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REVIEW

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Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C

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Abstract
Vitamin C (or ascorbic acid) is regarded as the most important water-soluble antioxidant in human plasma and mammalian cells which have mechanisms to recycle and accumulate it against a concentration gradient, suggesting that the vitamin might also have important intracellular functions. In this review we summarize evidence from human trials that have attempted an association between vitamin C supplementation and an effect on biomarkers of oxidative DNA damage. Most studies reviewed herein showed either a vitamin C-mediated reduction in oxidative DNA damage or a null effect, whereas only a few studies showed an increase in specific base lesions. We also address the possible beneficial effects of vitamin C supplementation for the prevention of cancer and cardiovascular disease. Finally, we discuss the contribution of cell culture studies to our understanding of the mode of action of vitamin C and we review recent evidence that vitamin C is able to modulate gene expression and cellular function, with a particular interest in cell differentiation.

Keywords: *Vitamin C, ascorbic acid, oxidative stress, DNA damage, cell differentiation, gene expression*

Introduction

In the human body, cells are constantly exposed to the effects of reactive oxidant species (ROS) deriving from either external sources or the endogenous metabolism. ROS are involved in normal biochemical processes, including the control of cell proliferation and cell signalling [1], but they can also be detrimental to cells by damaging cellular biomolecules, including DNA, proteins and lipids. The production of ROS can, however, be balanced by the existence of cellular antioxidant defences, including enzymes that remove ROS (superoxide dismutase, catalase, peroxidase, etc.), proteins that sequester transition metal ions (ferritin, transferrin), low molecular weight peptides and cofactors (glutathione, NADPH, thioredoxin, etc.) and lipid- and water-soluble low molecular weight dietary agents that scavenge reactive oxygen and nitrogen species (e.g. vitamin E, vitamin C and β-carotene). Nevertheless, when ROS production overwhelms the cellular antioxidant defences, cells are under oxidative stress. ROS are thought to be involved in the aetiology of a wide variety of diseases, including atherosclerosis, diabetes, neurodegenerative diseases, chronic inflammatory diseases, cancer and in ageing [2].

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Vitamin C (ascorbic acid) is a water-soluble micronutrient required for multiple biological functions. Vitamin C is a cofactor for several enzymes participating in the post-translational hydroxylation of collagen, in the biosynthesis of carnitine, in the conversion of the neurotransmitter dopamine to norepinephrine, in peptide amidation and in tyrosine metabolism. In addition, vitamin C is an important regulator of iron uptake. It reduces ferric Fe^{3+} to ferrous Fe^{2+} ions, thus promoting dietary non-haem iron absorption from the gastrointestinal tract, and stabilises iron-binding proteins. Most animals are able to synthesise vitamin C from glucose, but humans, other primates, guinea pigs and fruit bats lack the last enzyme involved in the synthesis of vitamin C (gulonolactone oxidase) and so require the presence of the vitamin in their diet. Thus the prolonged deprivation of vitamin C generates defects in the post-translational modification of collagen that cause scurvy and eventually death [3,4].

Redox metabolism of vitamin C

In addition to its antiscorbutic action, vitamin C is a potent reducing agent and scavenger of free radicals in biological systems (previously reviewed in [5–7]). Briefly, the mono-anion form (ascorbate) is the predominant chemical species at physiological pH. Ascorbate readily undergoes two consecutive, yet reversible, one-electron oxidations to generate dehydroascorbate (DHA) and an intermediate, the ascorbate free radical (AFR) (Figure 1). AFR is, however, a relatively unreactive free radical, with a reduction potential considerably low compared to the α-tocopherol radical, the glutathione radical and virtually all reactive oxygen and nitrogen species that are thought to be involved in human disease (e.g. superoxide anion, hydroxyl radical, hydroperoxyl radicals, singlet oxygen, nitrogen dioxide, nitroxide radicals and hypochlorous acid). These properties make ascorbate an efficient electron donor in many biological redox reactions, capable of replacing potentially highly damaging radicals by the poorly reactive ascorbate radical, according to Equation 1 where $AscH^-$ represents ascorbate, $Asc^{\bullet -}$ represents the AFR and $X^\bullet$ represents the oxidising species:

$$AscH^- + X^\bullet \rightarrow Asc^{\bullet -} + XH \qquad (1)$$

Furthermore, vitamin C has the ability to recycle other important antioxidant molecules such as α-tocopherol and glutathione from their respective radical species. Ascorbate can also be recycled by chemical and enzymatic mechanisms. AFR can be converted back to vitamin C by an NADH-dependent reductase or by dismutation of two molecules of the radical into one molecule of vitamin C and one molecule of DHA. DHA, in turn, is unstable at physiological pH and, unless it is reduced back to ascorbate, it may be irreversibly hydrolysed to 2,3-diketogulonic acid. DHA can be reduced back to ascorbate either directly by glutathione [8] or enzymatically by a glutathione-dependent DHA reductase, glutaredoxin, or the NADPH-dependent thioredoxin reductase [9].

Vitamin C availability and transport

Vitamin C is water-soluble and is well absorbed from the gastrointestinal tract. Mean plasma vitamin C levels are 50–60 μM for healthy, well-nourished, non-smoking individuals (e.g. [10,11]). Plasma levels can be increased by long-term vegetarian diet [12] and by oral supplementation up to approximately 100 μM; [10,13,14]. Higher plasma levels are not observed even with supplemental doses higher than 500 mg/day due to efficient vitamin C excretion in the urine [15]. Some studies have shown that the increase in plasma vitamin C was accompanied by an increase in the intracellular levels of the vitamin (e.g. [13]); however, this increase is often not dose-dependent [16], presumably due to cellular saturation. Thus it is known that the intracellular vitamin C concentrations of neutrophils, monocytes and lymphocytes saturate at lower supplementation doses than human plasma [15].

Cellular vitamin C transport has been studied *in vitro* and occurs by two distinct mechanisms. Ascorbate enters mammalian cells via a family of specific transporters (SVCT1 and SVCT2) in a process driven by the sodium electrochemical gradient [17]. Notably,

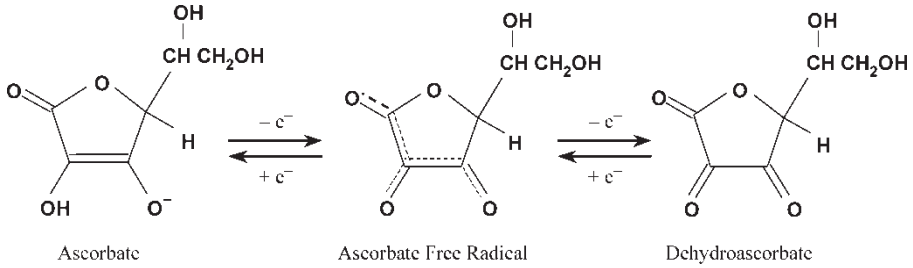

Figure 1. Redox metabolism of vitamin C. The one electron oxidation of ascorbate generates the ascorbate free radical, with one electron delocalised between three oxygen atoms, which on further oxidation originates dehydroascorbate (DHA). DHA is an unstable molecule and can be decomposed or reduced back to ascorbate.

the oxidised form (DHA) is transported into the cells faster than the reduced form by facilitated diffusion through several isoforms of the glucose transporter (GLUT) [18–20], a process that can be inhibited by glucose in some but not all cell types [21]. Once inside the cells DHA is readily reduced back to ascorbate by enzymatic mechanisms already mentioned, contributing to the intracellular accumulation of the reduced form. This recycling process allows many tissues to accumulate vitamin C up to millimolar levels against a concentration gradient. It has been recently shown that the DHA reductase activity of cultured human keratinocytes can be augmented in response to oxidative stress, simultaneously with an induction in catalase activity [22]. The authors speculated that vitamin C recycling might be part of an adaptive response that renders cells more resistant to oxidants in general. Thus it is likely that such an effect would be beneficial by sparing intracellular ascorbate in cells undergoing oxidative stress, in which ascorbate is being consumed and otherwise lost in an effort to neutralise ROS.

It is still unclear which of the two mechanisms of vitamin C transport predominates *in vivo*. Even though vitamin C seems to be present in human plasma mostly in the reduced form, a mechanism has been recently proposed to explain DHA uptake and subsequent intracellular vitamin C recycling by means of a bystander effect [23]. It was suggested that activated host-defence cells undergoing the oxidative burst promote oxidation of extracellular ascorbate to DHA and this can be transported into neighbouring cells through the glucose transporters and immediately reduced back to ascorbate.

Pro-oxidant effect of vitamin C

Paradoxically, vitamin C is also known to act as a pro-oxidant *in vitro*. Mixtures of vitamin C and copper or iron have been used for decades to induce oxidative modifications of lipids, proteins and DNA [2]. Vitamin C may contribute to oxidative damage formation by reducing ferric Fe^{3+} to ferrous Fe^{2+} ions (and Cu^{2+} to Cu^{1+}), which in turn can reduce hydrogen peroxide (H_2O_2) to hydroxyl radicals. However, in general these vitamin C-mediated Fenton reactions should be controlled in the human body due to efficient iron sequestration by metal binding proteins such as ferritin and transferrin. Consequently, it has been argued that the pro-oxidant effect may not be relevant *in vivo* [24,25]. Nevertheless, vitamin C supplements have not been recommended in people with high iron levels or in pathological conditions associated with iron overload such as thalassaemia or haemochromatosis [26]. Moreover, it is also possible that not all the undesired effects vitamin C depend on the presence of transition metals. Indeed, a mechanism has been provided by which vitamin C induces the decomposition of lipid hydroperoxides to genotoxic bifunctional electrophiles *in vitro* without the need for free transition metal ions [27]. It is still unknown, however, if this mechanism is relevant *in vivo*, and other authors have argued that in the more physiological context of human plasma incubated *in vitro*, vitamin C prevents lipid peroxidation even in the presence of added redox-active transition metals and H_2O_2 [28].

Human intervention studies

The controversy around the *in vivo* anti- or pro-oxidant nature of vitamin C has been the subject of several human intervention studies in the recent years. The ability of vitamin C to modulate oxidative DNA damage *in vivo* is of particular interest because some oxidative DNA lesions are thought to be pre-mutagenic [29]. Therefore, in this review we will focus on those trials that have attempted an association between vitamin C consumption and an effect on biomarkers of oxidative DNA damage, including DNA base lesions and strand breaks. In this respect, it is useful to note that most studies to date have looked at the effects of vitamin C supplementation on oxidative damage to DNA of blood cells. The most popular base lesion has been 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG). The detection of 8-oxo-dG is relevant because not only it is one of the most abundant DNA lesions formed during oxidative stress, it is also mutagenic causing GC → TA transversions and it is implicated in carcinogenesis [30]. In addition, it can be quantitatively measured as either the base product (8-oxo-G) or as the deoxynucleoside (8-oxo-dG) in tissues, serum and urine following exposure to oxidative stress. Measurement of 8-oxo-dG is commonly achieved with the use of high performance liquid chromatography with electrochemical detection (HPCL-EC), gas chromatography-mass spectrometry (GC-MS) or with antibody-based immunoassays [31]. Unfortunately, a number of methodological difficulties have been associated with the measurement of this lesion. This is especially due to the artefactual oxidation of guanine in those methods that require DNA extraction and derivatisation, which tends to produce overestimated baseline levels of the lesion. The consequence was the formation of the European standards committee on oxidative DNA damage (ESCODD), which has aimed at establishing standard protocols and quality control steps during sample manipulation and ultimately reaching consensus over the basal level of DNA damage in human cells [31,32].

DNA strand breaks measured by the single-cell gel electrophoresis (SCGE) or comet assay have been another popular biomarker in vitamin C intervention studies. It is important to note though that DNA single-strand breaks are not a specific biomarker of oxidative stress. Nevertheless, the specificity of the assay can be improved by including a digestion step

with lesion-specific repair enzymes, namely formamidopyrimidine (FAPY) DNA glycosylase (FPG) and endonuclease III, which recognise oxidised purines and pyrimidines, respectively. This modified version of the assay is able to detect low levels of damage with the advantage of avoiding artefactual oxidation [33]. Other studies have measured DNA single-strand breaks as an indirect indicator of antioxidant status. For this purpose, white blood cells have been challenged *ex vivo* with a DNA strand breaking oxidant (usually H_2O_2 or ionising radiation) to assess the donor's antioxidant status, assuming that the intracellular antioxidants would prevent DNA breakage.

We include in this review studies in which vitamin C has been administered to healthy or diseased individuals as a dietary supplement or intravenously, either alone or in combination with other antioxidants. Also, we include studies looking at the effects of a single vitamin administration and those where individuals were supplemented for several weeks or months. A comprehensive list of intervention studies where DNA damage was measured as either nucleotide base lesion or DNA strand breakage is supplied in Table I.

Regarding the measurement of DNA base lesions, an early report has shown that vitamin C content in the semen was inversely related to the level of 8-oxo-dG in sperm DNA [34] and more recently it was reported that 8-oxo-dG in lymphocyte DNA from human healthy volunteers was negatively correlated with the intracellular vitamin C levels [53]. But the question still remained of whether vitamin C supplements could contribute to reduce the levels of the lesion *in vivo*. Podmore et al. [41] supplemented healthy individuals with 500 mg vitamin C for 6 weeks and observed a decrease in 8-oxo-G in lymphocyte DNA relative to both placebo and baseline levels, suggesting that vitamin C was acting directly as an antioxidant *in vivo*. However, these workers found a simultaneous increase in the level of another base oxidation product, 8-oxoadenine (8-oxo-A), suggesting a concomitant pro-oxidant effect. 8-oxo-A is, however, much less mutagenic than 8-oxo-G, so the authors argued that the study has shown an overall protective effect of vitamin C [54]. In a later publication, but as part of the same study, Cooke et al. [42] found significant decreases in DNA levels of 8-oxo-dG that were strongly correlated with increase in plasma vitamin C concentration *in vivo*, and reported significant subsequent increases in serum and urinary 8-oxo-dG levels, which could be products of DNA repair. The authors suggested that vitamin C did not inhibit 8-oxo-dG formation but rather promoted its removal. It was hypothesised that vitamin C would initially cause oxidative DNA damage via a pro-oxidant activity and consequently cause the up-regulation of DNA repair processes that promote removal of highly mutagenic lesions. More recently, Cooke et al. [50] reported that vitamin C supplementation increased the levels of deoxycytidine glyoxal (gdC), a putative product of lipid peroxidation and autoxidation of vitamin C and glucose, which was also suggestive of a pro-oxidant effect *in vivo*. Deoxycytidine glyoxal levels were, however, significantly reduced upon continued vitamin supplementation, suggesting once again that vitamin C may promote lesion removal by up-regulating repair processes.

Further evidence for a pro-oxidant effect of vitamin C *in vivo* came from Rehman et al. [43]. The authors observed a significant rise in several oxidative DNA base damage products (5-OH methylhydantoin, 5-OH hydantoin and FAPY guanine) in the white blood cells of healthy human volunteers with a high initial plasma vitamin C concentration after 6 weeks of co-supplementation with iron and vitamin C. On the other hand, levels of 8-oxo-G decreased following 12 weeks of supplementation. In individuals with lower initial levels of plasma vitamin C, pre-supplemental levels of oxidative DNA damage were higher and decreased on supplementation. These studies were, however, criticised for not including a true placebo control [55]. Thus the trial reported by Podmore et al. [41] was sequential, with the placebo period preceding the vitamin C supplementation period, whereas the study by Rehman et al. [43] did not include a placebo group at all, leaving a possibility of false positive results caused by seasonal variation or changes in food habits and lifestyle of the subjects. In fact, when the same authors attempted to reproduce the latter study by including a more appropriate placebo control group, no increase in oxidative DNA lesions was found following vitamin C supplementation alone or in combination with iron [46]. It is noteworthy that, as in their previous study, vitamin C supplementation decreased the levels of 8-oxo-G when compared with pre-supplemental levels; however, a similar decrease in 8-oxo-G was observed in the placebo group. This observation reinforces the importance of including a placebo-controlled, parallel design rather than a sequential study design.

In two other placebo-controlled supplementation trials with healthy individuals, Welch et al. [45] claimed that vitamin C supplementation alone or in combination with vitamin E for 4 weeks did not have an effect on oxidative damage, but their results show a 50% reduction in leukocyte 8-oxo-dG, while Vojdani et al. [16] observed a reduction in lymphocyte 8-oxo-dG following vitamin C supplementation with 1000 mg per day for 2 weeks. In addition, four placebo-controlled supplementation trials were performed with smokers, a condition that is known to be associated with increased oxidative damage to DNA. Lee et al. [40] supplemented smokers with 500 mg vitamin C per day for 4 weeks and observed a decrease in 8-oxo-dG levels in white blood cells, even though this effect was not statistically significant. Jacobson

Table I. Human intervention studies relating vitamin C supplementation with biomarkers of oxidative DNA damage.

Study description	Measured endpoint	Effect	Reference
10 men initially kept on a controlled diet (250 mg/day) were subsequently given depletion (5 mg/day) and repletion (250 or 60 mg/day) doses for periods of 1 month	8-oxo-dG in sperm and lymphocyte DNA and 8-oxo-G in urine by HPLC-EC or HPLC-UV	Increase in 8-oxo-dG levels following depletion that was reduced upon repletion of dietary vitamin C; no effect on urine and lymphocyte DNA	[34,35]
6 healthy individuals who had fasted overnight were given a single 500 mg dose with breakfast; blood samples were taken immediately before and 1 h later	DNA breaks in white blood cells with or without *ex vivo* ionising radiation challenge by SCGE	Decrease in endogenous and radiation-induced DNA strand breaks	[36]
100 healthy males (smokers and non-smokers) were randomly assigned to receive 100 mg/day in combination with vitamin E (280 mg) and β-carotene (25 mg) or placebo for 20 weeks	DNA breaks and oxidised pyrimidine bases in lymphocytes with and without *ex vivo* H_2O_2 challenge by SCGE	Decrease in oxidised pyrimidines (but not in strand breaks) in smokers and in non-smokers; decrease in strand breaks produced by *ex vivo* exposure to H_2O_2	[37]
12 individuals (smokers and non-smokers) were given a single 1 g dose	DNA breaks in lymphocytes with *ex vivo* H_2O_2 challenge by SCGE	Decrease in H_2O_2-induced DNA breaks in smokers and in non-smokers 6 h after vitamin supplementation	[38]
48 individuals with a range of serum cholesterol levels were assigned to and cycled through one of the supplements (60 or 6000 mg/day) and placebo for 2 weeks each, all separated by 6-week washout periods	DNA breaks in lymphocytes with or without *ex vivo* H_2O_2 challenge by SCGE	No effect on endogenous or *ex vivo* H_2O_2-induced levels of DNA breaks	[39]
6 healthy smokers were randomly assigned to receive 500 mg/day or placebo for 4 weeks; 5 healthy non-smokers were used as a control	8-oxo-dG in PBMC by HPLC-EC	Decrease in 8-oxo-dG levels (but not statistically significant)	[40]
30 healthy individuals were on placebo for 6 weeks and then given 500 mg/day for the next 6 weeks	8-oxo-A and 8-oxo-G in lymphocytes by GC/MS and HPLC-EC; 8-oxo-dG in serum and urine by immunoassay	Increase in 8-oxo-A and decrease in 8-oxo-G levels followed by increases in serum and urine 8-oxo-dG	[41,42]
38 healthy individuals stratified by plasma vitamin C levels given 60 or 260 mg/day in combination with ferrous sulphate (14 mg/day) for 12 weeks	Twelve different oxidative base lesions in DNA isolated from whole blood by GC/MS	Increase in 5-oxomethylhydantoin, 5-oxohydantoin and FAPY guanine at 6 weeks and decrease in 8-oxo-G at 12 weeks in individuals with higher initial plasma levels	[43]
30 individuals exposed to environmental tobacco smoke were given 60 mg/day in combination with β-carotene (3 mg), vitamin E (30 I.U.), zinc (40 mg), selenium (40 mg) and copper (2 mg) for 8 weeks; 36 non-exposed individuals were used as a control	8-oxo-dG in DNA isolated from whole blood by HPLC-EC	Exposed individuals had significantly higher levels of 8-oxo-dG than the control (non-exposed) group, but these decreased to levels below those in the control group upon antioxidant supplementation	[44]
21 healthy individuals (9 smokers and 12 non-smokers) were randomly assigned to and cycled through one of the supplements (350 mg/day alone or in combination with 250 mg vitamin E for 4 weeks) and placebo (consisting of a 8-week washout period)	8-oxo-dG in mononuclear leukocyte DNA by HPLC with colorimetric detection and DNA breaks with or without *ex vivo* X-ray challenge by SCGE	No effect on endogenous and radiation-induced DNA strand breaks in smokers and in non-smokers; decrease in 8-oxo-dG levels in non-smokers (but not statistically significant)	[45]
20 healthy individuals were randomly assigned to and cycled through one of the supplements (260 mg/day alone or in combination with 14 mg/day ferrous sulphate) and placebo for 6 weeks, all separated by 8-week washout	Twelve different oxidative lesions in DNA isolated from whole blood by GC/MS	Decrease in 8-oxo-G and 5-oxomethyluracil in both treatments and increase in 5-oxomethylhydantoin and 5-oxocytosine in ascorbate + iron, but all changes also present in the placebo	[46]
111 healthy smokers were randomly assigned to receive 500 mg/day in combination with vitamin E (200 IU) and β-carotene (6 mg) or placebo for 6 months	8-oxo-dG in oral cells and mononuclear cells by immunoperoxidase staining	Decrease in 8-oxo-dG levels in both treated and placebo groups	[47]
15 SLE female patients were on placebo during 6 weeks and were given 500 mg/day for the next 6 weeks	8-oxo-dG in lymphocytes by HPLC-EC and in serum and urine by immunoassay	No effect on 8-oxo-dG levels in DNA or urine, increase in serum 8-oxo-dG	[48]

TABLE I (*Continued*)

Study description	Measured endpoint	Effect	Reference
7 healthy individuals given 1 g/day for 6 weeks	DNA breaks in lymphocytes with and without *ex vivo* H_2O_2 challenge by immunoassay	Decrease in H_2O_2-induced (but not in endogenous) DNA damage	[13]
20 healthy individuals were were randomly assigned to receive either 500, 1000 or 5000 mg/day or placebo for 2 weeks	8-oxo-G in lymphocytes by HPLC-EC	Decrease in 8-oxo-G only in the group supplemented with 1000 mg/day	[16]
30 asymptomatic HIV-seropositive patients were randomly assigned to receive 50 mg/day in combination with vitamin A (5000 units) and vitamin E (100 units) or placebo for 6 months	8-oxo-G, 5-oxouracil and 5-oxocytosine in lymphocyte by GC/MS	Decrease in 8-oxo-Guanine and 5-oxouracil levels	[49]
40 healthy individuals received placebo during the 5 weeks and then received 400 mg/day for 16 weeks	gdC in PBMC DNA by enzyme-linked immunosorbent assay	Increase in gdC levels at 6–16 weeks and a subsequent decrease relative to baseline on continued supplementation	[50]
9 healthy men were given 60 mg every other day for 3 weeks	DNA breaks and DNA repair capacity in lymphocytes by SCGE and cell-free DNA repair assay, respectively	No effect on baseline or *ex vivo* H_2O_2-induced levels of DNA breaks or on DNA repair capacity	[11]
12 healthy individuals were randomly assigned to and cycled through one of the supplements (single 500 mg dose alone or in combination with 400 units of vitamin E) and placebo, all separated by at least 10 days washout	DNA breaks in lymphocytes with or without *ex vivo* H_2O_2 challenge by SCGE	No effect on endogenous or H_2O_2-induced DNA breaks 90 or 180 min after supplementation	[14]
51 chronic haemodialysis patients were randomly assigned to receive intravenous 300 mg/day or placebo postdialysis 3 times/week for 8 weeks	8-oxo-dG in peripheral blood lymphocytes by HPLC-EC	Decrease in 8-oxo-dG level relative to baseline and placebo group	[51]
48 smokers were randomly assigned to receive either plain release or slow release tablets corresponding to 500 mg/day in combination with vitamin E (182 mg) or placebo for 4 weeks	DNA breaks and oxidised bases in mononuclear blood cells determined as EndoIII or Fpg sensitive sites with the SCGE	Decreased levels of EndoIII and FPG sensitive sites at 4 h, 8 h and 4 weeks following supplementation with slow release tablets, whereas plain release tablets only decreased EndoIII sites at 4 h and 8 h. No effect on endogenous DNA breaks	[52]

8-oxo-A, 8-oxoadenine; 8-oxo-G, 8-oxo-Guanine; 8-oxo-dG, 8-oxo-7-hydro-2′-deoxyguanosine; EndoIII, endonuclease III; FPG, formamidopyrimidine DNA glycosylase; GC/MS, gas chromatography/mass spectrometry; gdC, deoxycytidine-glyoxal; HPLC-EC, high performance liquid chromatography with electrochemical detection; HPLC-UV, high performance liquid chromatography with ultraviolet detection; n.a., PBMC, peripheral blood mononuclear cells; SCGE, single-cell gel electrophoresis; SLE, systemic lupus erythematosus.

et al. [47], on the other hand, supplemented smokers with vitamin C in combination with vitamin E and β-carotene and reported a decrease in 8-oxo-dG levels both in treated and in placebo groups. But a combination of the same antioxidants was able to decrease the endogenous levels of oxidised pyrimidines (assessed as endonuclease III sensitive sites with the use of the comet assay) in lymphocyte DNA of smokers and non-smokers [37]. Recently, Moller et al. [52] have also reported that oral supplementation with 500 mg vitamin C in combination with 182 mg vitamin E per day protected blood mononuclear cells of smokers against oxidative DNA damage by decreasing the amount of endonuclease III and FPG sensitive sites. Interestingly, the protective effect was only evident for a few hours after ingestion when the vitamin C was supplied as plain release tablets; however, supplementation with slow release tablets afforded a longer-term protection that was still evident at the end of the trial (4 weeks). In a different study, whole blood DNA from individuals exposed to environmental tobacco smoke contained increased levels of 8-oxo-dG, which decreased to levels below those in the control group on supplementation with an antioxidant cocktail containing vitamin C [44]. Finally, three trials have assessed the effect of vitamin C supplementation on DNA base oxidation in other conditions that have been associated with oxidative stress. Evans et al. [48] reported no effect of vitamin C supplementation on lymphocyte DNA 8-oxo-dG of systemic lupus erythematosus (SLE) patients. Human immunodeficiency virus (HIV)-infected patients had significantly higher levels of two oxidised DNA bases (8-oxo-G and 5-OH-Ura) that were decreased upon vitamin C supplementation in combination with vitamins A and E [49]. More recently, a randomised, placebo-controlled study with chronic haemodialysis patients showed additional evidence for a role of vitamin C in promoting removal of 8-oxo-dG. In these subjects, vitamin C intravenous supplementation for 8 weeks was able to reduce the lymphocyte 8-oxo-dG levels [51].

Regarding the measurement of DNA strand breaks, only one study shows some protection [36], whereas all other seven studies show a null effect (Table I). Hence there is no evidence that vitamin C supplementation can alter the endogenous levels of DNA strand breakage. This is perhaps not surprising considering that DNA single-strand breaks are not a specific biomarker of oxidative stress. Results from studies where white blood cells were collected from patients and subsequently challenged *ex vivo* with an oxidant insult are more promising but still conflicting. Three studies investigated the effect of a single-dose vitamin C supplementation. Green et al. [36] reported a protection against *ex vivo* exposure to ionising radiation that started as early as 1 h after a single vitamin C ingestion (500 mg) and peaked at 4 h. Panayiotidis et al. [38] reported that a single high dose (1 g) vitamin C supplement afforded protection against oxidative DNA damage caused by *ex vivo* exposure to H_2O_2. In agreement with the study of Green et al. [36], this protection peaked at 2–4 h after the ingestion. However, a recent study failed to show any effect of a single dose of vitamin C alone, or in combination with vitamin E, on resistance to an *ex vivo* oxidative challenge, despite the clear increase in plasma ascorbate [14]. Other trials have investigated the effects of long-term vitamin C supplementation. Antioxidant supplementation with a combination of vitamin C, vitamin E and β-carotene for 20 weeks has decreased the damage induced when lymphocytes were challenged *ex vivo* with H_2O_2 [37]. Brennan et al. [13] supplemented individuals with vitamin C on its own and observed a similar protective effect, which correlated with increases in patient plasma and lymphocyte intracellular vitamin C levels. However, other studies have failed to show a protective effect [11,39,45].

To summarise, more than twenty studies have been carried out so far to determine the effect of vitamin C supplementation on biomarkers of oxidative DNA damage in humans. Most of the studies reviewed herein showed either a vitamin C-mediated reduction in oxidative DNA damage or a null effect. The studies involving the measurement of base lesions have generally supported the idea that levels of 8-oxo-dG are negatively correlated with ascorbate plasma concentration, whereas a few studies measuring other base lesions have shown an increase in DNA oxidation following vitamin C supplementation. The latter ones suggest that the pro-oxidant effect may occur *in vivo* but this hypothesis remains controversial and more studies are required before a conclusion is reached. Also, it is well known that oxidative stress causes a plethora of DNA base lesions [56], so future studies should include the measurement of different base lesions, instead of relying only on 8-oxo-dG.

Some evidence showing that vitamin C supplementation protects blood cells against an *ex vivo* oxidant insult suggests that vitamin C may exert its protective action by increasing the cellular antioxidant status. Nevertheless, it is feasible that vitamin C may have other actions *in vivo* besides a classical antioxidant role. Indeed, as discussed earlier, it has been suggested that vitamin C is able to increase the cellular repair capacity [42]. Tarng et al. [51] have recently supported this hypothesis by measuring mRNA levels of human 8-oxo-Guanine DNA glycosylase (hOGG1), the enzyme that promotes repair of 8-oxo-G, following vitamin C supplementation *in vivo*. In their recent study with chronic haemodialysis patients, the authors have reported not only a decrease in 8-oxo-dG levels in lymphocyte DNA, but also an up-regulation of hOGG1 mRNA expression at 24 h after vitamin C administration. Even though it would be important to know if this observation has any

correspondence in hOGG1 protein expression and activity, it suggests that vitamin C may induce a transient up-regulation of hOGG1 and hence promote DNA repair. In a different study, vitamin C supplementation of smokers for 4 weeks had no effect on the levels of hOGG1 mRNA, despite the increase in plasma vitamin C [52]. This observation further suggests that the induction of hOGG1 may be a transient rather than a stable effect.

Finally, a possible reason for the discrepancies encountered between different studies might be the vitamin C intracellular saturation. It is known that blood cells saturate at lower vitamin C concentrations than human plasma and that this intracellular saturation occurs at plasma concentrations that can easily be obtained from the diet [15]. However, most studies only report increase in plasma levels. It is likely that if tissue saturation is achieved, then the additional beneficial effects are small and difficult to detect, leading to non-significant or null effects. Consistent with this notion, most of the studies that have shown a protective effect were carried out with smokers or patients with pathological conditions associated with oxidative stress and low plasma vitamin C levels. This has also been observed for other biomarkers of oxidative damage that have not been covered in this review. Thus, we have focused on oxidative modifications to DNA but other biomolecules like proteins and lipids are also susceptible to oxidation by ROS and naturally vitamin C protection to these biomolecules would also appear beneficial. In this respect, vitamin C supplementation was able to reduce *in vivo* levels of protein carbonyls, a biomarker of protein oxidation, even though this effect only occurred in subjects with low baseline ascorbate levels [57]. It is thus possible that the initial level of vitamin C in the cell predetermines whether supplementation trials may have a positive or null response. In this case, the lower the vitamin C level the more positive the response would be.

Vitamin C in human disease

Low levels of plasma vitamin C are known to occur in several conditions of increased oxidative stress, such as cancer, diabetes mellitus, cataract, HIV infection, SLE and smoking habits [48,49,58]. The possible use of vitamin C in cancer therapy and prevention has been an area of great interest. Thus it is tempting to speculate that vitamin C supplements, if able to prevent the formation and/or promote the repair of pre-mutagenic oxidative DNA lesions, could be of use in cancer prevention. In addition, an early report showed that daily supplementation with vitamin C at high doses (grams) increased the survival time of terminal cancer patients [59] and it was suggested that vitamin C could have important anticancer properties [60]. Indeed, vitamin C kills or inhibits growth of many tumour cell lines (reviewed in [61]) and potentiates the cytotoxicity of radiosensitising drugs [62]. There are also several reports showing that cancer cell lines are more sensitive to vitamin C than their non-malignant counterparts (e.g. [63,64]). However, so far only a limited number of studies have established an association between vitamin C administration and survival of advanced cancer patients and these have been heavily criticised for the lack of appropriate controls and randomisation [65]. Regarding cancer prevention, several epidemiological studies have linked the consumption of a diet rich in fruit and vegetables (and therefore in antioxidants) with lower incidence of many types of cancer (reviewed in [66]). In a very recent report, Bjelakovic et al. [67] presented the results of their systematic review of all randomised trials relating antioxidant supplements with the incidence of gastrointestinal cancers, which included meta-analyses of outcomes such as cancer incidence and mortality. The authors found no evidence that antioxidants can prevent gastrointestinal cancers. On the contrary, certain antioxidant combinations (β-carotene with vitamin A or vitamin E) results in increased patient mortality. Vitamin C, when added alone or in combination with other antioxidants, did not seem to have an effect on the incidence of gastrointestinal cancers or on overall mortality.

Epidemiological evidence has also associated fruit and vegetable consumption with lower risk of cardiovascular disease (CVD) [68]. Notably, low plasma levels of vitamin C were associated with death from CVD [69] and it has been speculated in the literature that vitamin C may protect against CVD through several mechanisms. Vitamin C enhances endothelium-dependent vasodilation, thereby preventing endothelial dysfunction associated with atherosclerosis, hypercholesterolemia, hypertension, diabetes and smoking. This process seems to involve the ability of vitamin C to increase the atheroprotective nitric oxide (NO) [70]. Thus vitamin C was shown to enhance the activity of endothelial NO synthase by keeping its cofactor, tetrahydrobiopterin, in a reduced state and thereby increasing its intracellular availability [71,72]. In addition, vitamin C prevents oxidation of low-density lipoproteins (LDL), a critical process during atherosclerosis and cardiovascular disease [73], and decreases damage caused by oxidised LDL to endothelial cells. Indeed, pre-treatment of cultured human arterial smooth muscle cells with vitamin C protected against apoptotic cell death induced by oxidised LDL [74]. It was speculated that, by protecting against vascular cell death, vitamin C could limit plaque instability in advanced atherosclerosis and consequently protect against thrombosis. Vitamin C has also an anti-inflammatory action in decreasing leukocyte adhesion to the endothelium. Thus individuals with low plasma

vitamin C levels have greater monocyte adhesion to endothelial cells [10] and express higher levels of monocyte ICAM-1 mRNA [75], but supplementation with 250 mg vitamin C/day for 6 weeks was able to reduce monocyte adhesion and ICAM-1 expression. However, despite all the proposed mechanisms and the epidemiological observations, data from clinical trials relating vitamin C with different CVD endpoints were inconsistent [76] and there is still no clear evidence that vitamin C (as well as other dietary antioxidants) may prevent CVD [77].

In summary, results from several epidemiological studies associate low levels of plasma vitamin C with increased death from CVD and cancer. However, whilst it seems likely that an increase in consumption of antioxidant-rich foods such as fruits and vegetables would offer some degree of protection, clinical trial data are not conclusive as to whether vitamin C supplements are beneficial in well-nourished individuals.

Studies with cell culture models

As discussed earlier, studies with cultured cells have helped elucidating the mechanisms of vitamin C cellular uptake. Vitamin C is also known to modulate proliferation of cells in culture. For example, it can act as growth promoter for human diploid fibroblasts, when supplied at confluent density, whereas at low cell density or above certain concentrations it becomes cytotoxic [78,79]. The cytotoxicity is associated with its autoxidation in the culture medium. Thus it is well known that, in the presence of oxygen, vitamin C autoxidises and is rapidly lost from aqueous solutions (including salt and buffer solutions and cell culture media) at physiological temperature and pH [80]. This autoxidation apparently results from the presence of trace levels of contaminant catalytic transition metals in most salt and buffer solutions employed in research [81]. The oxidation of vitamin C mediated by transition metal ions produces H_2O_2 and it has been suggested that some, if not all the effects of vitamin C on cells in culture is due to H_2O_2 formation by interaction of vitamin C with the cell culture medium [82]. In addition, it has been shown in numerous occasions that vitamin C addition to cells in culture increases their H_2O_2 content (e.g. [83,84]). H_2O_2 itself is a poorly reactive molecule, however, in the presence of transition metal ions it can be converted to the highly reactive hydroxyl radical by Fenton reaction. Thus the cytotoxicity of vitamin C is enhanced with the addition of metal ions [85] and prevented by catalase in mouse neuroblastoma cells and in human fibroblasts [63,86]. Likewise, millimolar (non-physiological) levels of vitamin C cause re-differentiation and growth inhibition in human hepatoma cells and it has been suggested that this effect may be due to an increase in the H_2O_2 content of these cells [83]. Moreover, we have mentioned before that vitamin C selectively suppresses the growth of several tumour cell lines; however, this effect is at least in some cases due to H_2O_2 production in the medium (e.g. [84,87]). Thus the toxicity of vitamin C towards cancer cells has been explained in terms of their low levels of catalase activity [61]. The involvement of H_2O_2 is also able to explain the controversy around some reports describing the effects of vitamin C on cell death by apoptosis. Vitamin C protects some cell types from apoptotic cell death induced by serum withdrawal [88] or by the apoptotic agents 6α-methylprednisolone (MPS), thapsigargin and etoposide [89]. In the latter study, protection was reproduced by incubation with H_2O_2 and suppressed if catalase was present during incubation with vitamin C, showing that the effects produced by vitamin C were mediated by H_2O_2 originating from vitamin C autoxidation. In other experiments, unstable vitamin C derivatives (L-ascorbic acid or sodium ascorbate) were found to induce apoptosis in human promyelocytic leukemic HL60 cells, whereas vitamin C derivatives that do not autoxidise such as L-ascorbic acid-2 phosphate (AA2P) magnesium salt and L-ascorbic acid 2-sulfate did not have any apoptotic activity [90]. Moreover, apoptosis was abolished in the presence of catalase, showing that vitamin C toxicity was due to extracellular H_2O_2 production [84,91]. In addition to causing cell death, vitamin C was also reported to enhance the differentiation of HL-60 cells to granulocytes and monocytes [61], presumably by modifying the cellular redox state of differentiating cells. Again, these effects were not achieved when using DHA or a stable vitamin C derivative [92] and the differentiation was abolished in the presence of catalase, showing that it was a result of H_2O_2 formation in the medium [93]. Some authors have also shown that vitamin C effects are not due to a pH change in the medium at least when using vitamin C concentrations up to 1 mM [64,94].

As a way to circumvent the problems associated with vitamin C autoxidation, some authors have used AA2P, a vitamin C derivative that remains stable in cell culture medium even after several days of incubation at 37°C until it is hydrolysed by intracellular phosphatases to ascorbic acid and inorganic phosphate [95]. Thus AA2P is taken up from the medium and accumulated intracellularly as ascorbic acid against a concentration gradient [96]. In human vascular endothelial (HUVE) cells, AA2P leads to a higher intracellular enrichment in ascorbate than equimolar concentrations of ascorbic acid itself [97]. AA2P is also superior to ascorbic acid in enhancing proliferation, collagen accumulation and extracellular matrix (ECM) formation in cultured human fibroblasts [95]. AA2P can also be useful in cell culture due to its efficiency in stabilising α-tocopherol concentration when both are added to cells in

culture medium [98]. Also, some evidence exists that AA2P may be superior to ascorbic acid as an antioxidant supplement to cells in culture. Thus, presumably by increasing the cellular antioxidant capacity, AA2P protects human keratinocytes from UVB-induced cell death [96,99] and primary cell lines from *in vitro* cellular ageing [97,100]. It is known that primary mammalian cells can undergo only a limited number of cell divisions when cultured in the laboratory, before reaching a non-proliferating G_0 senescent state. This loss of replicative potential is associated with telomere shortening and also with increased sensitivity to oxidative stress [101]. Indeed, oxidative stress has been implicated in the process of ageing and it is known that primary fibroblasts grown in the presence of high oxygen concentrations have a reduced lifespan and an increased rate of telomere shortening (reviewed in [102]). Likewise, higher steady-state levels of oxidative DNA damage have been observed in senescent diploid fibroblasts or in cells chronically exposed to low, non-cytotoxic levels of H_2O_2 [103]. Notably, AA2P reduced the rate of telomere shortening during the cellular ageing process of HUVE cells and fibroblasts and extended their replicative lifespan [97,100]. This effect has been attributed to its antioxidant properties, since AA2P decreased the level of intracellular ROS of control replicating cells as judged by a reduction in 2′,7′-dichlorofluorescein diacetate fluorescence.

Effects of vitamin C on gene expression

It is well known that ROS can act as subcellular messengers in several gene regulatory and signal transduction pathways, so it is not surprising that antioxidants, as well as oxidants, are able to activate certain genes and signalling pathways by modulating the redox state of the cell [104]. In addition, the binding activity of certain transcription factors is determined by the redox state of the cell. Indeed, NFκB and AP-1 are well-known mediators of redox-responsive gene expression [105]. ROS can activate NFκB presumably by causing release of the inhibitory subunit (IκB) from the NFκB complex, whereas redox regulation of AP-1 binding may occur through a conserved cysteine residue present in its Jun and Fos subunits [104].

In some cell culture conditions it has been shown that vitamin C, due to its pro-oxidant or antioxidant properties, can modulate nuclear binding of redox sensitive transcription factors such as AP-1 and NFκB. For example, vitamin C potentiates the phorbol 12-myristate 13-acetate (PMA)-induced AP-1 binding to DNA in murine macrophages [106]. AA2P, in turn, induces resistance of skeletal muscle cells to oxidative stress by modulating the binding of NFκB and AP-1 complexes, namely by increasing activity of the former and by inhibiting the latter [107].

Another possible mechanism by which vitamin C may modulate gene expression is related to its intracellular recycling process. A model has been proposed to explain the pro-oxidant effect of vitamin C in neuronal cells in which vitamin C is autoxidised extracellularly to DHA and the latter is rapidly transported into the cells by the GLUT transporters. Once in the cytosol, DHA is reduced back to ascorbate and this reduction process is thought to cause the oxidation of cellular components [108,109]. Likewise, it has been speculated that vitamin C recycling inside the cells could lead to the formation of intracellular ROS and consequently interfere with redox-sensitive signalling pathways, eventually inducing gene expression [110]. Even though the effects of vitamin C on gene expression are of particular interest, only recently they have gained more attention. Catani et al. [111] studied expression profiles of transformed human epidermal keratinocytes (HaCaT) exposed to a high dose of AA2P (1 mM) for 5 h using cDNA array technology. AA2P increased the expression of Fra-1, GST-pi and Mut L homologue-1 (MLH1). Fra-1 is a member of the Fos superfamily, which heterodimerises with members of the Jun family and acts as a negative regulator of AP-1 activity. When HaCaT cells were irradiated with UVB, pre-incubation with AA2P was able to modulate the binding of the transcription factor AP-1. This was achieved in part by changing the composition of the AP-1 complex in irradiated cells through an increase in the steady-state levels of Fra-1 protein and also by decreasing activation of the stress-activated JNK and consequently c-Jun phosphorylation. In a subsequent publication, the authors have confirmed that AA2P induces expression of MLH1, a member of the DNA mismatch repair machinery. In addition, AA2P specifically induced p73, an apoptosis-inducing protein that is a target of MLH1 and increases cellular susceptibility to apoptosis in response to cisplatin. The authors speculated that ascorbate, by inducing gene expression of MLH1 and p73, potentiates tumour cell susceptibility to apoptotic death by cisplatin, which in turn could explain its possible chemopreventive activity [112]. A similar effect was observed in a different study, where cervical carcinoma HeLa cells were sensitised to apoptotic cell death induced by cisplatin or etoposide by pre-loading with a low, non-toxic concentration of vitamin C (1 μM) [113]. In this work the effect was apparently associated with down-regulation of c-Jun and c-Fos, up-regulation of p53 and increased Bax/Bcl2 ratio.

Other authors recently looked at the effect of a pro-oxidant mixture of iron and a high (millimolar) dose of vitamin C on gene expression of intestinal epithelial cells [114]. Not surprisingly, these workers observed an increase in lipid peroxidation that was accompanied by the activation of transcription factor NFκB and an increased expression of the inflamma-

tory proteins ICAM-1, cyclooxygenase-2 and interleukin-8.

Recently, the effects of vitamin C on gene expression have also been studied in the context of cell differentiation. Vitamin C stimulates *in vitro* differentiation of several mesenchymal cell types such as adipocytes, chondrocytes, myoblasts, osteoblasts and odontoblasts [61]. Moreover, the differentiation of chondrocytes [115], myoblasts [116] and osteoblasts [117] requires vitamin C presumably due to its ability to induce collagen matrix synthesis and deposition. The ability of vitamin C to stimulate collagen secretion has been extensively studied in cultured fibroblasts. It was shown that vitamin C increases the post-translational hydroxylation of proline [118,119] and activates the transcription of procollagen genes [120].

Differentiation requires the commitment of mesenchymal stem cells to a given lineage, following by induction of tissue-specific gene expression patterns. During osteoblastic differentiation vitamin C induces expression of several osteoblastic marker proteins such as type I collagen, alkaline phosphatase [117], collagenase 3 [121], osteocalcin [122], osteonectin and tissue inhibitor of metalloproteinase 3 [123]. Osteoblastic differentiation of mesenchymal cells can also be achieved when using the stable vitamin C derivative AA2P (e.g. [117,123]), which suggests that it is an ascorbate-dependent process, rather than a possible non-specific effect resulting from vitamin C autoxidation *in vitro*. Ascorbic acid and AA2P can also stimulate differentiation of vascular smooth muscle cells (VSMCs) by increasing the expression of two smooth muscle-specific markers, smooth muscle-specific myosin heavy chain-1 and calponin 1 both *in vitro* and *in vivo* [124]. The differentiation of VSMCs is strongly implicated in the processes of atherosclerosis and restenosis after angioplasty, so the authors proposed that vitamin C, due to its ability to maintain VSMCs in the differentiated state in the vascular wall, could have an important cardioprotective action *in vivo*.

The recent availability of human ES cells that have the ability to be differentiated *in vitro* into specialised cell types has provided the scientific community with a unique opportunity to study the gene expression events related to many developmental processes. Vitamin C induces the differentiation of ES cells into cardiac myocytes and increases the expression of a number of cardiac marker genes (GATA4, α-MHC, β-MHC, ANF) [94]. As noted by the authors, this effect is apparently independent from its antioxidant properties. Thus, other antioxidants are known to inhibit cardiomyocyte differentiation, while H_2O_2 enhances it [125]. Very recently, another group has employed cDNA microarray technology to identify vitamin C-responsive genes in the differentiation of ES cells into neurons [126]. In agreement with the ability of vitamin C to enhance neuronal differentiation of ES cells, the authors reported that vitamin C induced the expression of genes involved in neurogenesis (neuronatin, brain derived neurotrophic factor and neurotrophic tyrosine kinase receptor), neuronal maturation (double cortin and calcium/calmodulin-dependent protein kinase-like 1, growth arrest specific 7 and DNA segment human D4S114) and neurotransmission (rabphilin 3, synuclein alpha, synaptotagmin 7 and receptors for the neurotransmitters glutamate, GABA and neurotensin 3). Likewise, vitamin C repressed the expression of genes associated with pluripotency of ES cells (developmental pluripotency associated five and embryonic stem cell specific gene). It was suggested that vitamin C might be useful to large-scale generation of neurons for future clinical treatment. Thus the production of dopaminergic neurons from ES cells may be an important cell source for cell replacement therapy of neurodegenerative diseases such as Parkinson's disease. In this respect, vitamin C was also shown to increase the yield of dopaminergic neurons differentiated *in vitro* from rat embryonic central nervous system precursors [127]. This effect could not be mimicked by any other antioxidants, suggesting a novel role for vitamin C independent of its antioxidant properties. The analysis of gene expression changes in the neuronal differentiation following vitamin C (200 μM) treatment was recently performed using cDNA microarray technology [128]. In contrast with the observations of Shin et al. [126], the authors reported that, despite causing a clear increase in differentiated neurons, vitamin C did not induce changes in the expression of genes previously known to be crucial for neuronal differentiation. Instead, vitamin C increased the expression of genes encoding for iron-binding proteins (transferrin and ferritin) and several genes that are known to be part of the cellular response to ROS, such as glutathione peroxidases, metallothioneins and glutathione-S-transferases. This suggests that the observed expression profiles may result from the pro-oxidant effect of vitamin C and, based on that evidence, the authors hypothesised that oxidative stress may play a role in inducing neuronal differentiation.

In summary, studies with cultured cells have shown that vitamin C can affect gene expression and this seems to be mediated by its redox effects. A schematic diagram of the proposed mechanisms of action of vitamin C is presented in Figure 2. Briefly, vitamin C enters cells as ascorbate directly through sodium-dependent transporters. Alternatively, DHA generated in the extracellular space upon oxidation of ascorbate by ROS or by free metal ions is taken up through the glucose transporters and, once inside the cells, it is reduced back to ascorbic acid. The intracellular ascorbic acid enrichment and the oxidation events putatively generated during the vitamin C recycling from DHA have the potential to change the cellular

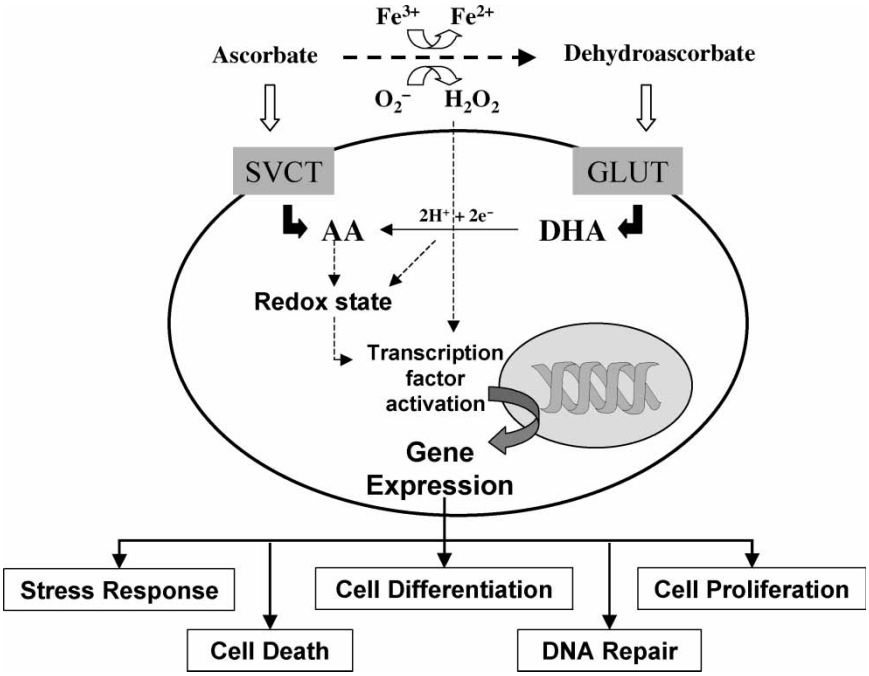

Figure 2. Proposed mechanisms of action of vitamin C in mammalian cells.

redox status. The H_2O_2 formed extracellularly during the autoxidation of ascorbic acid can modulate the binding activity of redox-sensitive transcription factors, resulting in the activation of gene expression. The endpoint effects reviewed herein are cell type-specific and include the activation of stress response genes, cell killing by apoptosis, promotion of cell differentiation, cell proliferation and possibly DNA repair. Some of the pro-oxidant effects discussed here can apparently be explained by vitamin C autoxidation because they were exclusive of those vitamin C derivatives that autoxidise and/or they could be mimicked by the addition of H_2O_2 to cells, whereas other effects seemed to be more specific to vitamin C. The biological relevance of the former is still unknown, partially because the effects of vitamin C on gene expression *in vivo* have not been thoroughly studied. In this respect, we mentioned before a study showing that individuals with low plasma vitamin C levels express higher levels of monocyte ICAM-1 mRNA, which were reduced upon vitamin C supplementation [75]. A recent report has investigated the effect of vitamin C on gene expression of rat liver during induced septic shock [129]. Rats were subjected to polymicrobial sepsis by cecal ligation and puncture (CLP) and immediately given either vitamin C or saline intravenously. The livers were removed 24 h later to evaluate oxidative stress and monitor expression of vasoregulatory genes that are induced in the liver in situations of oxidative stress and in inflammatory responses. CLP resulted in increased oxidative stress as demonstrated by a reduction in hepatic glutathione and a raise in lipid peroxidation. Notably, vitamin C treatment was able to attenuate these effects. Likewise, vitamin C inhibited or attenuated the sepsis-induced increase in the mRNA expression levels of inducible nitric oxide synthase, haem oxygenase-1, tumour necrosis factor-α and cyclooxygenase-2 mRNAs. As noted by the authors, oxidative stress has been implicated in sepsis, so the effect of vitamin C seems to correspond to that of an antioxidant. It is unknown, however, whether vitamin C inhibits the expression of oxidant-responsive genes indirectly by scavenging ROS (and thereby reducing oxidative stress), or directly by modulating the binding activity of redox-sensitive transcription factors. NFκB is a well-known activator of immune and inflammatory response genes [130], so the putative suppressive effects of vitamin C on the inflammatory responses associated with atherosclerosis or septic shock mentioned before suggest that vitamin C may be able to modulate NFκB *in vivo*.

Conclusions

ROS deriving from either external sources or the endogenous metabolism are thought to be involved in the aetiology of a wide variety of diseases, carcinogenesis and ageing. The production of ROS can be balanced by the existence of cellular antioxidant defences. Vitamin C is traditionally regarded as the most important water-soluble antioxidant in human plasma, where it is thought to scavenge reactive oxygen and nitrogen species. Health claims derived from observational epidemiological studies have associated diets rich in antioxidants with reduced

risks on certain cancers and CVD, increased function of the immune system and a reduction of stress. Based on these claims, consumers' interest for antioxidant supplements has been increasing rapidly. However, supplementation trials with single antioxidants have not been able to show a clear protective effect. Work reviewed herein can be summarised as follows:

- Data from intervention studies are contradictory and overall there is still not enough evidence that vitamin supplementation is beneficial by preventing DNA oxidation in humans consuming adequate amounts of vitamin C from their diet. It is thus possible that the beneficial effects of vitamin C supplementation are only relevant to those individuals with low levels of plasma vitamin C, such as smokers or in pathological conditions associated with high steady-state levels of oxidative stress. Likewise, clinical trial data have not been conclusive as to whether vitamin C supplements are beneficial in the prevention of cancer and CVD and more studies will be needed to determine the exact role of vitamin C in the repair of DNA damage *in vivo*.
- Vitamin C supplementation with doses above a certain threshold limit or under certain conditions (e.g. iron overload) may produce undesirable toxicological effects. Thus, in the presence of transition metal ions vitamin C acts as a pro-oxidant rather than as an antioxidant. However, while the vitamin C-driven Fenton reaction is well established *in vitro*, the relevance of its pro-oxidant chemistry needs to be assessed *in vivo*.
- It is now clear that vitamin C does not act only as a simple antioxidant. We have reviewed evidence that vitamin C is able to affect redox-sensitive signalling pathways and to modulate gene expression in cultured cells, but the relevance of these effects *in vivo* remains mostly unknown and should be the subject of future investigation.
- Vitamin C appears to have important regulatory effects in cell differentiation *in vitro* and *in vivo*. In this respect, vitamin C may protect some tissues by maintaining cells in a differentiated state and it might be useful in the *in vitro* production of specialised cells/tissues from ES cells for future clinical treatment.
- Vitamin C is unstable in aqueous solutions and some of its effects on cultured cells result from the susceptibility of a particular cell line to the H_2O_2 formed from its autoxidation. As an alternative, AA2P has been used as a long-acting and possibly non-toxic vitamin C analogue in cell culture systems. However, the use of AA2P has been limited, both *in vitro* and *in vivo*. Much more research is required in order to establish any potential beneficial or adverse effects associated with this compound.

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