack functional groups [31,32].

In this work, we demonstrated that adjuvant supplementation with megadoses of ascorbic acid (5 g) in patients submitted to EDTA chelation therapy increases acute free radical generation leading to deleterious oxidative damage immediately following one session of chelation therapy. Immediately following the standard treatment, we observed a large increase in lipid peroxidation monitored by plasma MDA concentrations, as well as an enhanced oxidation of proteins and DNA damage. Plasma thiols groups, whose oxidation is an early determinant of oxidative stress [33], were also decreased. Oxidized glutathione was higher after the treatment than before, also suggesting a burst of oxidative stress. The comet assay which measures DNA damage, particularly DNA strand breakage, demonstrated a significant increase of the tail extent moment, which strongly suggests oxidative alterations of DNA directly related to the treatment. None of these oxidative changes were observed in the absence of vitamin C.

In agreement with our observations, Cighetti et al. [34] reported the rapid formation of significant amounts of MDA despite desferoxamine chelation therapy in betathalassemia patients. The oxidative effects of 300 mg intravenous

ascorbic acid supplementation were measured as soon as 5 min after the injection [35] in hemodialysis patients in relation with their levels of serum ferritin. Cooke et al. reported possible prooxidant effects of vitamin C at intakes of roughly 400 [36] and 500 mg per day [16]. In healthy patients, but at oxidative risk, the prooxidant effects of high doses of vitamin C have also been reported. In athletes with muscle injury and thus in inflammatory situation, 12.5 mg of vitamin C/kg body weight resulted in increased lipid peroxidation [37]. In pregnant women receiving 14 mg/day of supplemental iron plus 500 mg/day of ascorbate, we observed increased plasma thiobarbituric acid reactive substances (TBARS) [38].

In animals, large doses of ascorbic acid administered intravenously promote lipid peroxidation in the serum of guinea pigs [39]. In slices prepared from cerebral cortex of rats receiving high doses of vitamin C, Song et al. [40] reported increased TBARS in a dose-dependent manner.

We also observed a higher rate of disulfide formation in patients receiving the EDTA cocktail with vitamin C. The decrease in plasma SH groups is known to be induced by a wide array of reactive oxygen species (ROS) and is one of the most immediate responses to an elevation in the level of oxidative stress. In parallel, the GSH/GSSG ratio is considered to be the most important redox couple that determines the antioxidant capacity of cells [41] and its decrease suggests strong prooxidant effects. Functional consequences of SH group losses include protein misfolding, catalytic inactivation, and decreased antioxidative capacity [42].

In our study, there were decreases in RBC antioxidant GPx and SOD activities in patients receiving the chelation cocktail containing vitamin C. This lowering effect could be related to losses of Se, Cu, or Zn during the EDTA chelation treatment [10] but this hypothesis is ruled out since in the group that did not receive the added vitamin C the activities remained unchanged before and after the chelation. The prooxidant effects of vitamin C are likely to be involved since an enzyme loses its catalytic activity after suffering oxidation [43]. It has been shown that NO directly inactivates GSH-Px, resulting in an increase in intracellular peroxides, which in turn are responsible for cellular damage [44]. It is also possible that the acute prooxidant effects of ascorbate in blood are due to ascorbate breakdown products, when ascorbate is oxidized to the free radical that dismutates to dehydroascorbate. The latter can be rapidly taken up by cells and reduced back to ascorbate. In erythrocytes, GSH is required for this reduction, which could further decrease intracellular GSH.

The prooxidant effects of high doses of vitamin C seem to be transitory and values returned to pretreatment values within 1 week (data not shown). Moreover, after multiple sessions, a significant antioxidant effect of standard EDTA chelation therapy on lipids was also observed. This was observed in patients that were not taking any form of

nutrient supplementation. Even larger effects of EDTA chelation therapy would be expected under standard conditions, since patients normally also receive elevated levels of nutrient supplements including antioxidants.

For patients suffering from cardiovascular diseases and diabetes, it is well known that decreasing lipid peroxidation is an important health challenge to avoid oxidative damage of the arterial walls and oxidative complications such as retinopathies or glomerulopathies. Thus, EDTA chelation therapy demonstrating *in vivo* protective effects against lipid oxidation should be considered in the treatment of such patients. The effects of multiple sessions of chelation therapy, without added vitamin C during the chelation session, may lead to even more beneficial effects and need to be determined.

In summary, our study demonstrates that the presence of 5 g of sodium ascorbate added to the EDTA chelation cocktail results in acute oxidative stress monitored by lipid, protein, and DNA oxidative markers immediately following the chelation session. However, these prooxidant and deleterious effects are transitory and after multiple EDTA chelation therapy sessions, there are also long-term protective effects on lipid peroxidation. Our results strongly suggest that caution should be exerted regarding the high amounts of ascorbic acid used in EDTA chelation therapy for patients at high risk of increased oxidative stress. Additional studies are needed to define the optimal type and amounts of antioxidants to be added to EDTA chelation cocktails and to demonstrate the maximal antioxidant effects of chelation therapy.

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